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Comparative analysis of aerobic and respiratory metabolism and genomic characterization of *Lactobacillus gasseri* strains

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CHAPTER I

OVERVIEW OF THE THESIS

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a functional group of microorganisms characterized as Gram-positive, non-sporulating, acid-tolerant, catalase negative, non-motile, rod or coccus-shaped bacteria, belonging to the phylum *Firmicutes* with low (≤55 mol %) G+C in the DNA (Carr et al., 2002). LAB are generally classified as oxygen-tolerant anaerobes and lack of cytochromes and porphyrins (components of electron transport chains, ETC), therefore, they get energy mainly through substrate level phosphorylation, producing lactic acid as major end-product. Orla-Jensen (1919) originally grouped LAB in four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, however, taxonomic revisions have proposed several new genera and the remaining group comprises the following: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactococcu*s, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. These genera include more than 300 species and they are found in nutritionally rich habitats such as food (dairy, meat and fish products, beer, wine, fruits, vegetables and silage), water, soil and they are part of the normal microflora in the mouth, gastro-intestinal (GI), and genital tracts of humans and many animals. Based on the metabolic pathways used to ferment glucose and the ability to metabolize pentoses, LAB are divided into two groups, homolactic and heterolactic. The first group, ferments glucose to exclusively lactic acid via the Embden-Meyerhof-Parnas (EMP). In this pathway, two molecules of ATP are generated from one molecule of glucose via substrate-level phosphorylation. The second group metabolize glucose via 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, producing one molecule each of lactic acid, CO_2 and ethanol from one molecule of glucose consumed (Carr et al., 2002). LAB have been used for centuries for their technological and functional properties in the manufacture of fermented foods. Besides food production, LAB are used in a variety of other industrial applications such as the production of lactic acid, high-value metabolites involved in flavour and texture development or health applications, probiotic products, and antimicrobial peptides.

1.2 The genera *Lactobacillus*

Among LAB, *Lactobacillus* is the most numerous genus of the *Lactobacillaceae* family and of the *Lactobacillales* order. The genus includes several Generally Recognized As Safe (GRAS) species and many strains have a remarkable significance in both food microbiology and human health field, due to their contribution to food fermentations or their use as probiotics. Members of the genus *Lactobacillus* are non-spore-forming, non-motile and generally rod-shaped bacteria; cells are sometimes organized in chains. They are usually considered aero-tolerant anaerobic, aciduric or

acidophilic bacteria and lacking of catalase enzyme, although, the presence of a heme and manganese-dependent catalase has been found in some strains of *Lb. plantarum*, *Lb. casei* and *Lb. sakei* species (Abriouel et al., 2004; Rochat et al., 2006; Guidone et al., 2013; Zotta et al., 2016). The genus *Lactobacillus* is composed by 200 species and, from a taxonomic viewpoint, it belongs to phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae* (Felis and Dellaglio, 2007). On the basis on the metabolic pathways, three physiological *Lactobacillus* groups were classified: (*i*) obligately homofermentative lactobacilli (group A), ferment glucose to exclusively lactic acid via EPM pathway but lack of 6-PG/PK pathway, therefore, neither gluconate and pentoses are fermented, (*ii*) facultatively homofermentative (group B), ferment glucose and pentose via EPM and 6-PG/PK pathway, respectively, with a resulting production of acetic acid and ethanol under glucose limitation, and (*iii*) obligately heterofermenative (group C) that metabolize glucose, pentoses and related compounds via 6-PG/PK pathway and produce lactic acid, CO_2 and ethanol (or acetic acid) (Hammes and Vogel, 1995). Over the past 20 years, the taxonomic analysis of *Lactobacillus* genus, based on the comparative analysis of 16S rRNA gene sequence, was characterized by a complex evolutionary history. Despite the taxonomy studies, conducted by Dellaglio and Felis (2005), Felis and Dellaglio (2007) and Salvetti et al. (2012), have make several changes in the phylogenetic structure of the *Lactobacillus* genus, recently further revision was carried out by Pot et al. (2014). To data, 17 phylogenetic groups can be discriminated, which are reported below: *Lb. delbrueckii*, *Lb. salivarius*, *Lb. reuteri*, *Lb. rossiae* and *Lb. siliginis* branch, *Lb. vaccinostercus, Lb. casei, Lb. sakei, Lb. alimentarius, Lb. plant, Lb. brevis, Lb. collinoides, Lb. kunkeci* and *Lb. ozensis, Lb. fructivorans, Lb. buchneri, Lb. coryniformis, Lb. composti* and *Lb. floricola cluster,* and *Lb. perolens.* Many species of this genus are widely used as starter or protective cultures in food fermentations and several strains, often of human origin, are actively used as probiotics. Furthermore, lactobacilli are under development as delivery systems in vaccines and therapeutics field.

1.2.1 Lactobacillus johnsonii *and* Lactobacillus gasseri *species: taxonomic, genomic and physiological aspects*

Lb. johnsonii and *Lb. gasseri* are Gram-positive, non-sporulating, rod-shaped,with a low G-C content (34.6%) in their DNA. Based on the results of DNA-DNA hybridizations, *Lb. johnsonii* and *Lb. gasseri* species belonging to *Lb. acidophilus* group that was recently embedded in *L. delbrueckii* group (Pot et al., 2014). These species are dominant bacteria in human gut and in vaginal microbiota and have received particular attention due to their reported probiotic activity (Pridmore et al., 2003). Likewise at other species of *Lb. acidophilus* group, *Lb. johnsonii* an *Lb. gasseri* are

defined as closely related bacteria (Singh et al., 2009). It is difficult to differentiate unambiguously these two species and several studies have explored a polyphasic strategy, based on one or more molecular techniques, necessary for accurate species identification (Pot et al., 1993; Plessis ad Dicks, 1995; Ventura and Zink, 2002; Berger et al., 2007; Singh et al., 2009). At time, 4 finished genomes and 3 in draft status of *Lb. johnsonii*, and 2 finished genomes and 12 in draft status of *Lb. gasseri* are available in IMG/M (https://img.jgi.doe.gov) database. Whole-genome sequencing and comparative genomic analysis of *Lb. johnsonii* NCC533 and *Lb. gasseri* ATCC 33323 strains, revealed a genomes similarity highly significant ($E \leq 1e\text{-}100$) between these species and a sequence identity of many houskeeping genes higher than 94%. However, several unique strains-specific genes were found (Pridmore et al., 2003; Berger et al., 2007). Moreover, these studies have revealed a high number of genome encoding traits that are not widely distributed among other *Lactobacillus* species but they could explain the successful adaptation of these bacteria to the gastrointestinal tract (GIT) (Peril et al., 2008). Both species share several metabolic capabilities. *Lb. johnsonii* and *Lb. gasseri* are described as strict anaerobes with homofermentative energy metabolism, so they are able to ferment hexose to acid lactic via EMP pathway. They have a partial citrate cycle (TCA) with fumarate reductase (*fccA*) and fumarate hydratase (*fumH*) enzymes, nevertheless, unlike *L. gasseri*, *Lb. johnsonii* lack of malate hydrogenase (*mdh*) enzyme. *Lb. gasseri* genome encodes for 21 putative phosphoenoltransferase system (PTS), compared to 16 PTS found in *L. johnsonii* genome (Pridmore et al., 2003; Peril et al., 2008), however, the *Lb. gasseri* carbohydrate utilization pattern resembles that of *Lb. johnsonii*. They are able to ferment glucose, fructose, cellobiose, trehalose, sucrose, mannose and *N*-acetyl glucosamine. Unlike *Lb. johnsonii*, *Lb. gasseri* lack of lactose permease, so its lactose uptake is only mediated by PTS transporters, which in turn are absent in *Lb. johnsonii.* Moreover, *Lb. gasseri* lack of *Lb. johnsonii*-specific metabolic cluster (LJ0635) predicted to encode a maltose 6-phosphate glucosidase, a maltose IIBC PTS, and a RpiR-type phosphosugar-responsive regulator (Pridmore et al., 2003). Regarding amino acids metabolism, both species are able to synthesize aspartate from oxalacetate and convert L-aspartate into L-asparagine by asparagine synthase. However, *Lb. johnsonii* and *Lb. gasseri* remain incapable to synthesize most amino acids, as well as, purine nucleotides and cofactors, therefore, they typically reside in the upper GI tract where it can obtain nutrients from the host.

1.2.1.1 Probiotic feature of Lb. johnsonii *and* Lb. gasseri *strains*

Lb. johnsonii and *Lb. gasseri* have been extensively studied for their probiotic properties. Recently, they have been object of various research investigations, many of which show the potential of *Lb. johnsonii* and *Lb. gasseri* as adjuvant therapy in various GIT human diseases. *Lb. johnsonii* NCC

533, formerly known as *Lb. johnsonii* LA1 (Nestlé culture collection), is a well documented probiotic strain and its DNA sequence have been fully demonstrated (Pridmore at al., 2003).

Lb. johnsonii LA1 has been shown to give many beneficial effects for the host, including stimulation of gut immune response (immunomodulation) and intestinal homeostasis, pathogen inhibition and improvement of *Helicobacter pylori* eradication regimens. The strain has been shown to strongly adhere to the intestinal epithelial monolayer (Ventura et al., 2002; Prindmore et al., 2003). It has been demonstrated that in *Lb. johnsonii*, lipoteichoic acids (LTA), the elongation factor Tu and the heat shock proteins (GroEL) can act as adhesin-like factors (Granato et al., 1999). These bacterial surface structures are able to bind the human epithelial lines and induce the secretion of different cytokines, involved in the immunomostimolatory mechanisms (Marteu et al., 1997; Granato et al., 2004; Bergonzelli et al., 2006). Moreover, in human studies, feeding of fermented milk containing *L. johnsonii* LA1 has been demonstrated to reinforce human leucocyte phagocytic activity, improve IgA immunoglobulin level in the serum and re-establish the homeostasis of the human faecal microbiota (Link-Amster, 1994; Schiffrin et al., 1995; Yamamoto et al., 2006; Garrido et al., 2005; Fukushima et al., 2007). An important mechanism that may explain the health-promoting effects of probiotic bacteria is their ability to modulate the intestinal microbiota and to maintain an equilibrium between the intestinal population of beneficial and potentially harmful bacteria. Disruption of the intestinal microbiota homeostasis is found in several GIT disorders such as the inflammatory bowel diseases (IBD), allergies and rheumatoid arthritis. It is well known that *Lb. johnsonii* LA1 plays an important role in inhibition of the gut colonization by pathogenic bacteria. This strains exert a competitive exclusion mechanism through (*i*) the production of antimicrobial substances and (*ii*) the competition for nutrients or specific receptor site, on epithelial cell surface, to prevent the pathogen adherence. *In vivo* and *in vitro* studies demonstrated the inhibitory effect of LA1 against several pathogens found in human diarrhea, including *E. coli* (EPEC), *E. coli* (ETEC) and *Salmonella typhimurium strains* (Bernet et al 1994; Livrelli et al., 1996; Camard et al., 1997; Neseer et al., 2000). Pridmore et al. (2008) and Massaudi et al. (2005) observed that the ability *Lb. johnsonii* to produce H_2O_2 and acetic acid could support this killing activity. Studies concerning intestinal cell lines, animal models and human voluntaries have shown that *Lb. johnsonii* LA1 exert an antimicrobial activity also against *Helicobacter pylori*. *In vitro* studies, LA1 exerts bacteriostatic or bactericidal activities against this pathogen and decrease its adhesion to cultured cells (Bernet et al., 1997). In animal model studies, the administration of LA1 could potentially affect the humoral immune response. In particular, it was observed a distinct attenuation in the neutrophilic polymorphonuclear inflammatory infiltration of the laminae propriae of the LA1-administered mice, as well as, a significant reduction of the

immunoglobulin IgG level in the LA1-treated mice, compared to untreated (Sgouras et al., 2005). In humans, a significant decrease in IgG antibody titer has been suggested as an indicator for successful eradication of *H. pylori* (Bergey et al., 2003). Finally, in clinical studies were also demonstrated that the regular ingestion of a dietary product containing LA1 may interfere with *H. pylori* colonization. In double-blind, placebo-controlled clinical trials, the administration of acidified milk containing LA1 strain modulated the *H. pylori* infection, decreasing the severity of gastritis in *H. pylori*–positive peoples during treatment (Cruchet et al., 2003; Pantoflickova et al., 2007; Gotteland et al., 2008). Similarly to *Lb. johnsonii*, different strains of *Lb. gasseri* are widely studied for their probiotic activity. To date, no dairy products contain *Lb. gasseri* probiotic strains, however, there are several pharmaceutical formulations that contain them. Several studies have explored the probiotic features of *Lb. gasseri* strains, including immunostimulation, pathogen inhibition, ability to re-establish intestinal homeostasis and prevention and treatment of hipercholesterolemia, and abdominal adiposity in rats and in humans (Martin et al 2005). Recently, Luongo et al. (2013) observed that *L. gasseri* OLLL2809 strain induced dendritic cells (DCs) to produce high levels of interleukines IL-10, IL-6, IL-12 and TNF-α, that play a kay role in IgA switching mechanism. Moreover, Sakai et al. (2014) demonstrated the increased IgA production in the mouse small intestine after oral administration with *Lb. gasseri* LG2055. The same authors elucidated also the detailed molecular mechanisms for the IgA production by lymphocyte B cells. They suggested that LG2055 is able to activate both (DCs) and B cells to induce the IgA production, and toll-like receptor 2 (TLR2) signal is critical for this production. Moreover, the authors showed that transforming growth factor beta (TGF-b), produced by LG2055-stimulated cell lines, induces the production of different interleukines (IL-6, IL-10, IL12) involved in the IgA pruduction. In clinical study, the consumption of dietary product containing *L. gasseri* CECT 5714 resulted in an increase of phagocytic cells, including monocytes and neutrophils, and in a significant increase of the total IgA concentration in serum (Olivares et al., 2006). Adhesion, aggregation, and co-aggregation are phenotypic traits that potentially provide a microbial colonization advantage of *Lb. gasseri* against pathogenic bacteria. Several studies, in fact, have demonstrated the ability of adhesion and aggregation of the potentially probiotic *L. gasseri* and a significant antagonism activity against *Clostridium difficile*, *Clostridium sakazakii* and *Staphylococcus aureus* (Otero and Macias, 2006; Ferreira et al., 2011). Moreover, Kawai et al. (1998) discovered a *Lb. gasseri* strain able to produce a bacteriocin, Gassericin A. This antimicrobial compound is active against several food-borne pathogenic bacteria, including *Listeria monocytogenes*, *Bacillus cereus*, and *S. aureus* (Kawai et al., 2001). An excess of visceral fat accumulation is considered a crucial factor in the development of a series of metabolic disorders. Recent studies have shown the involvement of *Lb.*

gasseri in body weight and adipose tissue level control (Kadooka et al., 2010; Kang et al 2010; Kung et al., 2013). Ogawa et al. (2015) demonstrated that mice fed with *Lb. gasseri* LG2055 showed a lower lymphatic lipid absorption and increased faecal fat excretion, compared to control group. Suppression of lipid absorption in the mice small intestine has been proposed as a potential mechanism for the anti-obesity effects of LG2055. Moreover, in clinic trial the same authors shown that the consumption of LG2055 decreased the BMI, and the serum triacylglycerol and cholesterol concentrations in peripheral blood (Ogawa et al., 2014).

1.3 Activation and regulation of aerobic metabolism in lactic acid bacteria.

Lactic acid bacteria (LAB) are generally recognized as anaerobic aerotolerant, that get their energy mainly through substrate level phosphorylation. Two major pathways for the hexoses metabolism are known: (*i*) Embden-Meyerhof-Parnas (EMP), in which lactic acid is the major fermentation end-product (homofermentative metabolism), and (*ii*) the phosphoketolase pathway, in which acetic acid, propionic acid, CO_2, ethanol are formed in addition to lactic acid (heterofermentative metabolism) (Kleerebezem & Hugenholtzy, 2003). LAB do not need oxygen for growth but they can grow under aerobic conditions and the pyruvate produced from glycolityc or phosphoketolase pathway may be aerobically metabolized into acetyl-phosphate and acetate (Petersen et al., 2012). It has been noted that, the presence of oxygen can have a remarkable effect on growth, metabolism and cell viability. Several LAB are able to consume oxygen through the action of flavoprotein oxidases, including NADH oxidase (NOX), pyruvate oxidase (POX), L-alpha-glycerophosphate oxidase, L-amino acid oxidase and lactate oxidase (LOX) that contributed to oxygen tollerance (Guidone et al., 2013). In particularly, in presence of oxygen, pyruvate can be catabolized in acetate via pyruvate oxidase-acetate kinase (POX-ACK) pathway with additional ATP generation. This pathway involved three enzymatic steps: (*a*) oxidation of lactate to pyruvate by the NAD-dependent lactate dehydrogenase (nLDH), (*b*) oxidative decarboxylation of pyruvate to acetyl-phosphate (acetyl-P) by POX (with production of CO_2 and H_2O_2) and (*c*) de-phosphorylation of acetylphosphate to acetate by acetate kinase (ACK) activity (Goffin et al., 2004). This metabolism can have a beneficial effect on cell viability for two reasons: (*i*) the accumulation of acetate instead of lactate could ensure the pH homeostasis and therefore increase cell survival during the stationary phase growth, (*ii*) the generated extra ATP can allow an additional cell metabolism after glucose exhaustion and an increased biomass production compared to anaerobic growth conditions (Goffin et al., 2004; Quatravaux et al., 2006). Previous studies have been demonstrated that the transcriptional regulators are directly involved in molecular mechanisms of oxygen regulation in

LAB. It was noted that the POX-ACK pathway is induced by oxygen presence and the expression of *pox* gene is controlled by carbon catabolite repression. In fact, several authors have been studied the role of CcpA protein (Catabolite control protein A) in the control of metabolism of different LAB, including *Lb. casei* (Gosalbes et al., 1999), *Lb. pentosus* (Mahr er al., 2000), *Lactococcus lactis* (Gaudu et al., 2003, Lopez de Felipe & Gaudu 2009) and *Lb plantarum* (Muscariello et al., 2001, Lorquet et al., 2004, Castaldo et al., 2006, Zotta et al., 2012). In particular, in *Lb. plantarum* *pox* activity (the key enzyme in oxygen metabolism) is induced by oxygen and hydrogen peroxide and strongly repressed in presence of glucose excess by CcpA action at the transcriptional level (Goffin et al., 2006; Muscariello et al., 2008). Lorquet et al. (2004) demonstrated that *pox* transcription was repressed by binding of CcpA protein with the *cre* sequence, located in the *pox* promoter, end the repression is relieved when glucose concentration becomes limiting for growth (at the end of the exponential phase and during the stationary phase growth).

