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GENOMIC AND FUNCTIONAL INSIGHTS INTO THE INTERACTIONS BETWEEN VAGINAL *LACTOBACILLUS* STRAINS AND PATHOGENS

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Abstract

The vaginal microbiota of healthy pre-menopausal women is typically dominated by one *Lactobacillus* species among *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners*. Thanks to a series of antimicrobial activities, strains belonging to these species represent the first barrier against infections and maintain niche homeostasis. On the other hands, the increase abundance in pathogen species is associated with the onset of numerous diseases, leading also to an increase risk of other infections acquisition. The deciphering of factors which influence *Lactobacillus* survival, as well as the interactions between lactobacilli-pathogens and pathogens-pathogens represent an important topic of study for improving woman health and investigating effective probiotic strategies.

Here, we investigated environmental factors and genetic traits that lead to the dominance of either *L. crispatus* or *L. gasseri* in the vaginal niche and the possible applications of liposomes loaded with *L. gasseri* biosurfactants for the treatment and prevention of *Staphylococcus aureus* biofilm infections. Furthermore, considering the increasing relevance acquired by bacterial extracellular vesicles (EVs) we analysed the role of EVs derived from vaginal lactobacilli and pathogens on both bacterial growth and HIV-1 infections. As a result, we reported for the first time i) common and species-specific genotypic and phenotypic features of *L. crispatus* and *L. gasseri* ii) significant antibiofilm activity of liposomes loading vaginal *Lactobacillus* biosurfactants against multi-drug resistant *S. aureus* strains iii) absence of growth regulation mediated by EVs derived from lactobacilli on pathogen cultures and vice versa iv) anti-HIV-1 activity of protein derived from *L. gasseri* EVs and unexpected antiviral effect of pathogen-derived EVs on HIV-1 infections *in vitro*. In conclusion, this PhD thesis explored characteristics and possible applications of vaginal lactobacilli for the human health, as well as promising antiviral effects of both lactobacilli and pathogen derived EVs.

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1. General introduction

1.1 Vaginal microbiota

The vaginal niche hosts several microorganisms which establish complex interactions and synergies within each other and with the host. The microbial community that colonises this niche is subject to deep rearrangements during women's life and strongly impacts on host health, influencing their physiology, immunity and onset of several diseases [1,2]. Indeed, the vaginal microbiota can prevent common human opportunistic pathogens through the creation of a selective environment and the secretion of different molecules[3]. The deciphering of vaginal microbiota composition, cross-sectional study done in around 400 healthy pre-menopausal women, revealed that there are five principal community groups, four of them are dominated by *Lactobacillus* species (I, II, III,V) while one is rich of facultative and strictly anaerobe bacteria (IV) [4,5]. This latter group IV includes a mixture of bacteria belonging to *Prevotella*, *Gardnerella* and *Atopobium* genus and has the highest Shannon diversity index as compared to the other four groups. Indeed, community groups I, II, III, and V are dominated by only one of the species of *Lactobacillus* (>50%) in particular, *L. crispatus* (I), *L. gasseri* (II), *L. iners* (III) and *L. jensenii* (V) [4,6,7] (Fig. 1). Overall more than 75% of women are dominated by *Lactobacillus* species and the frequency of each community group have been demonstrated to be associated with ethnic and geographical factors [4,8,9]. The vaginal microbiota of white and Asian women are more frequently dominated by community groups I and III, while group IV is more frequently found in both black and Hispanic women. To notice, women belonging to the group IV showed microbial communities similar to those detected in women affected by bacterial vaginosis; furthermore, the group IV microbial composition was associated to enhanced risk of bacterial and viral sexually transmitted infections[10–13]. Consequently, the presence of group IV might not be a symptom of bacterial vaginosis, but it is clearly associated with enhance risk of infections onset.

The highly dynamism of vaginal microbiome was demonstrated by longitudinal studies in which the microbial composition was evaluated twice every day for four or ten weeks [14,15]. The results of these studies highlighted that the community group transition varies individually, reporting some women with low or absent group transition and others with frequent group transition, including the group IV which is dominated by anaerobic bacteria. Some of these transitions were detected more frequently for example, transition from *L. crispatus* to *L. iners* as dominating group, while others are less frequent or absent (*L. gasseri* group ↔ *L. crispatus* group) [6,15]. The fluctuations from group to group were in some cases linked to sexual activity or menstruation while, in other cases, no specific factors were linked to group shifts, suggesting the involvement of still unknown *Lactobacillus* interaction

mechanisms [6]. These temporal dynamics studies pointed out the elevated variability of the vaginal microbial community and evidenced the need to identify crucial factors involved in microbial composition fluctuations.