1.4 Respiration metabolism in lactic acid bacteria

Although named and used in food fermentation for their main attribute, that is lactic acid production, several LAB have the potential to activate an aerobic respiratory pathway when exogenous heme or heme and menaquinones (vitamin K2) were provided (Petersen et al., 2012). In LAB, heme-induced respiration metabolism was firstly studied in *Leuconoctoc mesenteroides* specie (Bryan-Jones & Whittenbury, 1969; Antonie Van Leeuwenhoek, 1970), however, it remained little investigated until about fifteen years ago when aerobic respiration metabolism and its positive physiological impact has extensively studied in *Lactococcus lactis* (Blank et al., 2001; Duwat et al., 2001; Gaudu et al., 2002). To date, respiration metabolism has also been investigated in some strains of heterofermentative *Lb. reuteri* and *Lb. spicheri* (Ianniello er al., 2015), in *Lactobacillus casei* (Zotta et al., 2014a; Ianniello et al., 2015; Ricciardi et al., 2015) and *Lb. plantarum* groups (Brooijmans et al., 2009, Guidone et al., 2011; Watanabe et al., 2012; Zotta et al 2012; Zotta et al., 2013; Zotta et al., 2014b). These studies have revealed that the shift from fermentative towards respiratory metabolism increased biomass production, long-term survival in the storage and resistance to oxygen and acid induced stress in bacterial cells. Therefore, these findings opened new perspectives on LAB lifestyle. The respiration is the oxidative breakdown of organic molecules through the activation of a minimal electron transport chain (ETC) that generates a proton motive force (PMF) and produces energy (ATP) by F_0F_1-ATPase activity. In respiration-competent LAB, the ETC required three main membrane components: (*i*) an electron donator (NADH dehydrogense), (*ii*) an electron shuttle (usually menaquinones) that drive electrons from the

dehydrogenase to a terminal acceptor enzyme, and (*iii*) a heme-requiring final electron acceptor (Cytochrome oxidase) that allows the reduction of oxygen to water (Lechardeur et al., 2011). Despite the different type of cytochromes (*bo*-type cytochrome with a b-hemes, an o-heme and copper as cofactor), cytochrome *bd*-tipe oxidase (with two b-hemes and a single d-heme) is the only terminal oxidase used in ETC of some LAB (Minghetti et al., 1992; Borisov et al., 2011; Petersen et al., 2012). In particularly, cytochrome *bd* oxydase contains two-subunit integral membrane protein: (*i*) cytochrome *bd*-I ubiquinol oxidase subunit I (CydA) and (*ii*) cytochrome bd-I ubiquinol oxidase subunit II (CydB), and two ATP-binding cassette transporters (assembly subunits), called CydC and CydD (Borisov et al., 2011). Unlike cytochrome *bd* oxidase (encoded by *cydABCD* operon) that is synthesized by most of LAB, these bacteria lack enzymes for complete heme biosynthesis pathway and only some strains of *Lactococcus lactis*, *Enterococcus fecalis* and *Leuconostoc mesenteroides, Leuconostoc citreum* have the complete (mena)quinones biosynthesis pathway (encoded by *menFDHBEC* gene) (Lechardeur et al., 2011). Therefore, in LAB, the respiratory metabolism may occur only when heme or heme and menaquinone are supplied. On the basis of this consideration, Pedersen et al. (2012) classified LAB into three categories: (*i*) strains non able to activate respiration metabolism, (*ii*) respiration-competent in presence of heme, and (*iii*) respiration-competent in presence of heme and menaquinones. Even if do not synthesize heme, respiration competent LAB can able to assimilate heme and use it directly as a cofactor to activate aerobic respiration. Studies in *L. lactis* have explored a potential mechanism for heme uptake and homeostasis. Legardeur et al. (2010) demonstrated that a chaperone protein, named AhpC, may be involved both in insertion of heme in citochrome oxidase membranes and in protection of intracellular heme pool from degradation or efflux mechanisms. Moreover, the same authors revealed the key role of *hrtRBA* operon in heme homeostasis in order to avoid toxicity in *L. lactis*. In fact, deletion of *hrtRBA* operon results in a heme-sensitive phenotype with high production of reactive oxygen species (ROS). Therefore, respiration may be considered as a highly regulated process and it only occurs when exogenous heme are provided. Finally, unlike respiratory bacteria (i.e. *E. coli* and *B. subtilis*) that use the Krebs cycle to produce NADH, LAB have needed of glycolytic activity to generate NADH, required for NADH dehydrogenase activity in ETC. For this reason, Petersen et al. (2012), suggested that fermentation metabolism is likely required prior to, or during, respiration metabolism. As previously described, respiration metabolism have been widely investigated in *Lc. lactis, L. plantarum* and *L. casei* group, suggesting that respiration cultivation provided several advantages on bacterial robustness. Two major physiological consequences on LAB behaviour have been attributed to respiration metabolism:

(i) **Increased biomass production:** the respiration process is energetically more efficient than fermentation. The aerobic respiration process can involve glycolysis, citric acid cycle and ETC and produce up to 36 mol of ATP, compared to 2 ATP in fermentation alone.

(ii) **Increased survival to oxidative and acid stresses:** in some LAB, condition which promote aerobic and respiratory growth decrease ROS accumulation and oxidative damage compared to fermentation. Activation of respiration metabolism, through heme supplementation, may promote the synthesis of catalase antioxidant enzyme and cytochrome *bd* oxidase. Catalase can degrade H_2O_2 and protect the cell from oxidative damage, while CydAB can remove intracellular oxygen converting it in water. Moreover, respiratory cultivation led to the production of superoxide dismutase (SOD) in *Lc. lactis* (Duwat et al., 2001) and catalase, NADH oxidase (NOX) and NADH peroxidase (NPR) in *L. plantarum* (Guidone et al., 2013; Watanabe et al., 2012; Zotta et al., 2013).
The activation of respiration metabolism can generate less acid stress due to a change in metabolites pools production. In particularly, NADH is used by ETC and NOX/NPR activity instead by lactate dehydrogenase for lactic acid production, while, the pyruvate is converted into acetate, acetoin and diacetyl (Duwat et al., 2001; Pedersen et al., 2008). Thus, the accumulation of acetate instead of lactate could have a beneficial effect for the bacterial cells ensuring the pH homeostasis and long term survival during storage phase.

1.5 Oxidative stress response in lactic acid bacteria

The presence of oxygen in the growth environment of LAB is considered one of the main factors affecting cell survival, due to the induction of lethal oxidative damages (Ruiz et al., 2011) caused by the action of toxic by-products reactive oxygen species (ROS), including the superoxide anion radical (O_2^-), hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2). Moreover, ROS can further react with some cations (Fe^{2+} and Cu^{2+}) leading to highly reactive oxidants via Fenton reaction (De Angelis & Gobetti 2004; Kang et al., 2013). LAB are classified as anaerobes oxygen tolerant, therefore, lack effective oxygen scavenging enzymes and cellular repair mechanisms. Hence, the accumulation of toxic oxygen metabolites can cause protein damage, DNA mutations, oxidation of phospholipidic membrane, modification in low-density lipoproteins, as well as, the production of undesirable compounds in fermented food (Amaretti et al., 2013). However, most LAB can grow under aerobic conditions and their simplest way to utilize oxygen is through the action of flavoprotein oxidases (NOX, POX, LOX, α-glicerophosphate oxidase and L-amino acid oxidase) to oxidize substrates such as pyruvate or NADH (Sonomoto et al., 2011). Although these enzymes can eliminate oxygen from the environment, their activity results in the accumulation of H_2O_2 (> 1mM),

the primary cause of oxidative stress. On the other hand, under respiratory condition ROS can be generated due to leakage of electrons from intermediates of ETC (Watanabe et al., 2011). Hence, H_2O_2 and ROS scavenge ability is an important factor which contributes to aerotolerance of the strains. In order to detoxify ROS, LAB employ different mechanisms to protect against oxidative stress. The main enzymes involved in oxidative stress resistance in LAB are describe below.

1.5.1 Catalase

Catalase is a common antioxidant enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the degradation of hydrogen peroxide to water and molecular oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). LAB are generally considered as catalase-negative microorganisms (Mayo et al., 2008), however, a significant number of catalase sequences have been discovered in several LAB genome. Three classes of catalases are found: (*a*) monofunctional catalases, (*b*) manganese catalases (pseudocatalases) and (*c*) catalase-peroxidases (closely related by sequence and structure to plant peroxidases) (Frankenberg et al., 2002). Both monofunctional catalase and catalase-peroxidases (also named bifunctional catalase) have heme as prosthetic group, but they have significant differences in active site and tertiary and quaternary structures (Chelikani et al., 2004; Vlasits et al., 2007). Despite catalyzes the same reaction, catalases and catalase-peroxidases follow a different reaction mechanism. In a monofunctional catalase cycle, a first stage involves oxidation of the heme iron, using H_2O_2 as substrate, to form compound I (Reaction 1). In a second stage, peroxide molecule is used as a reducing agent for compound I regenerating the native enzyme and releasing molecular oxygen (Reaction 2). Catalase peroxidase exhibit a peroxidase activity similar to conventional peroxidases. Thus, a peroxidase cycle includes three reactions, (*i*) compound I formation (Reaction 1), compound I reduction to compound II, where electron donors (AH_2) are oxidized via one-electron transfers releasing radicals ($^{\bullet}AH$) (Reaction 3) and compound II reduction back to ferric peroxidase in a second one-electron reduction (Reaction 4) (Jakopitsch et al., 2003).

Reaction 1 $\quad Por\ Fe(III) + H_2O_2 \rightarrow {}^{\bullet+}Por\ Fe(IV)=O + H_2O$

Reaction 2 $\quad {}^{\bullet+}Por\ Fe(IV)=O + H_2O_2 \rightarrow Por\ Fe(III) + H_2O + O_2$

Reaction 3 $\quad {}^{\bullet+}Por\ Fe(IV)=O + AH2 \rightarrow Por\ Fe(IV)=O\ {}^{+}{}^{\bullet}AH$

Reaction 4 $\quad Por\ Fe(IV)=O + AH2 \rightarrow Por\ Fe(III) + H_2O + {}^{\bullet}AH$

The manganese catalases are not as widespread as the heme-containing catalases and so far have been identified only in bacteria. The crystal structures of the manganese catalases, one from *Thermus thermophilus* and the second from *Lb. plantarum*, reveal a homo-hexamer structure where each subunit contains a dimanganese group as catalytic center (Chelikani et al., 2004). Like heme-catalases, manganese catalase reaction occurs in two steps: (*i)* an oxidative reaction in which H_2O_2 is oxidized to dioxygen, followed by reduction of the manganese cluster, and (*ii*) a reductive reaction in which the reduced metallocluster delivers the electrons and protons to a second molecule of substrate, resulting in O-O bond cleavage and formation of two molecules of H_2O (Figure x) (Whittaker et al., 2012). Genome analysis in LAB indicated that, the gene *kat*A, encoding for monofunctional catalase, was found in several genomes of *Lb. sakei, Lb. plantarum, Lb. casei,* and *E. faecalis* strains (Knauf et al.; 1992; Frankenberg et al., 2002; Abriouel et al., 2004; Zotta et al., 2016), while, the gene Mn-*kat*, encoding for manganese catalase, is little distributed, and only encountered in a few *Pediococcus*, *Enterococcus* and *Lactobacillus* strains (Yamamoto et al., 2011). To date, the *Kat*G, encoding for catalase-peroxidase, was found only in a few strains of *Enterococcus fecalis* (https://www.ncbi.nlm.nih.gov/). Species belonging to *Lactococcus*, *Leuconostoc* and *Oenocuccus* do not have genes encoding for heme- manganese catalase and catalase peroxidase.

1.5.2 Superoxide dismutase

Superoxide dismutases (SODs) are metalloenzymes that catalyze the the dismutation reaction of the superoxide ($O2^{-}$) radical into molecular oxygen (O_2) or (H_2O_2). On the basis of metal cofactor present in their redox-active center, four types of SODs have been characterized: (*i*) manganese (Mn-SOD), (*ii*) iron (Fe-SOD), (iii) copper/zinc (Cu/Zu-SOD) and nickel (Ni-SOD) superoxide dismutases (Wuerges et al., 2004). However, only Mn-SODs have been found in several LAB, including streptococci, lactococci, enterococci and in some lactobacilli species (i.e. *Lb. sakei*, *Lb. sanfranciscensis*) (Yamamoto et al., 2011).

1.5.3 NADH oxidase-NADH peroxidase system

NADH oxidase and NADH peroxidase are supposed to be the major enzymes involved in the proposed NAD(P)-dependent H_2O_2 scavenging pathway in LAB. In a common oxidative resistance mechanism, intracellular oxygen is first used to oxidize NADH in NAD^{+} by NADH oxidase, generating H_2O_2. Afterwards, H_2O_2 is reduced to H_2O by NADH peroxidase. In particularly, two type of NADH oxidase have been found, (*i*) a NADH- H_2O_2 oxidase that reduce O_2 to form H_2O_2,

and (*ii*) a NADH- H_2O oxidase that promote the oxidation of O_2 directly in H_2O (Talkawar & Kailasapathy, 2004).

1.6 Impact of aerobic and respiratory metabolism in food technology

The biochemistry of aerobic and respiratory metabolism in LAB suggest a series of complex mechanisms that act in unison to confer several advances on cell physiology. As described above, the lifestyle of the aerobic and respirative LAB results in a greater biomass-yield, growth-efficiency, stress robustness (oxygen and acid) an long term survival compared to that under fermentation conditions. All these respiration-associated traits have a considerable industrial significance. The application of aerobic and respiratory metabolism in LAB can have a wide implications in three main field in LAB research: (*i*) starter and probiotic cultures production, (*ii*) development of food products with respirative phenotype, and (*iii*) human health application. These applications are discussed below and summarize in Table X. The respiration technology was firstly investigated in the 1990s and was implement by Chr. Hansen A/S industry and INRA (Institut National De La Recherche Agronomique), that entered into a patent license to explore the respiration ability of *Lactococcus Lactis* for the development and production of a new starter culture in presence of aeration and a porphyrin compound (Petersen et al., 2005). To date, this technology has been also used for R-604 starter culture production (Chr. Hansen A/S) with supplementation of precursors for the synthesis of nucleotides (Kringelum et al., 2008). However, despite aerobic and respiratory metabolism has improved growth and technological properties in several *Lactobacillus* strains, no patent is currently developed for the use of respirative lactobacilli as starter or probiotic cultures. Another interesting observation of the bacterial growth under respiration conditions is the ability to produce functional metabolites and aroma compounds.

1.7 Aim and outline of this thesis

Several authors have demonstrated that the presence of oxygen (aerobic growth) and heme and menaquinone (production of cytochromes and activation of an electron transport chain during respiratory growth) induces in some LAB species the expression of phenotypes with improved technological features. In recent years, several phenotypic and genotypic studies have been carried out to explore the aerobic and respiratory metabolism within the *Lactobacillus* genus. Despite the significant progress made in the knowledge on cellular mechanisms underlie the aerobic and/or respiratory nature of lactobacilli, a very limited data are actually available in *Lb. johnsonii* and *Lb.*

gasseri species, although, several strains of this species have been extensively studied for their technological and probiotic properties. Nowadays, only *Lb. johnsonii* NCC533 probiotic strain has been studied to provide a more global understanding of the molecular responses to the presence of oxygen. Therefore, no study on the respiratory growth was carried out in *Lb. johnsonii* and *Lb. gasseri* species. In view of the above considerations, the aim of this PhD thesis was to study and explore the adaptive response of *Lb. johnsonii/gasseri* strains to switch from fermentative to aerobic and respiratory metabolism, by phenotypic and genotypic approach. In particular, the effect of aerobic (presence of oxygen) and respiratory (presence of oxygen, heme end menaquinone) cultivation on the growth, oxidative and starvation stress tolerance, antioxidant activity and metabolic profile were investigated. Moreover, the study of the genetic basis underlie the metabolic changes occur during aerobic and respiratory growth was carried out.

The first experimental chapter of this thesis is focused on (*i*) the isolation and molecular characterization of strains belonging to *Lb. johnsonii/gasseri* species, and (*ii*) the assessment of anaerobic, aerobic and respiratory growth, tolerance towards oxidative stress and probiotic features (resistance to simulated oral gastrointestinal condition and antimicrobial activity) **(chapter II)**. Afterwards, to better understanding the genetic basis that can explain the phenotypic evidences observed such as, possible aerobic and respiratory metabolism activation and oxidative stress tolerance, whole-genome sequencing of 2 selected strains was carried out. All result were reported in **chapter III**. Finally, the adaptation to aerobic end respiratory metabolism of 2 selected strains was further investigated in batch cultivation experiments. All results concerning the effect of aerobic and respiratory cultivation on growth kinetics, glucose and oxygen consumption, metabolites production, as well as, tolerance to starvation stress and antioxidant activity were reported in **chapter IV.**

The availability of these phenotypic and genotypic data may be useful to understanding the mechanisms related to the aerobic and respiratory metabolism in *Lb. johnsonii/gasseri* species and to exploit them for further scientific and applicative studies. Moreover, the addition of our findings to the currently available data set can extend the scientific knowledge on the aerobic lifestyle and oxygen tolerance in *Lactobacillus* genus.

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CHAPTER II

Aerobic environmental adaptation of promising probiotic strains belonging to *Lactobacillus johnsonii/gasseri* species

2.1 Abstract

Oxygen is considered one of the main factors affecting probiotic bacteria survival due to the induction of lethal oxidative damages caused by the action of toxic by-products (reactive oxygen species). It has been shown that oxidative stress resistance in lactic acid bacteria is strongly dependent on the type of cell metabolism. The shift from fermentative to respiration metabolism (through the addition of heme and menaquinone and in presence of oxygen) was associated with increase in biomass, long term survival and production of antioxidant enzymes. The aim of this work was to investigate the effect of aerobic (presence of oxygen) and respiratory (presence of oxygen, heme end menaquinone) cultivation on the growth kinetic, catalase production and consequent oxygen uptake and oxidative stress response of *Lactobacillus johnsonii* and *Lactobacillus gasseri* strains isolated from breast-fed babies stools. Moreover, their probiotic features were assessed. For many strains, the aerobic and respiratory growth increased pH and biomass production compared to anaerobic cultivation. However, only seven of them showed to consume oxygen under aerobic and respiratory conditions, allowing the selection of oxygen-tolerant or respiratory strains. Surprisingly, one strain showed a catalase activity in all growth conditions, while another one showed this activity only in respiratory condition. Nevertheless, for both strains only respiratory condition improved their tolerance to oxidative compounds (hydrogen peroxide and reactive oxygen species generators) and they showed also proper probiotic features, in term of antimicrobial activity and ability to survive under oral-gastrointestinal simulated environment. The exploration of respiratory competent phenotypes with probiotic features may be extremely useful for the development of competitive starter or probiotic cultures.

2.2 INTRODUCTION

The greatest challenge of the probiotic bacteria is enduring stresses encountered during food processing and gastrointestinal transit (Mils et al., 2001). Probiotic performances and robustness can be compromised by exposure to various environmental stresses, including acid, cold, drying, starvation, oxidative and osmotic stresses, that may affect the physiological status and the functional properties of bacterial cells (Zhang et al., 2013). Survival to harsh conditions is an essential prerequisite for probiotic bacteria before reaching the target site where they can exert their health promoting effects. Several probiotics, in fact, have shown a poor resistance to technological processes, limiting their use to a restricted number of food products. The presence of oxygen is considered one of the main factors affecting the survival of probiotic lactic acid bacteria (LAB), anaerobic aerotolerant microorganism, which lack the capability to synthesize an active electron transport chain. The aerobic environment in LAB may induce the production of toxic oxygen by-products (reactive oxygen species, ROS, such as superoxide anion radical, hydroxyl radical and hydrogen peroxide) that may damage DNA, proteins and lipids, resulting in cellular death (Amaretti et al., 2013). Moreover, hydrogen peroxide can further react with some cations (Fe^{2+} and Cu^{2+}) leading to highly reactive oxidants via Fenton reaction (De Angelis and Gobetti, 2004; Kang et al., 2013). LAB get their energy mainly through substrate level phosphorylation, and lack both heme containing enzymes and active cytochrome oxidases, which are essential components for oxygen-linked energy metabolism (Kang et al., 2013). However, most LAB can grow under aerobic conditions and their simplest way to utilize oxygen is through the action of flavoprotein oxidases (NADH oxidase, NOX; pyruvate oxidase, POX; lactate oxidase, LOX; α-glicerophosphate oxidase and L-amino acid oxidase) that use substrates such as pyruvate or NADH (Yamamoto et al., 2011). The activity of these enzymes, however, may result in the accumulation of toxic H_2O_2. LAB are able to overcome the oxidative damage by producing several ROS-degrading enzymes (catalase, superoxide dismutase, flavin-dependent oxidase and peroxidases) and different redox and repair systems (glutathione and thioredoxin systems) and antioxidant enzymes (catalase, pseudo-catalase, superoxide dismutase, and NADH-peroxidases) (Amaretti et al., 2013; Kullisaar et al., 2010; Rochat et al., 2006; Ruiz et al., 2011). Recently, several authors have demonstrated that oxidative stress resistance in some LAB species dependent on the type of metabolism and that the shift from fermentative towards respiratory metabolisms may increase growth, long-term survival and stress tolerance (Pedersen et al., 2012; Guidone et al., 2013; Ianniello et al., 2015). These effects are likely associated to the activation of the electron transport chain by growing cells in presence of oxygen, heme and menaquinone (vitamin K2). Heme is an essential cofactor in heme-dependent catalase and

cytochrome *bd* oxidase (CydAB) synthesis (Pedersen et al., 2008). Catalase can degrade H_2O_2 and protect the cell from oxidative damage, while CydAB can catalyse the rapid reduction of oxygen to water as well as to increase the ATP production. Instead, menaquinone may act as a central respiratory chain component delivering electrons from reducers (such as NADH dehydrogenase) to terminal oxidases (such as CydAB) (Pedersen et al., 2008). However, while respiration metabolism and oxidative stress response have been extensively studied in in *Lactococcus lactis* (Gaudu et al., 2002; Miyoshi et al., 2003; Rezaiki et al., 2004) and *Lactobacillus casei* (Zotta et al., 2014; Ianniello et al., 2015) and *Lb. plantarum* groups (Zotta et al., 2012; Watanabe et al., 2012; Guidone et al., 2013; Zotta et al., 2013), limited data are available for the strains of *Lb. johnsonii* and *Lb. gasseri*. These species are genetically correlated and belonging to *L. delbrueckii* group (Sun et al., 2014) *Lb. johnsonii* and *Lb. gasseri* are reported as the dominant bacteria in human gut and in vaginal microbiota and are described as strict anaerobes with fermentative energy metabolism (Pridmore et al., 2003). Current data on oxygen tolerance and oxidative stress response in *Lb. johnsonii* are limited to the probiotic strain NCC 533 (Hertzberger et al., 2013; Hertzberger et al., 2014). These authors have observed that the endogenous production of H_2O_2 is the main cause of oxidative stress in *L. johnsonii* NCC 533 during its aerobic growth, even though the presence of oxygen relieves its carbon dioxide (CO_2) and acetate dependence, compared to anaerobic growth. On the contrary, no data on the capability to activate a minimal respiratory chain are available for *L. johnsonii* and *L. gasseri* species. The aim of this work was to evaluate the effect of aerobic (presence of oxygen) and respiratory (presence of oxygen, heme end menaquinone) cultivation on the growth ability of *Lb. gasseri* and *Lb. johnsonii* strains. Tolerance of oxidative stress and other functional features (i.e. survival to simulated oral-gastrointestinal transit and antimicrobial activity) were also evaluated in order to select new promising probiotic strains.

2.3 MATERIALS AND METHODS

2.3.1 Samples, strains and culture conditions

Faecal samples (n. 6) were obtained from healthy breast-fed babies, which had not used antibiotics prior to the sampling date and had no recent history of gastrointestinal disorders. They were collected from babies of personal friends of the authors. These latter were informed on the purpose of the research and that no clinical aspect is involved. Indeed, authors received an oral informed consent by the parents of babies to use faecal samples exclusively as sources of lactobacilli. Donors reckoned that a written consent was not necessary. On the other hand samples were de-identified

prior the authors received them. Ethical approval was not requested because of any clinical aspect involved in this study. Samples were diluted in de Man Rogosa Sharpe (MRS, Oxoid) broth, pH 6.2, supplemented with 0.5 g/L of the reducing agent L-cysteine hydrochloride (Sigma) and incubated in anaerobic condition at 37°C for 48 h (Hartemink et al., 1997). At the end of the incubation the pre-cultures were inoculated (1% v/v) in MRS broth supplemented with 2 mg/ml of vancomycin hydrochloride (Sigma-Aldrich) (Hartemink et al., 1997), acidified at pH 4.5 and incubated in anaerobiosis at 37°C for 24 h. The pre-cultures were ten-fold diluted in quarter strength Ringer solution (Ringer, Oxoid), streaked on both MRS Agar and Rogosa Agar (Oxoid) plates and incubated in anaerobiosis at 37°C for 48 h. All isolates considered presumptively belonging to *Lactobacillus* genus were stored at -25°C in MRS broth (Oxoid) with 20% (v/v) glycerol. *Lb. johnsonii* DSM 10533^T, *Lb. johnsonii* DSM 20533, *Lb. gasseri* DSM 20243^T, *Lb. gasseri* DSM 20077 and *Lb. rhamnosus* ATCC 53103 (commercially known like GG strain) were used as reference strains. All lactobacilli were routinely propagated in Weissella Medium Broth (WMB) (Zotta et al., 2012) pH 6.8 or in MRS Agar and incubated in anaerobiosis at 37°C for 24 h. Nine strains belonging to potential pathogen and spoilage species (Table 1) were used for antimicrobial activity tests.

2.3.2 Molecular characterization of isolates

Genomic DNA was extracted using the Insta-Gene matrix (Bio-Rad, Milan, Italy) according to the manufacturer's protocol, with some modifications. Briefly, 2-3 colonies of each microorganism were suspended in 0.05 M phosphate buffer solution (PBS) pH 7.0 and centrifuged for 1 min at 10,000 *g*. Pellet was dissolved in 200 µl of InstaGene matrix and incubated at 56°C for 30 min (Thermomixer Comfort, Eppendorf). After vortexing for 10 seconds, sample was treated for 8 minutes at 100°C. Mixture was centrifuged at 10,000 *g* for 3 min and the resulting supernatant, containing the bacterial DNA, was used for PCR reaction. Quality and quantity of DNA was assessed using a NanoDrop spectrophotometer 1000 (Thermo Scientific, Milano, Italy). In order to avoid the presence of clones, the isolates were firstly analysed by rep-PCR using oligonucleotide GTG_5 (5′-GTG GTG GTG GTG GTG-3′) primer (Invitrogen, Life Technologies, Milan, Italy). The reaction was performed in 20 µl mixtures containing: 50 ng DNA template, 2.5 µl of 10X PCR Buffer (Invitrogen, Milano, Italy), 50 mM $MgCl_2$, 10 mM dNTPs mix, 10 µM primer and *Taq* Polymerase (Bio-RAD) 5 U/µl. PCR was carried out using an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C each, and by a final extension of 8 min at 72°C. PCR products were separated by electrophoresis (3 h at 130 V) on

1.7% (w/v) agarose gel stained with 0.1 µl/ml SYBR safe (Invitrogen) and visualized by UV transillumination. Rep-PCR profiles were analysed by BioNumerics 5.0 software (Applied Maths) using Pearson's correlation coefficient with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering of averaged profile similarities. Universal primers (Invitrogen) fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1990) were used to amplify the 16S rRNA gene of isolates. PCR reaction mixture (final volume 50 µl) contained 50 ng of DNA template, 5 µl of 10X buffer (200 mM Tris HCl pH 8.4, 500 mM KCl), 25 mM $MgCl_2$, 10 mM dNTPs mix, primers 50 pM and *Taq* Polymeras 5 U/µl. PCR amplification was performed using an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C and 1 min at 72°C each, and by a final extension of 5 min at 72°C. The PCR products were separated by on agarose gel 1.5% (w/v), containing 0.1 µl/l SYBR safe, purified using QIAquick PCR Pufication Kit (Qiagen, Milan, Italy), and sequenced by Primm srl (Milan, Italy). Research for DNA similarity was performed using the BLAST program of the National Centre of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) GenBank. Strains showing a % similarity higher than 98% with *Lactobacillus johnsonii/gasseri* were used for further analyses.

2.3.3 Preliminary evaluation of probiotic potential of isolates

The ability of *Lb. johnsonii/gasseri* strains to survive to simulated OGIT was performed according to Vizoso et al. (2006) with some modifications. Briefly, overnight cultures were recovered by centrifugation (6500 *g* for 10 min), washed twice with sterile saline (NaCl 0.85%) and suspended in equal volume of simulated saliva juice (SSJ: NaCl 5 g/l, KCl 2.2 g/l, $CaCl_2$ 0.22 g/l and $NaHCO_3$ 1.2 g/l, lysozyme 100 mg/l, pH 6.9) and incubated for 5 min at 37°C. The suspension was then centrifuged as above, re-suspended in equal volume of simulated gastric juice (SGJ: NaCl 5 g/l, KCl 2.2 g/l, $CaCl_2$ 0.22 g/l, $NaHCO_3$ 1.2 g/l, pepsin 3 g/l, pH 2.5) and incubated at 37°C for 120 min under gentle agitation (200 rpm) to simulate peristalsis. After centrifugation, pellet was re-suspended in equal volume of simulated pancreatic juice (SPJ: $NaHCO_3$ 6.4 g/l, KCl 0.239 g/l, NaCl 1.28 g/l, 0.5% bile salts and 0.1% pancreatin, pH 7.0) and incubated at the same condition of SGJ. Survival (%) was calculated after each treatment. *Lactobacillus rhamnosus* GG was used as positive control in this experiment.

Antimicrobial activity was assayed using an agar spot test and a well diffusion agar test, as previously described by Banerjee et al. (2013). In the first case, 10 µl of each overnight culture were spotted onto a MRS Agar plate. After incubation at 37 °C for 24 h, plate was overlaid with 10 ml of Tryptone Soya Broth (TSB, Oxoid, Milan, Italy) supplemented with 0.75% agar (TSB soft

agar) previously inoculated with the indicator strains (Table 1) to reach a final concentration of $1x10^6$ CFU/ml. After 24 h of incubation at optimal growth temperature of indicator strains the antimicrobial activity was detected by the presence of a clear growth inhibition zone around the colony of tested strain. In the well diffusion agar test, a cell free supernatant was recovered by centrifugation (6500 *g* for 10 min), adjusted at pH 6.5 with NaOH 1 M, heat-treated for 10 min at 80°C and sterilized by a low-binding protein 0.22 µm pore size filter. Fifty µl of supernant were placed into a 6 mm-diameter well done in a TSB soft agar plate previously inoculated with the indicator strain at a final concentration of $1x10^6$ CFU/ml. After incubation at optimal growth temperature of indicator strain, the antimicrobial activity was determined by measuring the diameter (cm) of the inhibition zone around the wells. In both agar spot test and a well diffusion agar test results were calculated as the mean of three experiments. The strains that showed antimicrobial activity in well diffusion agar test were further tested to investigate the nature of the antimicrobial substance produced. The sensitivity to different enzymes was tested using 10 mg/ml (final solution) of lipase, catalase, papain, trypsin, α-chymotrypsin, pronase E and pepsin in PBS. Ten µl of solution used in well diffusion assay were spotted onto TSB soft agar plates previously inoculated with the indicator strain. Afterwards, 8 µl of enzyme solution were deposited adjacent the spot of supernatant to inhibit the activity of antimicrobial substance.

2.3.4 Aerobic and respiratory growth and catalase production

The assessment of aerobic and respiratory growth as well as the presence of catalase activity were performed as reported by Zotta et al. (2014). Strains were cultivated for 24 h at 37°C in 24-well microplates in different growth conditions: anaerobic (AN, static cultivation in modified WMB with 10 g/L of glucose, pH 6.8, with AnaeroGen bags, Oxoid), aerobioc (AE, in modified WMB, shaken on a rotary shaker at 150 rpm), and respiratory (RS, AE growth in modified WMB, supplemented with 2.5 µg/ml of hemin and 1 µg/ml of menaquinone) cultivations. Micro-plates were inoculated (2% v/v) with standardized (OD_{650}= 1.0) overnight anaerobic pre-cultures and incubated for 24 h at 37 °C. At the end of incubation the optical density at 650 nm (OD) and pH values were measured. Catalase activity was qualitatively evaluated by re-suspending the washed biomass derived from 1 ml of culture in all different conditions (AN, AE and RS) in 100 µl of a 3% (v/v) H_2O_2 solution. Catalase production was revealed by an evident formation of bubbles in the cell suspension. Three independent replicates were carried out for each experiment.

Strains that showed H_2O_2-degrading capability were further tested to quantify the enzymatic activity. Five ml of AN, AE and RS cultures standardized at OD_{650}=1 were centrifuged at 13000 *g* for 5 min and the resulting pellet mixed with 1.0 ml of 60 mM H_2O_2 in 50 mM PBS pH 7. The

activity was measured at 240 nm after 3 min incubation at room temperature. Results were expressed as units per ml of solution (U) and calculated according to the following formula:

$U = (\Delta OD * \varepsilon * df)/(t * v)$

where,

ΔOD: decrease in absorbance after 3 minutes at 240 nm;

ε: 39.4 $M^{-1}cm^{-1}$, extinction coefficient of H_2O_2 at 240 nm;

df: dilution factor;

t: time of analysis in min;

v: volume of sample.

2.3.5 Oxygen uptake

The consumption of oxygen in AN, AE and RS growing cells was measured as described by Ricciardi et al. [29]. Briefly, washed and standardized (OD_{650} = 1) biomass was re-suspended in air-saturated solution of 5.5 mM glucose and 0.002 g/l of resazurin sodium salt (Sigma) in 0.1 M PBS pH 7. The discoloration time (DT, expressed in minutes) from blue oxidized form (resazurin) to colorless reduced form (dihydroresofurin) was used as indicator of oxygen uptake. The strain *Pseudomonas fragi* SP1 was used as positive control.

2.3.6 Effect of aerobic and respiratory cultivation on oxidative stress tolerance.

AN, AE and RS cultures, washed twice and standardized (OD_{650} = 1) in 0.1 M PBS pH 7.0, were loaded in 96-well microplates and exposed to different concentrations (from 0.62 to 320 mM serial two-fold dilution in 0.1 M PBS pH 7.0) of H_2O_2, menadione and pyrogallol (Table 4) for 30 min at 37 °C in anaerobiosis. Stressed cultures were inoculated (10% v/v) in sterile WMB broth (20 g/l glucose, pH 6.8; 96-well microplates) and the surviving cells were detected by evaluating the medium turbidity (presence/absence) after 24 h of incubation at 37°C in anaerobic conditions. For each strain the results were expressed as Maximum Tolerated Concentration (MCT; mM) of oxidative stress compounds.

2.3.7 In silico *analysis of genes involved in aerobic-respiratory pathway and oxidative stress response*

The presence of genes coding for the main enzymes involved in oxygen utilization (pyruvate oxidase, *pox*; acetate kinase, *ack*; lactate oxidase, *lox*; NADH oxidase, *nox*), electron transport chain (NADH dehydrogenase, *ndh;* cytochromes *bd* oxidase, *cydABCD* operon; menaquinone biosynthesis complex, *menFDXBEC*, and ubiquinone/menaquinone biosynthesis methyltransferase,

ubiE) and oxidative stress response (heme- and manganese-dependent catalases, *kat* and *Mnkat*; manganese-dependent superoxide dismutase, *sodA*; thioredoxin/thioredoxin reductase system, *trx-trxB*; glutathione peroxidase/reductase system, *gop* and *gor;* NADH peroxidase, *npr*) was evaluated in the finished, draft and permanent draft genomes of *Lb. johnsonii* and *Lb. gasseri* from IMG/M (https://img.jgi.doe.gov) database (Table 2). The occurrence expressed in % (Occ) of each gene in the *L. johnsonii* and *L. gasseri* group was calculated. The sequences retrieved from the genomes of *Lactococcus lactis* subsp. *cremoris* MG1363 (*menFDXBEC*), *Lb. plantarum* WCFS1 (*pox*, *ack*, *lox*, *nox*, *npr*, *cydABCD*, *ubiE*, *kat*, *trxA-trxB*, *gop-gor*), *Lb. plantarum* ATCC 14431 (*Mn-kat*) and *Lb. sakei* 23K (*sodA*) were used as queries. Sequence similarity was detected using the default cut-off parameter (% of identity) obtained by ClustalW2 multiple sequence alignment tool.

2.3.8 Data analysis

Analyses were carried out in triplicate and all values were expressed as mean and standard deviation. Two-way Anova test and t-test analysis (Microsoft Excel for Mac version 11.5) were performed to evaluate significant differences ($P<0.05$) between averages.

2.4 RESULTS

2.4.1 Isolation and molecular characterization of strains belonging to Lb. johnsonii/gasseri *species*

A pool of 145 isolates potentially belonging to *Lactobacillus* genus was obtained by healthy breast-fed babies. On the basis of genotypic (rep-PCR analysis) and phenotypic (i.e. cell and colony morphology, growth performance, planktonic or aggregated growth in MRS broth) characteristics, a total of 80 strains were selected and subjected to 16s rRNA gene sequencing analysis. Results of BLAST analysis showed that 55 strains belonging to *Lb. johnsonii/gasseri* species (≥98% homology), while the remaining 25 strains to *Lb. casei* (15 strains) and *Lb. plantarum* groups (10 strains). Since 16S rRNA gene sequence is not discriminative for *Lb. johnsonii* and *Lb. gasseri* species, in all experiments we decided to indicate the strains as *Lb. johnsonii*/*gasseri*. On the basis of the different rep-PCR profiles, 34 strains of *Lb. johnsonii/gasseri* were selected and used to investigate the potential probiotic features and the capability to grow in presence of oxygen and oxidative stress conditions.

2.4.2 Survival to simulated oral gastro-intestinal transit (OGIT)

Results of viable counts of the strains after simulated OGIT showed that all strains were significantly ($P<0.05$) resistant (at least the 50% of initial population) to SSJ exposure (Table 3). On

the contrary, the number of survivors to SGJ and SPJ dramatically decreased. Only 13 and 6 strains had at least 50% of viable cells ($P<0.05$) after the exposure to SGJ and SPJ, respectively (Table 3). Specifically, the 6 strains tolerant of SPJ showed more than 90% of survival (data not shown). As expected, the probiotic strain *Lb. rhamnosus* GG showed a high resistance to OGIT (>90% of final survival).

2.4.3 Antimicrobial activity

Results of agar spot test showed that 22 strains of *Lb. johnsonii*/*gasseri* exhibited antimicrobial activity against at least 2 of the 9 indicator strains used in this study (data not shown). In Table 1 are reported the strains with the widest inhibitory pattern. The strains AL5, AL3 and ALA had the highest antimicrobial activity against the most of indicators (diameter of inhibition halos > 2 cm). On the other hand, results of well-diffusion agar assay showed that only the strains BM4, AL5, BR32, AL3, ID5AN, BM1CM and BM61CG were able to produce antimicrobial substances, although they inhibited only *Staphylococcus aureus* DSM 20231 (data not shown). Inhibition was not associated to the production of bacteriocin-like substances or H_2O_2 since the antagonistic activities were not affect by the action of proteolytic enzymes and catalase.

2.4.4 Aerobic and respiratory promoting growth, oxygen uptake and catalase production

Ratios between OD and pH values (AE/AN, RS/AN) measured in the different growth conditions were calculated and used to identify the phenotype of each strain. Specifically, when both OD and pH ratios in AE/AN were >1 (Fig 1, upper right side), strains were indicated as oxygen tolerant phenotypes (OTP); when OD and pH ratios in RS/AN were >1 (Fig 2, upper right side), strains were indicated as respiration-competent phenotypes (RCP). Results showed that 14 strains had both OTP and RCP, while 2 strains had only the RCP. For these 16 strains the OD and pH ratios in RS/AE were also calculated (Fig 3) and the oxygen uptake was tested. Results showed that 12 strains grew better when hemin and menaquinone were supplied (Fig 3, upper right side), while 4 strains grew better when only oxygen was supplied (Figure 3, upper left side). Interestingly, the four strains showing OD ratio RS/AE<1 (AL5, AL15, BM32 and BM6CG) and the three strains showing the best growth performance in RS (ALJ, AL3 and BM4) resulted to be the strains that consumed oxygen during their growth (DT<180 min, Table 3). Consistently, ALJ, AL3 and BM4 consumed more oxygen in RS than in AE, AL5 consumed more oxygen in AE and BM6CG, unexpectedly, consumed oxygen only in AE cultivation. Instead, AL15 and BM32 showed the same DT in both AE and RS (Table 4). Catalase activity was detected only in 2 strains. Specifically, AL5 showed

11.30 U, 12.20 U and 12.00 U of enzymatic activity in AN, AE and RS, respectively, while AL3 showed catalase activity only in RS with 13.50 U.

Table 1. Results of agar spot test on the strains showing a remarkable antimicrobial activity against the selected indicator strains.

	Indicators								
Strains	*Brochothrix thermosphacta* ATCC 11509	*Brochothrix thermosphacta* 7R1[a]	*Pseudomonas fragi* 6P2[a]	*Listeria monocytogenes* ATCC 7644	*Listeria innocua* ATCC 1770	*Micrococcus luteus* ATCC 10240	*Bacillus subtilis* DSM 5547	*Staphylococcus aureus* DSM 20231	*Escherichia coli* 1634*
BM4	+	++	-	++	++	++	+	++	+
AL5	+++	+++	++	+++	+++	+++	+	++	-
AL9	+	+	+	++	+++	+	+	-	+
BR32	++	++	+++	++	+	++	+	+	-
AL3	++	+++	+++	+++	+++	++	+	+++	+
ALA	+++	+++	+++	+++	+++	+++	+++	+	-
ID5AN	+	+	+	++	+	+	+	+	-
10533^T	+	+	-	++	++	-	+	+	+

[a]Provided by Department of Agriculture, Division of Microbiology, University of Naples FedericoII.

(-) no inhibition halo

(+) inhibition haloes ≥ 0.5 and ≤ 1.5 cm

(++) inhibition haloes > 1.5 and ≤ 2.0 cm

(+++) inhibition haloes > 2.0 cm

Table 2. Results of *in silico* analysis of genes (query sequence from *Lactobacillus plantarum* WCFS1) involved in aerobic and respiratory metabolism and oxidative stress response in *Lactobacillus johnsonii* e *Lactobacillus gasseri* genomes from Integrated Microbial Genome and Microbiomes database (IMG/M).

Strains	Completeness level of genome	*pox3* (pyruvate oxidase)[a]			*ack2* (acetate kinase)			*loxL* (lactate oxidase)			*ndh* (NADH dehydrogenase)		
		Occ %[b]	Logus Tag	% ID	Occ %	Logus Tag	% ID	Occ %	Locus Tag	% ID	Occ %	Locus Tag	% ID
L. johnsonii		100			100			42			14		
FI9785	Finished		FI9785_1805	51		FI9785_502	60		FI9785_1781	50		-	-
NCC 533	Finished		LJ_1853	51		LJ_0912	60		-	-		-	-
DPC 6026	Finished		LJP_1790c	51		LJP_1241c	60		-	-		-	-
N 6.2	Finished		T285_09130	51		T285_06205	60		-	-		-	-
ATCC 33200	Permanent Draft		HMPREF0528_0499	51		Ga0106082_104191	62		-	-		-	-
pf01	Permanent Draft		PF01_01488	45		PF01_00963	47		-	-		PF01_00600	51
16	Permanent Draft		Ga0081980_100422	52		Ga0081980_1045116	47		-	-		-	-
L. gasseri		100			100			14			7		
ATCC 33323	Finished		LGAS_1893	55		LGAS_0431	51		-	-		LGAS_1626	52
130918	Finished		Ga0069304_121971	52		Ga0069304_12681	61		-	-		-	-
224-1	Permanent Draft		HMPREF9209_1596	45		HMPREF9209_0556	61		-	-		-	-
MV-22	Permanent Draft		LBGGDRAFT_02078	52		LBGGDRAFT_00613	46		-	-		-	-
JCM 1131	Permanent Draft		JCM1131DRAFT_00094	55		JCM1131DRAFT_00560	51		-	-		-	-
L32	Permanent Draft		LGS32_00996	58		LGS32_01619	60		LGS32_02417	52		-	-
SJ-9E-US	Permanent Draft		HMPREF0516DRAFT_01456	51		HMPREF0516DRAFT_00713	51		-	-		-	-
202-4	Permanent Draft		HMPREF0890_1112	45		HMPREF0890_1123	61		-	-		-	-
2016	Permanent Draft		M497_03520	45		M497_08405	44		-	-		-	-
SV-16A-US	Permanent Draft		HMPREF5175DRAFT_01637	51		HMPREF5175DRAFT_00801	52		-	-		-	-
K7	Permanent Draft		LK7_00106	39		LK7_00555	61		-	-		-	-
JV-V03	Permanent Draft		HMPREF0514_11618	52		HMPREF0514_10278	61		-	-		-	-
L3	Permanent Draft		LGS03_01236	52		LGS03_00282	61		-	-		-	-
CECT 5714	Permanent Draft		A131_64062	52		A131_63426	44		-	-		-	-

Strains	*cydA* (cytochrome D ubiquinol oxidase subunit I)			*cydB* (cytochrome D ubiquinol oxidase subunit II)			*ubiE* (ubiquinone /manaquinone biosynthesis methyltrasferase)			*trxA* (thioredoxina peroxidase)		
	Occ %	Logus Tag	% ID	Occ %	Logus Tag	% ID	Occ %	Locus Tag	% ID	Occ %	Locus Tag	% ID
L. johnsonii	100			100			100			100		
FI9785		FI9785_1761	62		FI9785_1762	59		FI9785_116	53		FI9785_496	61
NCC 533		LJ1810	62		LJ1811	59		LJ0053	53		LJ0480	61
DPC 6026		LJP_1743	62		LJP_1744	55		LJP_0061	53		LJP_0466	43
N 6.2		T285_08935	62		T285_08940	59		T285_00320	53		T285_02375	55
ATCC 33200		HMPREF0528_0541	62		HMPREF0528_0540	50		HMPREF0528_0446	53		HMPREF0528_0036	61
pf01		PF01_01448	64		PF01_01449	55		PF01_01575	54		PF01_00129	61
16		Ga0081980_10171	62		Ga0081980_10172	62		Ga0081980_10155	41		Ga0081980_104717	38
L. gasseri	100			100			100			100		
ATCC 33323		LGAS_1841	64		LGAS_1842	59		LGAS_0051	53		LGAS_0427	62
130918		Ga0069304_121909	62		Ga0069304_121910	55		Ga0069304_1257	53		Ga0069304_121562	56
224-1		HMPREF9209_1524	51		HMPREF9209_1525	55		HMPREF9209_1667	54		HMPREF9209_2113	57
MV-22		LBGGDRAFT_02156	50		LBGGDRAFT_02155	40		LBGGDRAFT_00757	41		LBGGDRAFT_01384	62
JCM 1131		JCM131DRAFT_00038	64		J CM1DRAFT_00040	59		JCM1131DRAFT_00780	43		JCM1131DRAFT_00556	57
L32		LGS32_02557	50		LGS32_02558	46		LGS32_02172	54		LGS32_01524	60
SJ-9E-US		HMPREF0516DRAFT_01509	50		HMPREF0516DRAFT_01508	40		HMPREF0516DRAFT_00577	43		HMPREF0516DRAFT_00804	45
202-4		HMPREF0890_0547	58		HMPREF0890_0546	40		HMPREF0890_0917	54		HMPREF0890_1432	62
2016		M497_03250	65		M497_03255	55		M497_01865	53		M497_04780	57
SV-16A-US		HMPREF5175_01604	50		HMPREF5175_01603	40		HMPREF5175DRAFT_00605	53		HMPREF5175DRAFT_01158	62
K7		LK7_00037	63		LK7_00038	55		LK7_01321	49		LK7_01550	61
JV-V03		HMPREF0514_11680	53		HMPREF0514_11679	40		LGS32_01525	53		HMPREF0514_10102	49
L3		LGS03_01171	62		LGS03_01172	55		LGS03_00422	45		LGS03_00194	48
CECT 5714		A131_64178	50		A131_54100	40		A131_63987	53		A131_35963	62

Strains	***trxB* (thioredoxina oxidase)**			***gor* (glutathione reductase)**			***gop* (glutathione peroxidase)**			***npr* (NADH peroxidase)**		
	Occ %	Logus Tag	% ID	Occ %	Logus Tag	% ID	Occ %	Locus Tag	% ID	Occ %	Locus Tag	% ID
L. johnsonii	100			100			0			0		
FI9785		FI9785_517	51		FI9785_107	47		-	-		-	61
NCC 533		LJ0501	51		LJ0042	48		-	-		-	61
DPC 6026		LJP_0493c	50		LJP_0052	48		-	-		-	43
N 6.2		T285_02480	61		T285_00250	47		-	-		-	55
ATCC 33200		Ga0106082_104129	51		HMPREF0528_0013	44		-	-		-	61
pf01		PF01_01020	64		PF01_01566	48		-	-		-	61
16		Ga0081980_1047	65		Ga0081980_10276	48		-	-		-	38
L. gasseri	100			85			7			28		
ATCC 33323		LGAS_0447	43		LGAS_0040	46		-	-			62
130918		Ga0069304_121542	63		Ga0069304_1244	47		-	-			56
224-1		HMPREF9209_0485	62		-	-		-	-			57
MV-22		LBGGDRAFT_00549	62		-	-		-	-		LBGG_00050	62
JCM 1131		JCM1131DRAFT_00577	43		JCM1131DRAFT_00140	45					-	57
L32		LGS32_00137	68		LGS32_02102	48		LGS32_00080	59		-	60
SJ-9E-US		HMPREF0516DRAFT_00773	54		HMPREF0516DRAFT_01411	45		-	-		HMPREF0516_00383	45
202-4		HMPREF0890_1451	53		HMPREF0890_0197	46		-	-		-	62
2016		M497_08685	62		M497_01805	46		-	-		-	57
SV-16A-US		HMPREF5175DRAFT_00863	49		HMPREF5175DRAFT_01591	45		-	-		HMPREF5175_00403	62
K7		LK7_00572	43		LK7_00152	46		-	-		-	61
JV-V03		HMPREF0514_10221	62		HMPREF0514_11575	47			-		-	49
L3		LGS03_00225	62		LGS03_01284	46		-	-		-	48
CECT 5714		A131_63398	43		A131_64012	46		-	-		A131_62641	62

Table 3. Strains showing a survival ≥50% after exposure to the different conditions of oral gastro-intestinal transit.

SSJ	SGJ	SPJ
All strains	AL5, BR32, ID5AN, ID7AN, AL8, ALJ AL3, BM1CM, BM1CP, BR35, AL15 *Lb. johnsonii* 10533^T, *Lb. gasseri* 20243^T	AL5, BR32, ID5AN, ID7AN, AL3 *Lb. johnsonii* 10533^T

Table 4. Consumption of oxygen in *Lactobacillus johnsonii*/*gasseri* strains expressed as discoloration time in minutes (DT) of the redox indicator resazurin.

Strains	Growth conditions		
	AN	AE	RS
AL9	>180	>180	>180
AL5	>180	90	120
AL15	>180	100	100
AL8	>180	>180	>180
BR32	>180	100	100
BM4	>180	130	75
BM7CP2	>180	>180	>180
BR35	>180	>180	>180
BM6CG	>180	120	>180
BM1CP	>180	>180	>180
BR36	>180	>180	>180
BM2	>180	>180	>180
BM7CP1	>180	>180	>180
BM1CM	>180	>180	>180
ALJ	>180	120	100
AL3	>180	110	75
Pseudomonas fragi SP1	ND	65	ND

Growth conditions: AN, anaerobiosis; AE, aerobiosis; RS, AE growth supplemented with 2.5 µg/mL of hemin and 1 µg/mL of menaquinone.

2.4.5 Effect of aerobic and respiratory conditions on the oxidative stress tolerance

In order to investigate the effect of growth conditions on oxidative stress tolerance the strains cultivated in AN, AE and RS conditions were exposed to generators of superoxide anion (menadione and pyrogallol) and H_2O_2. Strains cultivated in AE exhibited the lowest tolerance of all oxidative compounds (Table 5). For several strains, the respiratory growth increased the resistance to H_2O_2. Most of respiratory growing cultures had H_2O_2-tolerance similar to that of cells grown under anaerobic conditions, while only AL9 had a lower resistance when cultivated in presence of oxygen, hemin and menaquinone. Compared to anaerobic conditions, respiration promoted the menadione resistance only in 5 strains and impaired the survival in AL9, BM6CG and BM1CM. With exception of AL9, the resistance of respiratory cultures to pyrogallol was similar to that of anaerobically growing cells.

2.4.6 In silico *analysis of genes involved in aerobic-respiratory metabolism and oxidative stress response*

Results of *in silico* analysis are reported in Table 2 where the occurrence and % of identity (% ID) of each gene is indicated. Interestingly, we found 100% of occurrence of genes *pox*, *ack*, *cydA*, *cydB*, *ubE*, *trxA* and *trxB* both in *Lb. johnsonii* and in *Lb. gasseri*. Instead, remaining genes were found at very low % in both species, with exclusion of *gor* gene that was found in all *Lb. johnsonii* genomes and lacking in only two *Lb. gasseri* genomes. Moreover, an ID in the range 45-55% was registered for most of genes. Finally, the genes *kat*, *Man-kat*, *sodA, menFDXBEC* and *nox*, not reported in the table, have been never annotated in genomes of *L. johnsonii* and *L. gasseri*.

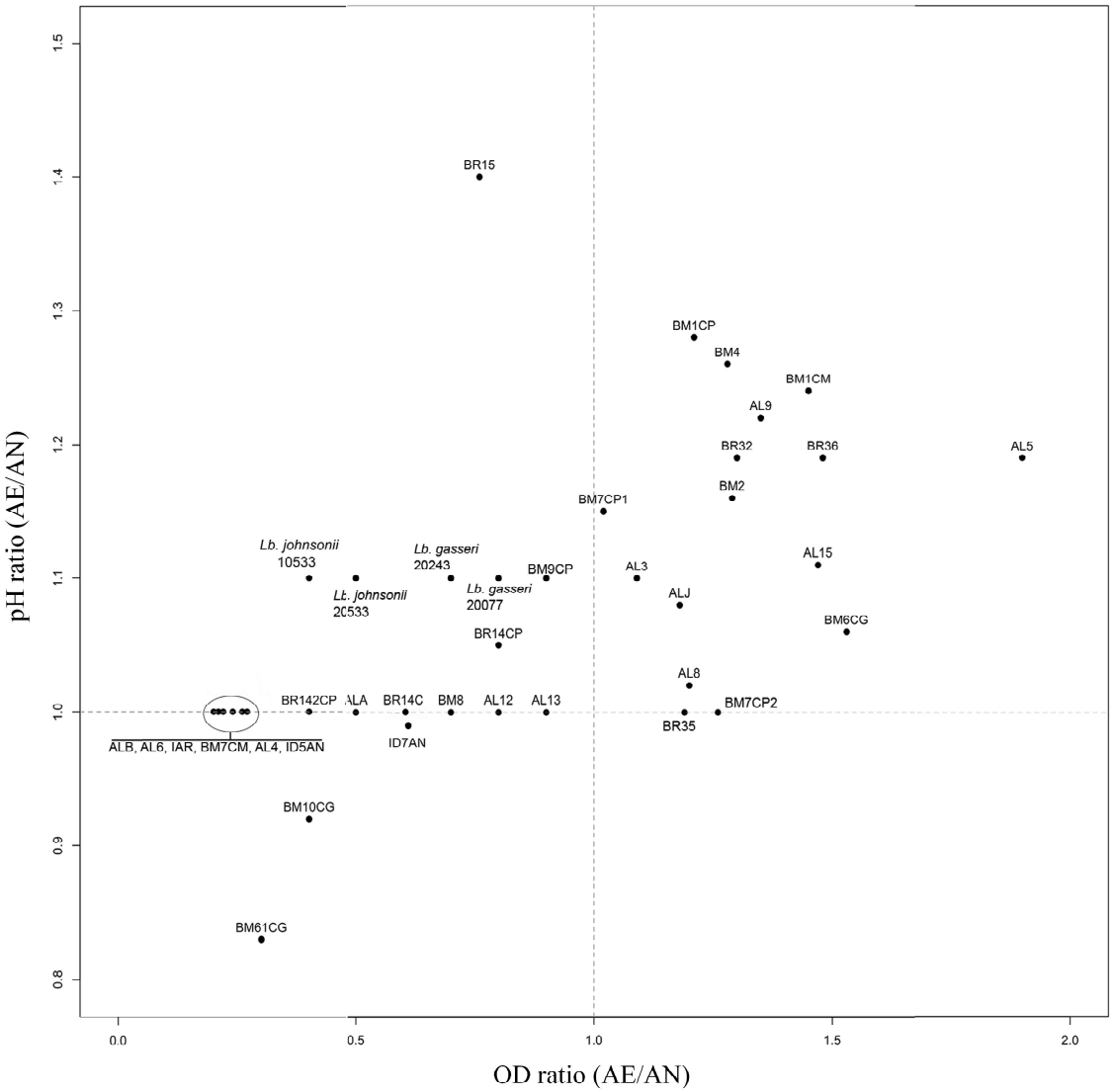

Figure 1. Scatter plot of OD_{650} ratio against pH ratio in AE/AN of *Lb. johnsonii/gasseri* strains.

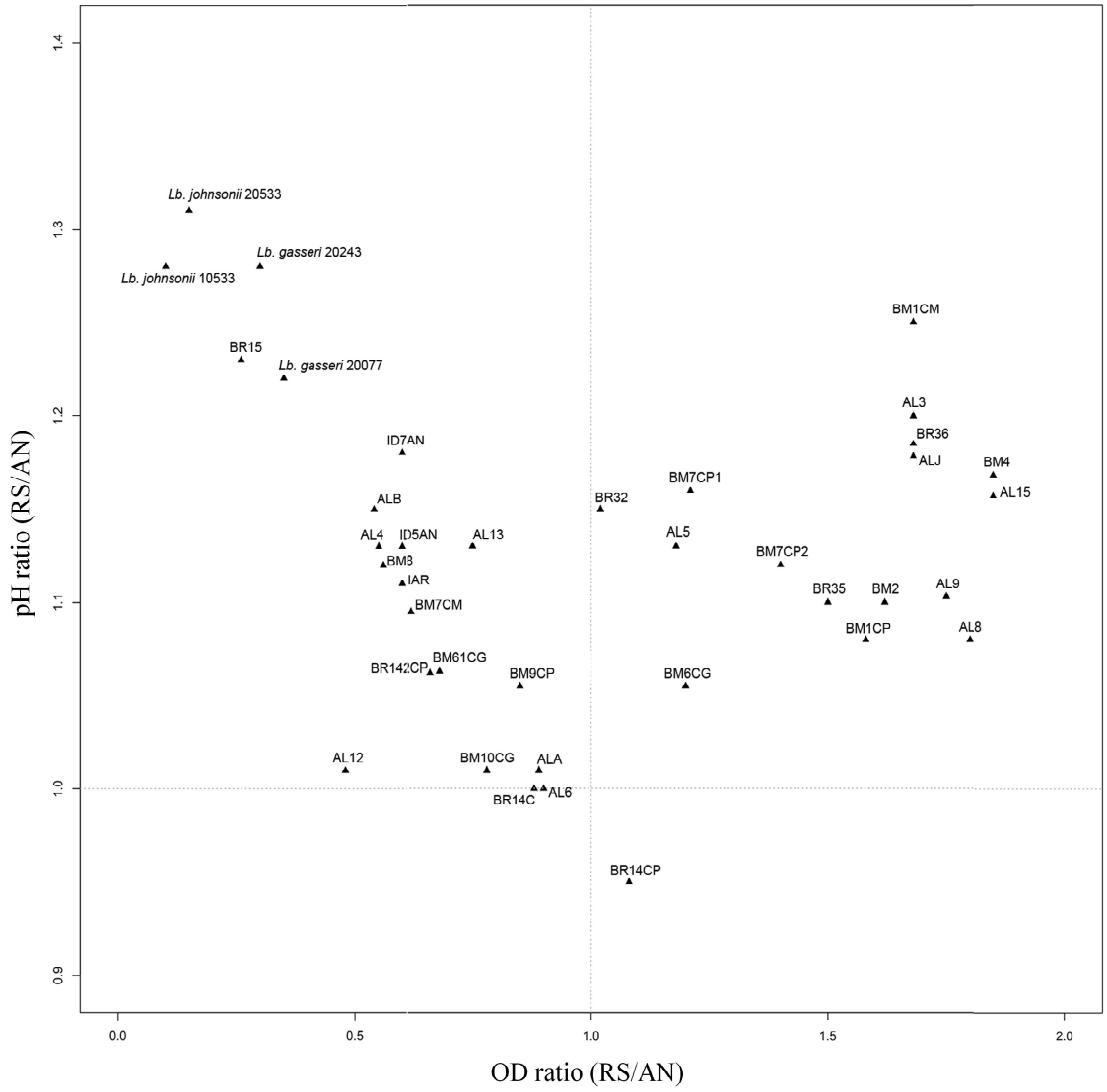

Figure 2. Scatter plot of OD_{650} ratio against pH ratio in RS/AN of *Lb. johnsonii/gasseri* strains

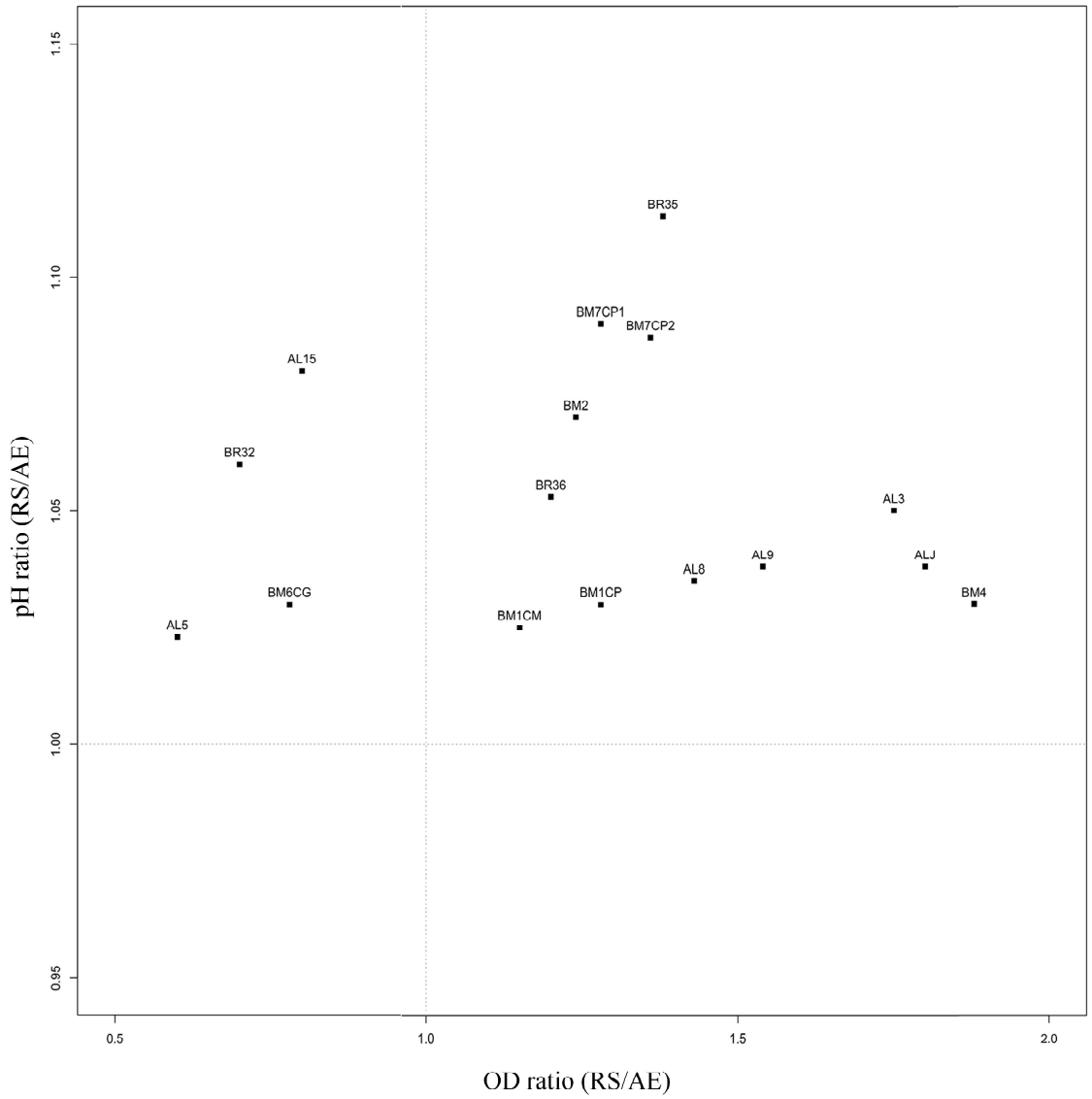

Figure 3. Scatter plot of OD_{650} ratio against pH ratio in RS/AE of *Lb. johnsonii/gasseri* strains.

Table 5. Tolerance of H_2O_2, menadione and pyrogallol (expressed as Maximum Tolerated Concentration MCT; mM) in *Lb johnsonni/gasseri* strains grown under AN, AE and RS conditions.

Strains	H_2O_2 (mM)			Menadione (mM)			Pyrogallol (mM)		
	AN	AE	RS	AN	AE	RS	AN	AE	RS
AL9	20	0	1.25	10	0	0	160	10	10
AL5	10	10	40	2.5	5	20	320	80	320
AL15	20	10	40	0.62	1.25	5	320	80	320
AL8	20	5	20	5	0	5	80	2.5	80
BR32	20	2.5	20	1.25	1.25	5	80	40	80
BM4	20	20	20	5	2.5	5	320	80	320
BM7CP2	20	5	20	2.5	0.62	2.5	80	20	80
BR35	20	5	20	5	0	2.5	80	20	80
Growth condition									
BM6CG	20	0	20	10	0	5	80	0	80
BM1CP	20	5	20	5	1.25	5	80	2.5	80
BR36	10	5	40	10	5	20	80	20	80
BM2	20	10	40	2.5	2.5	2.5	320	40	80
BM7CP1	20	10	20	2.5	2.5	2.5	80	40	80
BM1CM	20	2.5	20	10	0	5	80	0	80
ALJ	5	2.5	20	2.5	0	2.5	40	40	80
AL3	20	10	40	2.5	0	20	80	40	80

Growth conditions: AN, anaerobiosis; AE, aerobiosis; RS, AE growth supplemented with 2.5 µg/mL of hemin and 1 µg/mL of menaquinone.

2.5 DISCUSSION

This study investigated the adaptive response of promising probiotic *Lb. johnsonii/gasseri* strains to switch from fermentative to aerobic and respiratory metabolism and the effect of this metabolic pathway on oxidative stress response. We found that about 70% of lactobacilli isolated from baby stools belonged to *Lb. johnsonii/gasseri* species. This result is in agreement with findings of many authors (Wall et al., 2006; Mitsou et al., 2008; Morelli et al., 2008) who demonstrated that *Lb. johnsonii/gasseri* species are the more commonly homofermentative lactobacilli isolated in new-borns and infant faeces. According to the FAO/WHO definition, probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). In order to perform their physiological role, probiotics bacteria must overcome a number of stresses before they reach the target site (Kwarteng et al. 2015). The ability to survive to gastro-intestinal transit and the antimicrobial activity are two important features of probiotics (Vizoso et al., 2006). In this study, 34 *Lb. johnsonii/gasseri* strains were screened for the ability to pass through OGIT. All strains showed a strong resistance to saliva juice, suggesting a high ability to survive in the presence of lysozyme. On the contrary, for most strains the viability decreased when exposed to simulated gastric and intestinal juice. Six strains, however, showed a great resistance to OGIT, with levels of survival comparable to those of *Lactobacillus rhamnosus* GG, suggesting their possible use as probiotic supplements.

The capability to inhibit the growth of pathogenic and spoilage bacteria varied among *Lb. johnsonii/gasseri* strains. The inhibitory activity demonstrated with agar spot test could be due to a lowering of pH due to organic acids production; indeed, it disappeared when free cell supernatants were neutralized. However, the ability to produce organic acids should be a useful feature to reduce colonisation of pathogenic microorganisms in human GIT. Tejero-Serinena et al. (2012) showed that the production of organic acids by different probiotic strains reduced the growth of potential pathogenic microorganisms. In presence of low O_2 concentration, acetic acid and H_2O_2 may be produce by some probiotic strains. Pridmore et al. (2008) described the ability of some *Lb. johnsonii* and *Lb. gasseri* strains to produce H_2O_2 and acetic acid, with antimicrobial activity against *Salmonella* Typhimurium SLI344. In this study the neutralized cell-free supernatants had inhibitory activity against *Staphylococcus aureus*. Since bacteriocin-like and H_2O_2 activities were excluded, we hypothesized that the inhibition may be due to the production of neutral compounds or some undissociated short chain free fatty acids with antimicrobial activity (Huang et al., 2011; Ricke et al., 2014; Aldunate et al., 2015). However, further investigations are needed to reveal the nature of antimicrobial substances produced by *Lb. johnsonii* and *Lb. gasseri* active against *Staphylococcus*

aureus. A large diversity on the capability to grow in presence of oxygen and/or respiratory cofactors was present within the *Lb. johnsonii* and *Lb. gasseri* strains and on the basis of growth performances and oxygen consumption it was possible to group the strains in: *i)* oxygen-tolerant anaerobes, capable to grow in aerobic and respiratory conditions, but not able to consume oxygen (i.e. AL8, AL9, BM2, BM1CM, BM1CP, BM7CP1, BM7CP2, BR35 and BR36); *ii)* aerobic phenotypes, able to consume oxygen and for which aerobiosis was the best growth condition compared to respiration (i.e. AL5, AL15, BM32 and BM6CG); *iii)* respiratory phenotypes, able to consume oxygen and for which respiration was the best growth condition compared to aerobiosis (i.e. ALJ, AL3 and BM4); *iv)* oxygen-sensitive anaerobes, unable to grow in both aerobic and respiratory conditions (remaining strains). Hertzberger et al. (2013) evaluated for the first time the response of *Lb. johnsonii* NCC533 to oxidative conditions. Compared to anaerobiosis, the presence of oxygen relieved the acetate and CO_2 growth dependencies of the strain, but induced more rapidly the entry in stationary phase. In this study, we found four strains (AL5, BM6CG, AL15 and BR32) with aerobic phenotype that showed increased biomass production, reduced acidification and oxygen uptake capability, suggesting a possible activation of aerobic metabolism. In several LAB strains, the stimulatory effect of oxygen was shown to be dependent by pyruvate oxidase (POX) and acetate kinase (ACK) activities (Goffin et al., 2006; Quatravaux et al., 2006). According to results of *in silico* analysis, *Lb. johnsoni* and *Lb. gasseri* possess genes predicted to encode for both POX and ACK (Occ=100%), the main enzymes involved in aerobic pathway (Pridmore et al., 2008). In presence of oxygen and at low glucose concentration, pyruvate can be metabolised by POX-ACK pathway, allowing the production of acetate, CO_2 end extra ATP biosynthesis (Pedersen et al., 2012). Therefore, in our strains the possible POX-ACK pathway activation could directly explain the observed physiological consequence, such as increase of pH due to a possible conversion of pyruvate into acetate and increase of OD due to extra ATP generation. As a matter of fact, Hertzberger et al. (2014) demonstrated that *pox* deletion in *Lb. johnsonii* NCC533 resulted in a slower growth rate and a growth arrest upon CO_2 depletion, confirming the positive role of POX-ACK pathway. The respiratory growth was never investigated in *Lb. johnsonii*/*gasseri* strains. Similarly to aerobic metabolism adaptation, the ability to shift toward respiratory pathway was strain-specific. In this study we found that the strains AL3, ALJ and BM4 had a respiratory phenotype. Like most LAB, the genomes of *Lb. johnsonii* and *Lb. gasseri* lack the genes for menaquinone and heme biosynthesis, but harbour those for the cytochrome oxidase production (Occ=100%). Thus, in these species, the respiratory metabolism may occur only when heme and menaquinone are supplied (Petersen et al., 2012). As in all lactobacilli, the mechanism of heme uptake is unknown. However, heme-binding proteins and heme homeostasis systems have been

investigated in several LAB competent for aerobic respiration (Gaudu et al., 2003; Lechardeur et al., 2010; Lechardeur et al., 2011; Sawai et al., 2012). In several LAB the growth in presence of oxygen may result in the production and accumulation of H_2O_2 and ROS. Pridmore et al. [36] and Hertzberger et al. (2014) demonstrated that the accumulation of H_2O_2 due to LOX and POX activity was the primary reason of oxidative stress in *Lb. johnsonii* NCC533. Therefore, the ability to scavenge H_2O_2 and ROS may contribute to the survival in aerobic conditions. Surprisingly, we found that two strains had a catalase like activity. Specifically, the strain AL5 was able to degrade H_2O_2 in all growth conditions, suggesting the presence of both heme and manganese-dependent catalases. On the contrary, the strain AL3 showed catalase activity only in RS conditions, suggesting the presence of heme-catalase. Although the heme and manganese-catalase activity has been previously studied in several LAB species (Knauf et al., 1992; Abriouel et al., 2004; Rochat et al., 2006; Guidone et al., 2013; Ianniello et al., 2015; Ianniello et al., 2016) this is the first study that demonstrated it in *Lb. johnsonii/gasseri* strains. However, genes encoding for heme-catalase or Mn-catalase were never annotated in *Lb. johnsonii* and *Lb. gasseri* genomes. Heme-dependent and Mn-dependent catalase activities were previously found in the respiration-competent strain *Lb. casei* N87 (Zotta et al., 2014; Ianniello et al., 2015; Ianniello et al., 2016) and the genome sequence confirmed the presence of both genes (Zotta et al. 2016). In our study, several strains showed a higher resistance to H_2O_2 and radical generators when cultivated in RS, compared to AN and AE conditions. The H_2O_2 robustness of respirative cells of AL5 and AL3 (up to 40 mM of H_2O_2) could be related to the catalase-like activity. The strains AL15, BR36, BM2 and ALJ, although lack catalase activity, showed higher H_2O_2 tolerance in RS condition. These results suggested that other mechanisms might be involved in H_2O_2 resistance. Ianniello et al. (Ianniello et al., 2015) demonstrated that in *Lb. rhamnosus* GG, *Lb. casei* Shirota and in some respiration-competent strains of *Lb. casei*, the increased resistance to H_2O_2 under RS condition was due to NADH peroxidase (NPR) activity. In our study all strains were able to cope to menadione and pyrogallol stress in both RS and AN, although they lack the *sod* gene encoding for superoxide dismutase (SOD) enzyme. The in silico gene distribution analysis revealed high % of occurrence in *Lb. johnsonii* and *Lb. gasseri* genomes of sequences encoding for glutathione reductase (*gor*), thioredoxin peroxidase (*trxA*) and thioredoxin reductase (*trxB*). These enzymes belong to flavoprotein disulfide oxidoreductases family and play a significant role in oxidative stress resistance, maintaining a high intracellular thiol/disulfide homeostasis in both prokaryotic and eukaryotic cells (Harel et al., 2000; Janschn et al., 2007). Glutathione and thioredoxin reductase systems and their role in oxygen and H_2O_2 tolerance have been explored in some *Lactobacillus* species (Li et al., 2003; Vido et al., 2005; Serrano et al., 2007; Jansch et al., 2007;

Kullisaar et al., 2010; Serata et al., 2012). The genes encoding for other enzymes of glutathione system, *gshA* (γ-glutamylcystiene synthetase) and *gshB* (glutathione synthetase), are absent in the genomes of *Lb. johnsonii* and *Lb. gasseri*, suggesting that they are not able to synthetize glutathione, but they may use it when supplied.

In conclusion, we investigated 34 *Lb. johnsonii*/*gasseri* strains from baby stools and found that some of them showed both typical probiotic features, like resistance to OGIT and antimicrobial activity, and aerobic environment adaptation. In particular, the strains AL5, BR32 and AL3 showed to tolerate very well the stress due to OGIT, in fact more than 90% of their population survived to this treatment. Furthermore, they showed to inhibit the growth of most of indicator strains used in this study by producing organic acids, and the growth of a *Staphylococcus aureus* strain by producing a sort of antimicrobial substance, not ascribable to a protein or to hydrogen peroxide, be further investigated. Additionally, they showed an interesting adaptation to aerobic environment with the AL5 and BR32 strains showing an OTP and the AL3 strain showing a RCP. Their overall enhanced resistance to oxidative stressors and the evidence of catalase production ability of AL5 and AL3 strains confirmed this adaptation. Probiotic strains of this type could effectively work both during biomass production and in food or processing in which the aerobic condition could be a limiting factor for a standard probiotic strain.

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CHAPTER III

Draft genome sequence of oxygen-tolerant *Lactobacillus johnsonii/gasseri* strains

3.1 INTRODUCTION

The study of the aerobic and respiratory metabolism within the *Lactobacillus* genus is limited to species of *Lb. plantarum* and *Lb. casei* group, while, no reports on the respiratory growth in *Lb. johnsonii* and *Lb. gasseri* species is currently available, even if these species include strains with interesting technological features. *Lb. johnsonii* and *Lb. gasseri* are dominant bacteria in human gut and in vaginal microbiota and have received particular attention due to their reported probiotic activities (Pridmore et al., 2003). In our previous screening study, we investigated 34 *Lb. johnsonii*/*gasseri* strains from baby stools and found that some of them showed an interesting adaptation to aerobic environment and typical probiotic features (resistance to oral-gastrointestinal transit and antimicrobial activity). The shift towards aerobic and respiratory growth has been largely investigated in heterofermentative lactobacilli (Brooijmans et al., 2009; Zotta et al 2012; Watanabe et al., 2012; Guidone et al., 2013; Zotta et al., 2014a; Zotta et al., 2014b; Ricciardi et al., 2014; Ianniello et al., 2015; Ianniello et al., 2016), while, limited data are available in homofermentative lactobacilli. Nowadays, only *Lb. johnsonii* NCC533 probiotic strain has been studied to provide a more global understanding of the molecular responses to the presence of oxygen (Hertzberger et al., 2013-2014). However, most of the knowledge obtained by genetic studies are related only to the consequences of O_2 and CO_2 exposure on *Lb. johnsonii* NCC533 physiology, but they not fully explain the complexity of mechanisms underlie of the aerobic metabolism and oxidative stress response. In our screening study, *Lb. johnsonii*/*gasseri* AL3 and AL5 strains were selected because of their ability to grow under aerobic and/or respiratory promoting conditions and scavenge hydrogen peroxidase and reactive oxygen species (ROS). Results of *in silico* analysis, performed with all *Lb. johnsonii* and *Lb. gasseri* genomes (finished, permanent draft and draft status) available in IMG (Integrated Microbial Genome) database, revealed the presence of several genes involved in oxygen utilization, electron transport chain activation and oxidative stress tolerance. However, many genes have been found with a very low % of occurrence. Therefore, at this research point it was considered necessary to proceed with the genome sequencing of *Lb johnsonii*/*gasseri* AL3 and AL5 strains toward a better understanding of the genetic basis that can explain the phenotypic features observed. Moreover, the genomes sequencing allowed the correct species-level identification of the strains.

3.2 MATERIALS AND METHODS

3.2.1 Strains and culture conditions

Lb. johnsonii/*gasseri* AL3 and AL5 strains were routinely propagated in de Man Rogosa Sharpe (MRS, Oxoid) broth and incubated in anaerobiosis at 37°C for 24 h.

3.2.2 Sample preparation for gDNA sequencing

The genomic DNA (gDNA) from AL3 and AL5 cultures were extracted by using MasterPure GramPositive DNA purification kit (Epicentre, Illumina company, Chicago, U.S.A) according to the manufacturer's protocol. The quality and quantity of gDNA was assessed using agarose gel electrophoresis and both NanoDrop spectrophotometer 1000 and a Qubit fluorometer (Thermo Scientific, Milano, Italy).

3.2.3 Genome sequencing

The construction of NGS libraries was performed using Nextera DNA library preparation kit (Illumina) and the quality of the obtained libraries was evaluated by Agilent on-chip electrophoresis. The whole-genome sequencing of the strains was performed by GATC Biotech AG company, (Germany) using Illumina HiSeq platform.

3.2.4 Bioinformatics analysis

Before assembly, the quality of the reads was analysed by FastQC bioinformatics tool. The reads were assembled with IDBA software and the assembly was further improved by using SSPACE software. The genes were predicted with PROKKA software. Predicted genes were queried against an amino acidic database containing genes involved in aerobic and respiratory metabolism and oxidative stress response, by using BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A match was considered valid when showing more than 30% of identity to the database amino acidic subject sequences over at least 90% of the length. The functional annotation was carried out using NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAP).

3.3 RESULTS AND DISCUSSION

3.3.1 General genome characteristics

The features of genome annotation of the strains AL3 and AL5 are summarized in Table 1. The resulting AL3 and AL5 draft genome sizes are 1,994,887 pb and 1,999,212 pb, respectively. The AL3 draft genome contained 16 scaffolds, with an overall G+C content of 34.81%, while AL5 draft genome contained 35 scaffolds, with an overall G+C content of 34.87%. A total of 1,945 and 1,998 genes were found for AL3 and AL5, respectively.

Table 1. Main features and statistics of AL3 and AL5 genome assembly and annotation

Genomic features	Strains	
	AL3	AL5
Total assembly length (bp)	1,987,677	1,985,485
Average coverage	279.0X	303.0X
Number of scaffolds (>200 bp)	16	35
G+C content (%)	34.81	34.87
N_{50} (bp)	1,106,151	1,125,240
N_{75} (bp)	559,690	571,948
Total genes	1,945	1,998
Protein-coding genes	1,874	1,832
Pseudogenes	77	100
tRNA genes	61	56
rRNA genes	71	66

The phenotypic evidences on the capability of AL3 and AL5 strains to grow under aerobic and respiratory conditions and to cope to oxidative stress response, are supported by genomic information. The results of genome analysis revealed the presence, in both strains, of main genes involved in oxygen utilization (pyruvate oxidase, *pox*; lactate oxidase, *lox*; NADH oxidase, *nox*; L-amino oxidase; acetate kinase, *ack*), in the synthesis of electron transport chain components (NADH dehydrogenase, *ndh*; cytochrome oxidase operon, *cydABCD*; ubiquinone/menaquinone biosynthesis C-methylase, *ubiE*), as well as in oxidative stress response (NADH-peroxidase, *npr*; superoxide dismutase, *sod*; glutathione reductase, *gor*; thioredoxina peroxidase, trxA; thioredoxina oxidase, trxB; γ-glutamylcystiene synthetase, *gshA*; DNA-binding protein from starved cells, *dpr*). The genes encoding for catalase (*kat*), pseudocatalase (*Mn-kat*), catalase-peroxidase (*katG*), glutathione peroxidase (*gop*), glutathione synthetase (*gshB*) and bifunctional glutamate-cysteine ligase/glutathione synthetase (*gshF*) were not found. As shown in figure 1, the genetic pattern is very similar among the strains, although, the strain AL5 lacks of L-amino acid oxidase (flavoprotein oxidase) and *ribH* gene encoding for riboflavin synthase enzyme. The analysis of genome

sequences has also allowed the correct species-level identification. In particularly, *ribA* (GTP cyclohydrolase II, involved in riboflavin metabolism) and *ribH* were used as *Lb. gasseri* species-specific genes, while, *galP* (galactose permease) was used as *Lb. johnsonii* species-specific gene. Therefore, results of blast analysis have shown that both strains belong to *Lb. gasseri* species. On the basis of our results, AL5 and AL3 strains have the genetic potential for the activation of both aerobic metabolism (presence of *pox* and *ack* genes involved in aerobic conversion of pyruvate to acetate) and respiratory pathway (presence of genes involved in minimal electron transport chain activation), as well as to tolerate oxygen for the presence of main flavoproteins oxidase (*nox*, *lox*). Noteworthy, in both strains we found sequences encoding for *lox*, *nox*, *ndh*, *npr* and *gop* genes that, in our previous *in silico* analysis (chapter II), they showed a very low % of occurrence in *Lb. johnsonii* and *Lb. gasseri* genomes. Moreover, as described before, a gene encoding for L-amino acid oxidase was found in AL3 strain. Interestingly, to date this gene has never been annotated in all *Lb. johnsonii* and *Lb. gasseri* genomes (https://www.ncbi.nlm.nih.gov/). In our previous screening study, the strains AL3 and AL5 showed catalase like activity, but the genes encoding for *kat*, *Mn-kat* and *katG* were not found. However, in both genomes we found a gene that showed a significant similarity (45%) with *oxyR* gene of *Lb. plantarum*. OxyR is a hydrogen peroxide-inducible protein activator (Teramoto et al., 2013). It has been demonstrated that OxyR acts as peroxide-sensing transcriptional regulator of gene encoding for *katG* in *Escherichia coli*, *Salmonella typhimurium, Corynebacterium diphtheriae* and *Caulobacter crescentus* under oxidative stress conditions (Tao et al., 1991; Papp-szabo et al., 1994; Belkini et al., 1996; Tkachenko et al., 2001; Italiani et al. 2011; Teramoto et al., 2013). Surprisingly, the results of genome analysis revealed that both genomes have sequences encoding for the antioxidant enzyme superoxide dismutase (SOD). Noteworthy, SOD sequences are relatively rare in *Lactobacillus* genus and occur only in some strains of *Lb. sanfranciscensis*, *Lb. sakei*, *Lb. curvatus* and *Lb. paracasei* species (Yamamoto et al., 2011; Zotta et al., 2017). This is the first time that *sod* gene was annotated in *Lb. gasseri* genome. Moreover, the genome analysis also revealed the presence of large pattern of genes involved in oxidative stress resistance mechanisms. In both genomes, in fact, genes encoding for NADH oxidase, NADH peroxidase (NOX, NPR), Dps-like peroxide resistance protein (DPR) and for the complete thioredoxin-thioredoxin (TrxA/TrxB) reductase system, were found. NOX and NPR are the main enzymes involved in the NAD(P)-dependent H_2O_2 scavenging pathway in LAB, however, the occurrence of NOX gene is very limited among lactobacilli compared to NPR (Zotta el al., 2017). Thus, the presence of *npr*, *trxA*, *dpr* and *sod* genes could explain the peculiar resistance of AL3 and AL5 strains toward H_2O_2 and menadione observed in our previous study. Regarding to glutathione (GSH) synthesis, the strains can be able to perform only the first reaction of the GSH synthesis and

regenerate glutathione from its oxidized form (glutathione disulfide, GSSH) because only *gor* and *gshA* genes, compared to the complete genes pattern of GSH reductase system (*gshA*, *gshB*, *gor*, *gop*,) were found. Interestingly, unlike *trxA*, *trxB* and *gor* genes that are widely distributed among *Lb. gasseri* and other *Lactobacillus* genomes, *gshA* and *dpr* genes are absent in all *Lactobacillus* genomes (Zotta et al., 2017). Dpr is a member of the DNA-binding proteins from starved cells (Dps) that are able to provide cell protection during exposure to harsh environmental stress, including oxidative stress and nutritional deprivation. The function of Dpr has been the object of numerous studies and its role in acid and oxidative stress (iron and hydrogen peroxide detoxification) resistance in *Escherichia coli,* has been proposed (Calhoun et al., 2011). While, regarding to LAB, the role of Dpr in the oxidative stress response was reported only in some species of *Streptococcus* and in *Lactococcus lactis* (Pulliainen et al., 2003; Cesselin et al., 2011). Finally, in both genomes we found *soxR* and *soxS* genes. *SoxR* and *soxS* are adjacent genes and their critical role in transcriptional regulation of the defence system for oxidative stress has been extensively studied in *Escherichia coli* (Seo et al., 2015). However, at best of our knowledge no evidence on the *soxR* and *soxS* role in oxidative stress resistance mechanisms in LAB has been reported. In conclusion, genomic information can support the phenotypic evidences of *Lb. gasseri* AL3 and AL5 strains observed in our screening study. Draft genome sequence of *Lb. gasseri* strains with a detailed analysis of the genes pattern involved in aerobic and respiratory pathway, as well in oxidative stress resistance have not been published at the time. Therefore, the availability of these genomic data may be useful to confirm the promising features of *Lb. gasseri* AL3 and AL5 strains and to exploit them for further studies to understanding the aerobic and respiratory lifestyle of this species. Moreover, the addition of our findings to the currently available data set can extend the genomic evidence of aerobic lifestyle and oxygen tolerance in *Lactobacillus* genus.

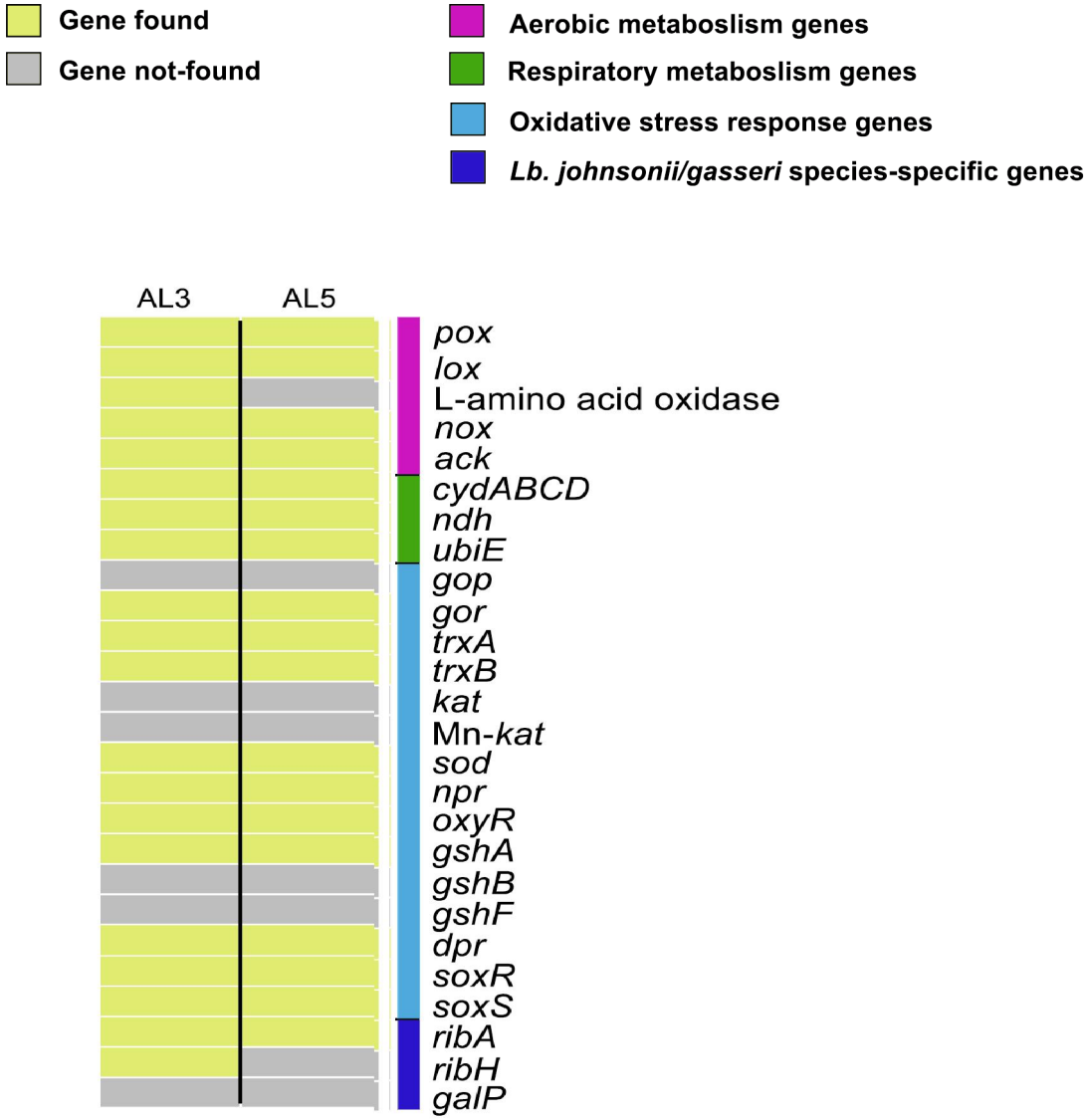

Figure 1. Graphical summary of genes presence/absence in the genomes of *Lactobacillus gasseri* AL3 and AL5.

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CHAPTER IV

Metabolic profiling and stress response of oxygen-tolerant *Lactobacillus gasseri* strains growth in batch fermentation

4.1 INTRODUCTION

The fermentative metabolism of lactic acid bacteria (LAB) has been intensively studied mainly due to its industrial relevance. However, in recent years several phenotypic and genotypic information indicated that many industrially relevant LAB are able to shift toward aerobic and respiratory metabolism (Zotta et al., 2017). Aerobic and respiratory pathway has been studied and characterized in several LAB species, allowing the selection of strains with improved technological and stress response properties. In particular, the advantages of aerobic and respiratory promoting growth have been successfully used by Chr. Hansen A/S industry that entered into a patent license to explore the respiration ability of *Lactococcus lactis* for the development and production of a new starter culture in presence of aeration and a porphyry compound (Petersen et al., 2005). However, the application of aerobic and respiratory metabolism in LAB can have a wide implication in three main field in LAB research: (*i*) starter and probiotic cultures production, (*ii*) development of food products with respirative phenotype, and (*iii*) human health application. The biochemistry of aerobic and respiratory metabolism in LAB suggest a series of complex mechanisms that act in unison to confer several advances on cell physiology. As described before, the lifestyle of the aerobic and respirative LAB results in a greater biomass-yield, growth-efficiency, stress robustness and a long term survival compared to that under fermentation condition. All these respiration-associated traits have a considerable industrial significance. Despite the advancements obtained on this topic research, less is known for the two closely related species *Lb. johnsonii* and *Lb. gasseri.* In our previous screening study, the adaptive response of promising probiotic *Lb. johnsonii/gasseri* strains to switch from fermentative to aerobic and respiratory metabolism and the effect of this metabolic pathway on oxidative stress response was investigated. Two strains (AL3 and AL5), belonging to *Lb. gasseri* species, were selected for their ability to grow under aerobic and/or respiratory promoting conditions and scavenge hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS). Genomic information supported the phenotypic evidences. Draft genome sequence analysis, in fact, revealed the presence, in both strains, of main genes involved in aerobic and respiratory metabolism as well as in oxidative stress response. At this research point we decided to investigate the adaptation to aerobic end respiratory metabolism of AL3 and AL5 strains in more controlled growth conditions using fed-batch cultivation. The metabolic profiles, in term of sugars consumption and metabolites production, were further investigated in order to provide additional biochemical evidences of a possible activation of aerobic and/or respiratory pathway. Moreover, the effect of this metabolic pathway on antioxidant activity and starvation stress resistance of the strains was evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Strains and culture conditions

Lb. *gasseri* AL3 and AL5 were routinely propagated in modified Weissella Medium Broth pH 6.8 (mWMB) (Zotta et al., 2012) and incubated in anaerobiosis at 37°C for 24 h.

4.2.2 Fermentation conditions

The growth of the strains was carried out in 1 L fermentation vessel (Applikon Biotechnology, Schiedam, the Netherlands) in mWMB at 37°C for 30 h, under different growth conditions: anerobiosis (AN, nitrogen flow at 0.1 vol/vol/min, stirrer speed 150 rpm), aerobiosis (AE, air flow at 0.1 vol/vol/min, stirrer speed 150 rpm) and respiratory (RS, AE growth supplemented with 2.5 µg/ml of hemin and 1 µg/ml of menaquinone) cultivations. Bioreactor was inoculated (2% v/v) with standardized (OD_{650}= 1.0) overnight anaerobic pre-cultures. Dissolved oxygen concentration (DO%) was measured using a polarographic electrode (Applikon Biotechnology). Fermentation parameters such as DO%, pH and temperature were controlled using ezControl controllers (Applikon, Schiedam, the Netherlands). The foam was controlled by adding at the start of the fermentation process 0.5 ml of Antifoam solution (Sigma-Aldrich). Growth of the strains under AN, AE and RS conditions was monitored by measuring the optical density of the cultures at 650 nm, (OD_{650}) at different time points (0, 3, 4, 5, 6, 7, 8, 9, 20, 22, 24, 26, 28 and 30 h). Moreover, after 0, 7, 9, 20, 22, 24 and 30 h of cultivation in the different growth conditions, samples were collected and used for HPLC analysis as described below.

4.2.3 HPLC analysis

Sugars consumption and metabolites production of *Lb. gasseri* AL3 and AL5 strains during AN, AE and RS cultivations were measured by HPLC analysis. An aliquot of 1 ml of AN, AE and RS cultures were collected at different time points and centrifuged at 13000 *g* for 5 min. The resulting supernatants were diluted in mobile phase (H_2SO_4 0.01 N) 1:5 (vol/vol) and filtered by AcroDisc millipore (0.2 µm). Sugars and metabolites were quantified by system (Gilson 307 Series HPLC system) fitted with column (MetaCarb 67 h column 6.5x300 mm, Varian) in an oven thermostated at 65°C. Column was eluted at 0.4 ml/min by a solution 1:9 (vol/vol) of H_2SO_4 in ultrapure water. A refractometer (RID 133, Gilson) was used as detector. Standards were used for quantification of different sugars s in the samples.

4.2.4 Tolerance to starvation stress

For both strains, cultures were collected at 20 h in AE condition and at 28 h in RS conditions. Samples were centrifugation (6500 g, 10 min)), washed twice in 20 mM potassium phosphate buffer pH 7 (PB7) and re-suspended in PB7 to obtain a final OD_{650} = 1. Tolerance to starvation was evaluated by storing cell suspensions at 4 °C and viable counting was performed on MRS Agar (Oxoid) at 0, 7, 14, 21 and 28 days.

4.2.5 Assay of scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazil) radical

The ability to scavenge DPPH by AL3 and AL5 under AE and RS conditions was evaluated in according to the protocols of Wang et al. (2009). For both strains, cultures were collected at 20 h in AE condition and at 28 h in RS conditions. Before the assay, cell suspensions were standardized to OD_{650} = 1.

4.2.6 Data analysis

Analyses were carried out in triplicate and all values were expressed as mean and standard deviation. Two-way Anova test and t-test analysis (Microsoft Excel for Mac version 11.5) were performed to evaluate significant differences ($P<0.05$) between averages.

4.3 RESULTS

4.3.1 Growth parameters and metabolites production

The growth curves of *Lb. gasseri* AL3 and AL5 strains in anaerobiosis (AN), aerobiosis (AE) and in respiration (RS) during fermentation experiments were obtained. All results shown below are related to the AL3 and AL5 growth in AE and RS conditions because both strains have not been able to grow in AN condition. The kinetics of growth, DO% and pH values of AL3 and AL5 during fermentation processes are shown in Figure 1 and 2, respectively. Results showed that the AE condition impaired the growth of AL3 compared to RS. In AE (Figure 1, panel A), AL3 has achieved the highest cell density (OD_{650}= 0.8) and was able to consume oxygen up to 20 h growth. Instead, in RS (Figure 1, panel B) AL3 continued to grow up to 28 h (final OD_{650} = 1.6) showing a high oxygen consumption in keeping with its growth. However, RS condition seemed to delay the entry in exponential phase for AL3 compared to AE condition. Moreover, in RS the growth curve of AL3 showed a like-diauxic trend. In AE, the growth of AL3 is associated with a decrease of pH values until the strain entered in stationary phase (20 h) (Figure 1, panel C). While, in RS condition a low decrease of pH was observed from 20 h onwards, despite the increased AL3 cell density (Figure 1, panel D).

AL5 strain showed the best growth performance in AE condition (Figure 2, panel A) compared to RS (Figure 2, panel and B). In AE condition, the growth of AL5 is associated with a significant decrease of both DO% (Figure 2, panel A) and pH (Figure 2, panel C) until the strain entered in stationary phase (20 h). In RS condition, the growth of AL5 continued from 20 h onwards but this growth has not been accompanied by oxygen consumption (Figure 2, panel B). Moreover, a further decrease of pH was observed after 20 h onwards (Figure 2, panel D). As shown in Figure 2 (panel B), even in this case, the growth curve of AL5 in RS condition showed a like-diauxic trend.

Results of the sugars consumption and metabolites production of AL3 and AL5 strains during their growth in AE and RS condition, are shown in Figure 3 and 4, respectively. In AE, AL3 strain was able to metabolized glucose, fructose and citric acid in the first 20 h of growth, and lactic acid was the main end product (2.3±0.1 g/L) (Figure 3, panel A). While, in RS condition (Figure 3, panel B), glucose was gradually metabolized until 30 h of growth, however, the highest lactic acid concentration (0.97 ±0.06 g/L) was produced at 20 h, and it was significantly lower than that found in AE condition (2.3±0.1 g/L). Moreover, unexpectedly, we also observed a significant production of acetic acid (1.56±0.05 g/L) from 20 h onwards. Regarding to AL5 strain in AE condition, glucose, fructose and citric acid was metabolized in the first 20 h, and a significant amount of acetic (1.85±0.08 g/L) and lactic acid (2.75±0.04 g/L) were produced (Figure 4, panel A). Unexpectedly, we also observed a significant consumption of citric acid (1.7±0.03 g/L). In RS condition (Figure 4, panel B), a lower glucose and fructose consumption was observed, compared to AE. Consequently, a lower concentration of acetic (0.89±0.05 g/L) and lactic (2±0.06 g/L) was produced. Noteworthy, no significant difference in citric acid consumption was found.

4.3.2 Survival of the cells during starvation stress and antioxidant capability

Results of viable counts of AL3 and AL5 strains after growth in AE and RS conditions are shown in Figure 5. In RS condition, only AL3 strain exhibited the highest tolerance of starvation condition, with no significant difference ($P<0.05$) in cell load after 28 h of storage. On the contrary, the AE cultivation strongly impaired the viability of the both strains. Viable count of aerobic AL3 and AL5 cultures decreased by 3 Log and 2 Log cycles, respectively. Similar results were found for the capability of the strains to scavenge DPPH radicals. As shown in Figure 6, the ability to remove DPPH radicals was affected by the different growth conditions. In RS, AL3 strain showed the greater degrading activity compared to AE condition. While, in AE both strains exhibited a low removal capability.

4.4 DISCUSSION

In our previous screening study, *Lb. gasseri* AL3 and AL5 strain were selected as respiratory-competent and aerobic phenotype, respectively. In this work the ability of AL3 and AL5 strains to shift towards aerobic and respiratory metabolism was further investigated in batch fermentations and the effect of these different growth conditions on growth performance and stress tolerance (oxidative and starvation) was evaluated. Moreover, the metabolic profiles were investigated in order to provide additional biochemical evidences of possible activation of aerobic and/or respiratory pathway. For AL3 strain, our results seemed to confirm its capability to grow better in RS condition and to activate a respiratory metabolism. In RS cultivation, AL3 showed increased biomass production, reduced acidification and oxygen uptake capability. The comparison between the kinetic of growth and kinetics of substrates consumption and metabolites production in RS condition could explain the simil-diauxic growth of AL3 strain. In the first 20 h of growth, glucose is metabolized to form mainly lactic acid and this could explain the observed pH decline. After this time, the glucose consumption continued but only acetic acid was produced. In fact, a very low decrease of pH was observed despite AL3 continued to grow and to consume oxygen. Taken together, these results suggest a double metabolism. The strain could grow first mainly via fermentation and then via respiration. Similar results were found by Duwat et al. (2001). These authors demonstrated that in *Lactococcus lactis* subsp. *lactis* the shift towards a respiratory metabolism was correlated to a biphasic metabolism. In particular, in respiration condition, *Lactococcus lactis* showed glucose consumption and acid lactic accumulation in the first 7 h of growth. On the contrary, after this time, a continued glucose consumption and subsequent reduction in lactic acid accumulation was observed. Moreover, high acetate level in respiratory cultures was found. Therefore, these authors suggest that respiration-like metabolism occurs only late in growth. These evidences could support our hypothesis. The activation of cytochrome *bd* oxidase (by heme addition) in the respiratory chain can explain the high oxygen consumption and the increased cell density (due to extra ATP generation) observed after 20 h of growth. Moreover, the production of acetate instead of lactate is one of typical metabolic changes associated with respiratory growth and it can have a beneficial effect on pH homeostasis (Duwat et al., 2001; Petersen et al., 2008; Guidone et al 2013). The ability to AL3 strain to shift toward a respiratory metabolism have also contributed to a remarkable improvement in survival under stress starvation and increased radical scavenging activity. This result suggest a strong link between respiratory metabolism and stress tolerance. The analysis of AL3 genome sequences revealed a large pattern of genes involved in oxidative stress resistance mechanisms, including superoxide dismutase (SOD), NADH peroxidase (NPR), complete thioredoxin-thioredoxin (TrxA/TrxB) reductase system, as well as, member of the DNA-

binding proteins from starved cells (Dps) that are able to provide cell protection during exposure to harsh environmental, ncluding nutritional deprivation. However, further studies are needed to better understand why AL3 showed these promising features only under RS condition. On the other hand, antioxidant activity and robustness to starvation stress was previously demonstrated in also respiration competent strains of *Lb. plantarum* (Zotta et al., 2013; Zotta et al., 2014), *Lb. casei* (Ianniello et al., 2016) and *L. lactis* (Razaiki et al., 2004; Casselin et al., 2010). Similarly, AL5 strain confirms the best growth performance in AE condition. According to results of draft genome analysis, AL5 possess genes predicted to encode for both POX and acetate kinase (ACK), the main enzymes involved in aerobic pathway. In presence of oxygen, the POX-ACK pathway activation can directly explain the observed physiological consequences, such as production to acetate (due to pyruvate conversion) and increase of OD due to extra ATP generation. Noteworthy, we observed a significant consumption of citrate acid. In AL5 draft genome we found sequences encoding for citrate lyase (CL), oxaloacetate decarboxylase (AOD), pyruvate carboxylase (PYC), malate dehydrogenase (MDH), and fumarate hydratase (FUM), the main enzymes involved in partial tricarboxylic acid (TCA) cycle of LAB. These genotypic data, therefore, can support the phenotypic evidence. In particular, the citric acid can be converted in oxaloacetate by CL activity and the oxaloacetate can be converted in pyruvate by AOD activity. The pyruvate, in turn, may be rerouted to lactate and acetate production and this could explain the presence of both end-products during the AL5 growth under AE condition. As described before, in RS condition, the growth curve of AL5 showed a like-diauxic trend. However, the possibility that the strain can activate a respiratory pathway is very limited, because, after 20 h of growth, the strain was not able to consume oxygen and lactic acid was the main end-product. Despite the aerobiosis was the best growth condition for AL5, the strain showed a very low resistance to stress starvation and a poor ability to scavenge DPPH radicals. It has been demonstrated that the activation of aerobic pathway (POX-ACK) and/or other enzymes involved in oxygen utilization (NADH oxidase lactate oxidase) may result in the high production of hydrogen peroxide (H_2O_2). Hertzberger et al. (2013) have found that the endogenous production of H_2O_2 is main cause of oxidative stress in *Lb. johnsonii* NCC 533 probiotic strain during its aerobic growth. On the other hand, in our previous study AL5 strain showed H_2O_2 robustness only under respiration condition. Moreover, in the draft genome sequence we not found sequence encoding for catalase or pseudocatalase enzyme, therefore, the possible toxic effect of H_2O_2 accumulation can explain the decreased AL5 survival during long term storage. In conclusion, this work contributed to major understating of the adaptive response of AL3 and AL5 strains to aerobic and respiratory metabolism and can provide important progress in the knowledge of this topic research field. To date, this is the first study in which the aerobic and respiratory

growth was evaluated in *Lb. gasseri* strains under batch fermentation. We demonstrated that both strains were not able to growth under anaerobiosis, their typical growth condition. During growth in batch fermentation, several differences in growth kinetics and in metabolite profiles between the different growth conditions were found. These results clearly suggest that several metabolic changes were underlie of the physiological characteristics observed. Moreover, this study evaluated for the first time the effect of respiratory conditions on the long term survival and radical scavenging activity in *Lb. gasseri* strains. We demonstrated that a possible activation of respiratory pathway can provide several advantages, such as improved biomass production and robustness of respiration-competent AL3 strain to oxidative and starvation stress. This features may have relevant technological consequences. In particular, the starvation stress tolerance and the antioxidant capability of some LAB are gaining very interest because of their implication in several technological and health-promoting applications. It has been noted that, the presence of oxygen in the growth environment of probiotic bacteria is one of the main factors affecting cell survival due to the induction of lethal oxidative damages. Moreover, recent studies reported that among probiotic health-promoting effects, the protection against oxidative stress and the ability to decrease the risk of reactive oxygen species (ROS) accumulation have a remarkable relevance (Amaretti et al., 2013; Zhang and Li, 2013). In this direction, the selection of specific strains with antioxidant capability can be exploited to formulate novel probiotic foods or supplements that can exert a role in the control of several free radical-related disorders. However, a further investigation on the genetic basis underlie the respiratory pathway and related stress response are needed. The set of all these results could allow significant progress in the understanding the cellular mechanisms involved aerobic and respiratory metabolism in homofermentative lactobacilli with promising scientific and applicative impact.

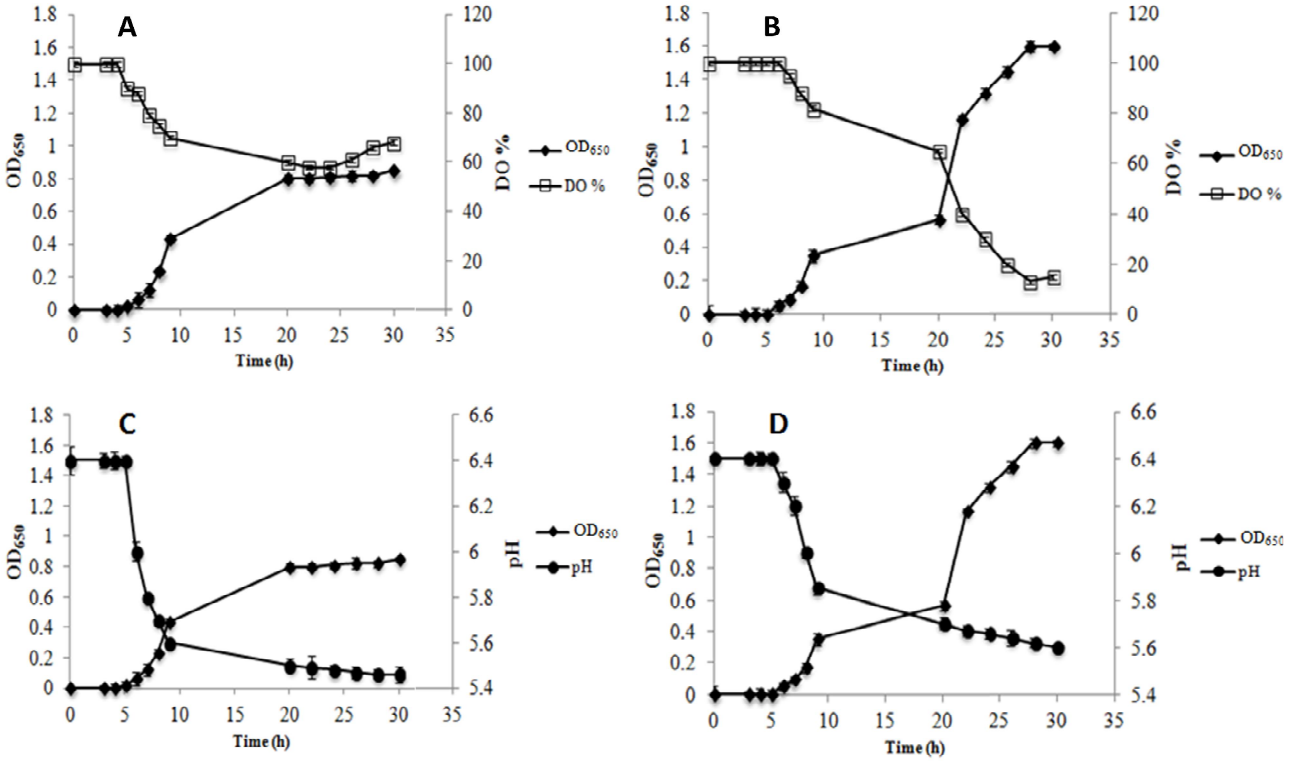

Figure 1. Kinetics of growth and dissolved oxygen concentration (DO%) of *Lb. gasseri* AL3 strain under aerobic (AE, panel A) and respiration (RS, panel B) conditions.

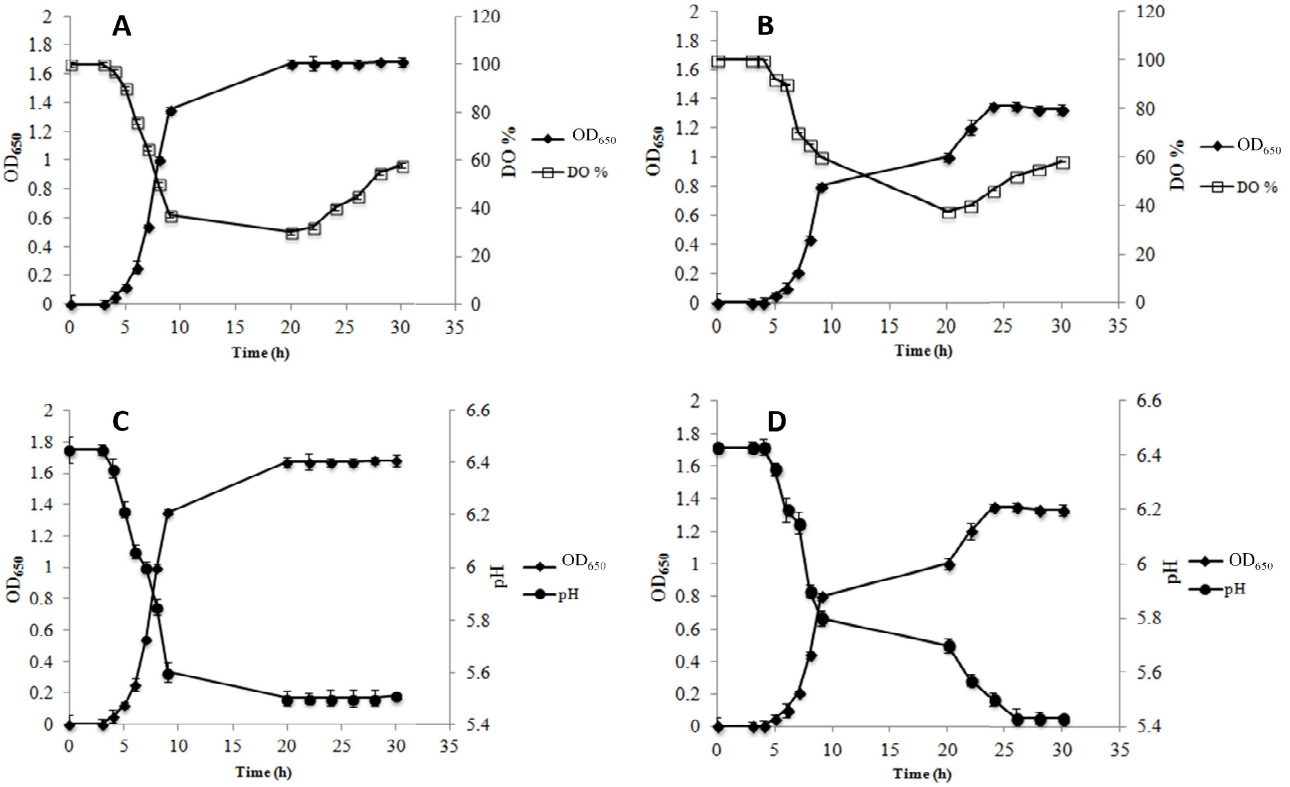

Figure 2. Kinetics of growth and dissolved oxygen concentration (DO%) of *Lb. gasseri* AL5 strain under aerobic (AE, panel A) and respiration (RS, panel B) conditions.

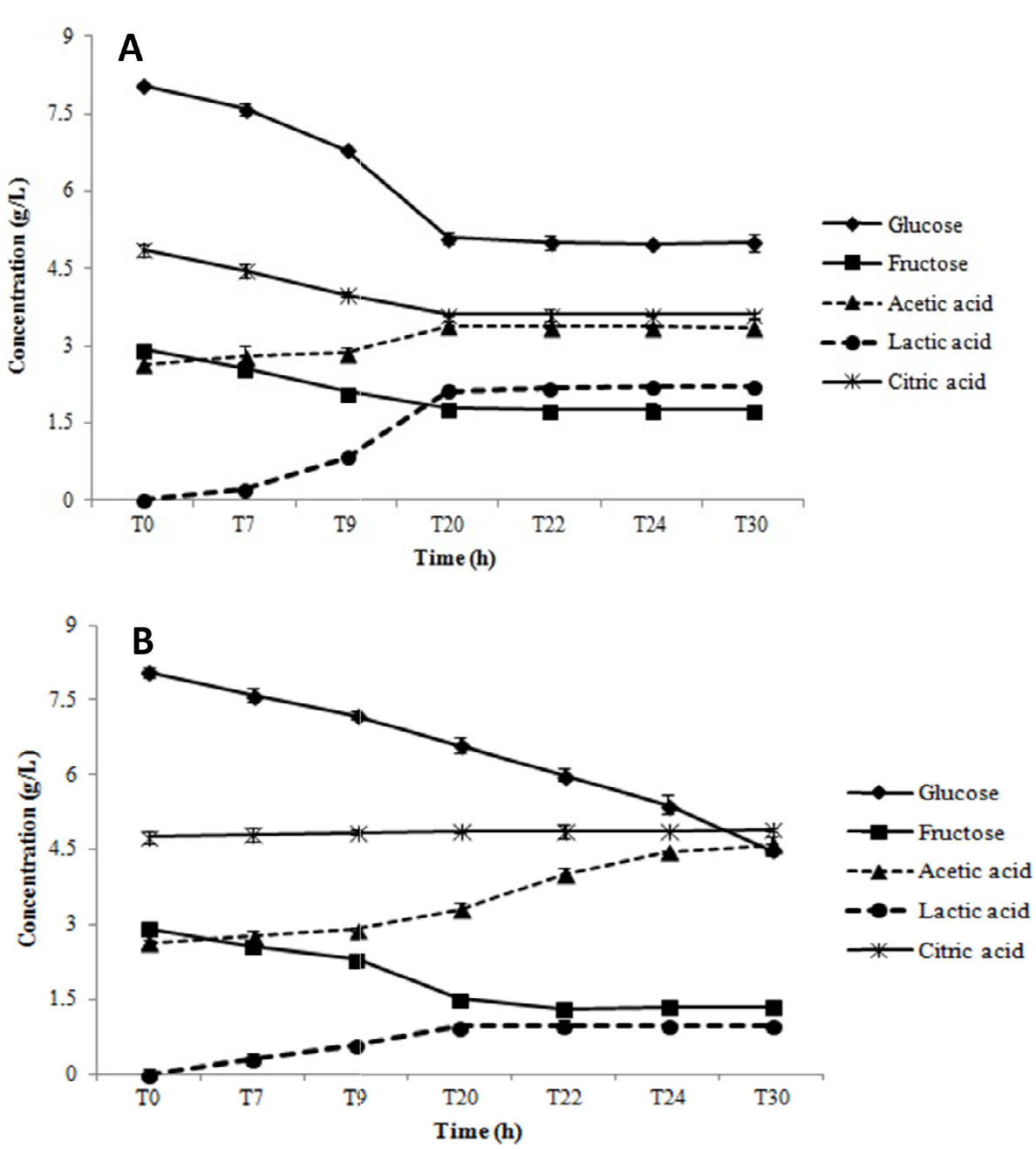

Figure 3. Sugars consumption and metabolites production of *Lb. gasseri* AL3 strain under aerobic (AE, panel A) and respiration (RS, panel B) conditions.

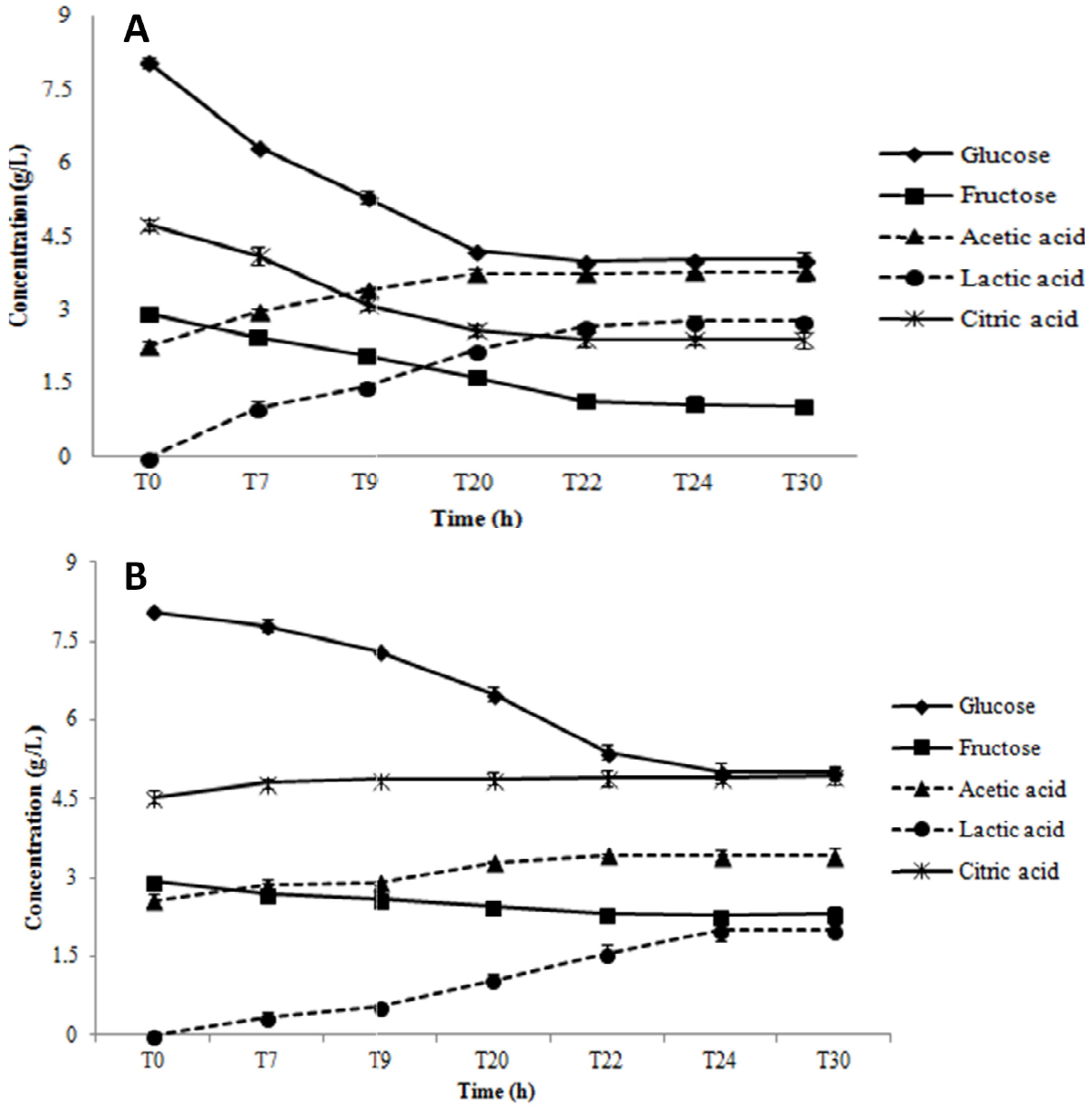

Figure 4. Sugars consumption and metabolites production of *Lb. gasseri* AL5 strain under aerobic (AE, panel A) and respiration (RS, panel B) conditions.

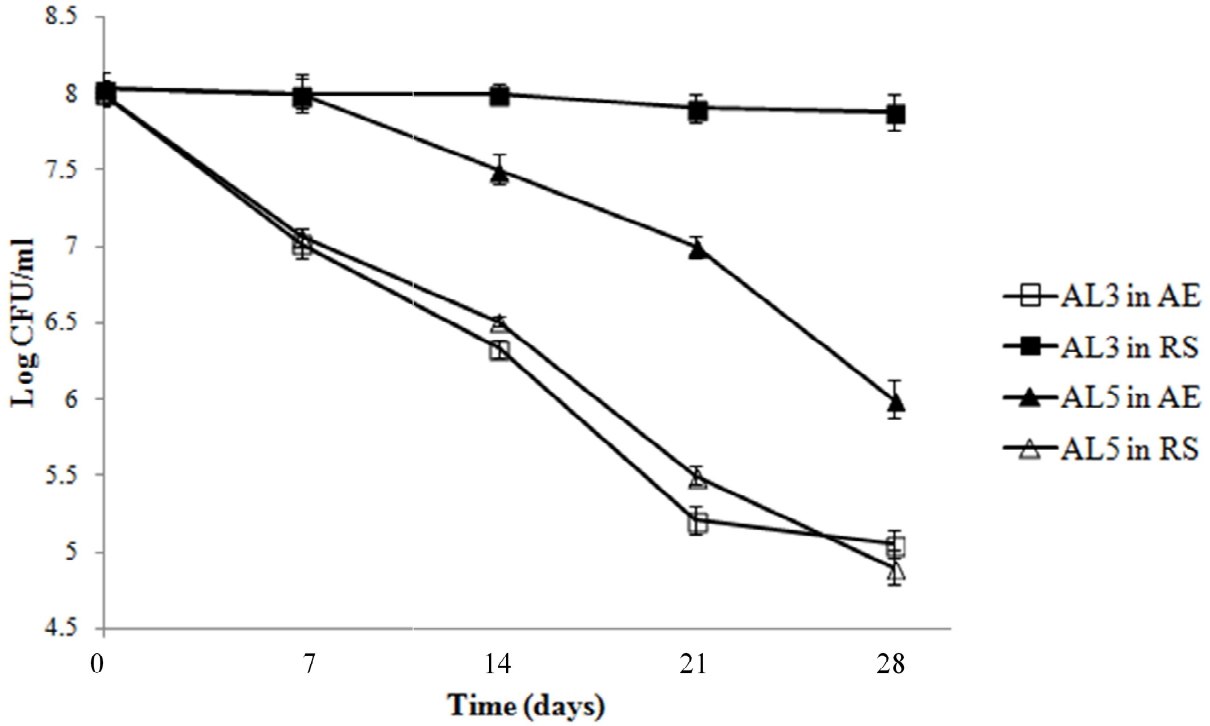

Figure 5. Viable counts (Log CFU/ml) of *Lb. gasseri* AL3 and AL5 strains, cultivated under aerobic (AE) and respiration (RS) conditions, along 28 days of starvation stress.

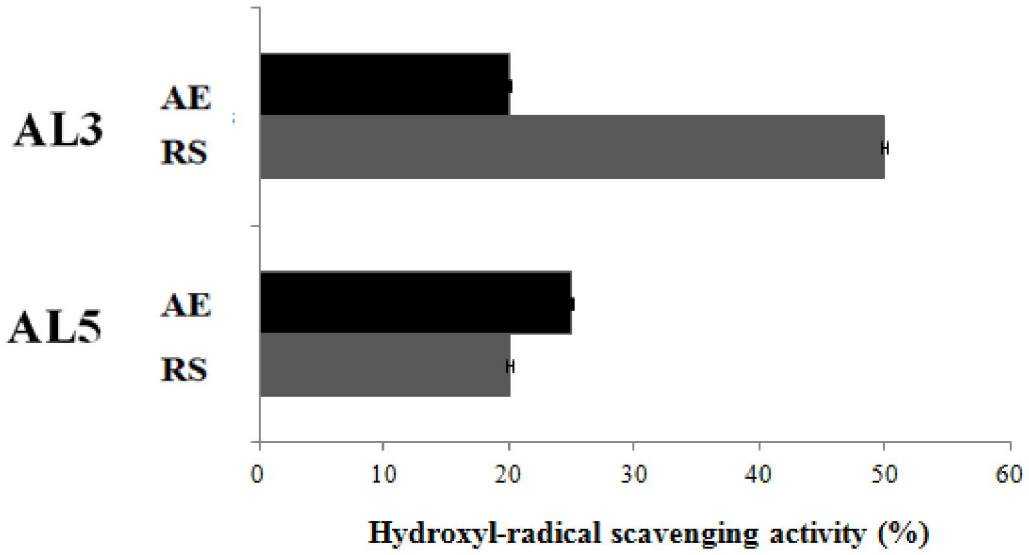

Figure 6. Percentage of hydroxyl radical scavenging activity in *Lb. gasseri* AL3 and AL5 strains cultivated under aerobic (AE) and respiration (RS) conditions.

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5. CONCLUSION

Lb. johnsonii and *Lb. gasseri* species are closely related bacteria dominant in human gut and in vaginal microbiota. These species have received particular attention due to their reported technological and probiotic properties. *Lb. johnsonii* and *Lb. gasseri* species are generally described as strict anaerobes with fermentative energy metabolism (Pridmore et al., 2003).

It was been noted that, the performance and robustness of LAB, including probiotic bacteria, can be compromised by exposure to various environmental stresses including acid, cold, drying, starvation, oxidative and osmotic stress that affect the physiological status and the functional properties of the bacterial cells. In particular, the accumulation of toxic oxygen metabolites can cause proteins damage, DNA mutations and membrane phospholipids oxidation, resulting in cellular death (Amaretti et al., 2013). To overcome the harmful effects, some LAB have developed various defense systems. Several authors have demonstrated that the growth condition and the type of metabolism significantly affect the stress responses in LAB. In particular, it was been demonstrated that in several LAB species the shift from fermentative to aerobic and respiration metabolism is associated with higher cell yield, robustness and lower oxidative stress. In this contest this PhD thesis was focused on the study, the understanding and the exploitation of aerobic and respiratory metabolism in *Lb. johnsonii* and *Lb. gasseri* species.

In our first study, thirty four *Lb. johnsonii/ gasseri* strains were isolated from best feed baby stools and screened for their ability to grow under aerobic and respiratory conditions. Moreover, oxidative stress response and functional features (i.e. survival to simulated oral-gastrointestinal transit and antimicrobial activity) were also evaluated in order to select new promising probiotic strains. We found a large diversity on the capability to grow in presence of oxygen and/or respiratory cofactors within the *Lb. johnsonii* and *Lb. gasseri* strains. As expected, most of strains grew better under anaerobiosis condition, while, remaining strains were able to cope whit aerobic condition. For many strains the aerobic and respiratory condition conferred several physiological advantages such as, increased biomass production, oxidative stress resistance and prevention of oxygen accumulation. Results allowed the selection of promising probiotic strains with antioxidant capability and respiratory and/or aerobic phenotypes. The strains *Lb. johnsonii/gasseri* AL5 and AL3 were selected as aerobic and respiratory phenotype, respectively, and used as model for the study of the genetic basis involved in aerobic and respiratory metabolism. Therefore, whole-genome sequencing of both strains was performed. The results of genome analysis revealed that both strains have the genetic potential for the activation of aerobic metabolism (presence of *pox* and *ack* genes involved in aerobic conversion of pyruvate to acetate), respiratory pathway (presence of genes involved in minimal electron transport chain activation), as well as to tolerate oxygen for the presence of main

flavoproteins oxidase (*nox*, *lox*). The analysis of genome sequences has also allowed the identification of genes with a very low % of occurrence (*lox*, *nox*, *ndh*, *npr* and *gop*) and genes (*sod, gshA, dpr*) that have never been found in *Lb. johnsonii* and *Lb. gasseri* genomes. Moreover, analysis of genome sequences revealed that both strains belong to *Lb. gasseri* species. The selected *Lb. gasseri* AL3 and AL5 strains were further used to study the basic mechanisms of the aerobic and respiratory metabolism. In particular, the effect of aerobic and respiratory cultivation on the growth, oxidative and starvation stress tolerance, antioxidant activity and metabolic profile were investigated during batch fermentations. Results contributed to major understating of the adaptive response to the aerobic and respiratory metabolism of AL5 and AL3, respectively. Moreover, this study evaluated for the first time the effect of respiratory growth on metabolites production, the long-term survival and radical scavenging activity in *Lb. gasseri* strains. We demonstrated that a possible activation of respiratory pathway can provide several advantages, such as improved biomass production and robustness to oxidative and starvation stress. In view of the above phenotypic and genotypic evidences, we provided important progress in the knowledge of this topic research. However, further studies need to be undertaken to exploit the respiratory phenotypes for the development of competitive starter and probiotic cultures for use in foods and/or in health applications. The preparation of starter or probiotic cultures under respiratory conditions can allows not only a greater biomass production but also more robust cultures with improved ability to survive during storage.