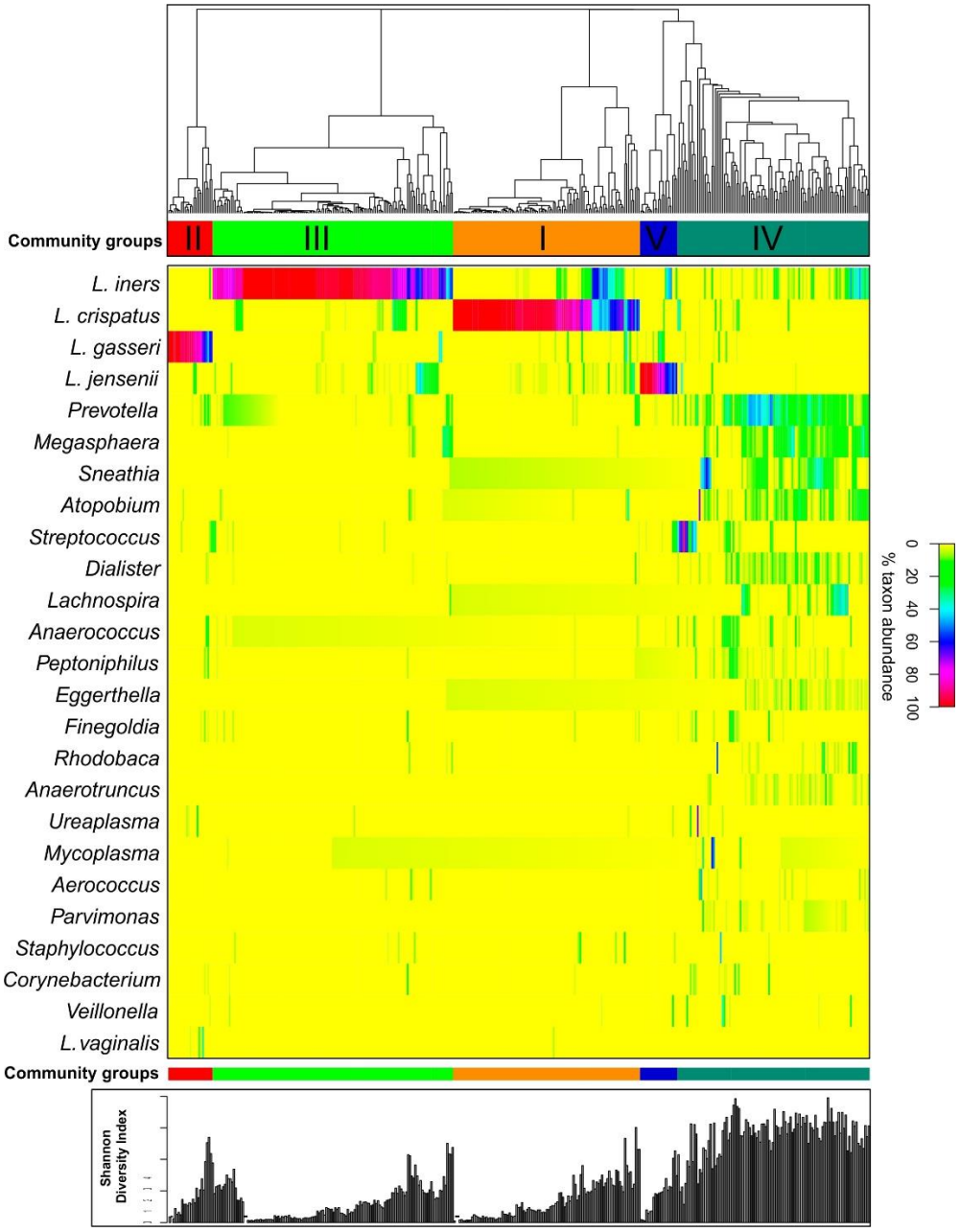

Fig. 1. Microbial taxa composition generated sequencing the 16S rRNA gene amplified from vaginal swabs collected from 394 healthy premenopausal women. From the top down, the figure panels show the clusters created on the basis of species abundance in the community, community groups, heatmap with % of taxon abundance and the Shannon diversity index calculated for each vaginal microbiota analysed. Modified from [4]

1.2 Vaginal lactobacilli

Historically, the vaginal niche of pre-menopausal women was considered healthy when populated by *Lactobacillus* spp. which inhibit the overgrowth of pathogens and prevent the colonisations of different opportunistic microorganisms [16]. However, the assumption that vaginal microbiota dominated by lactobacilli is considered as "normal" in healthy women has been questioned in the last years, as suggested by detection of community group IV, non-dominated by lactobacilli, in 25% of women [4,5,17]. Moreover, the abundance of *L. iners* strains (group III) was correlated with higher risk of bacterial vaginosis development [18,19]. Some strains of *L. iners* are also equipped with pathogenic gene clusters encoding for inerolysin, cytolisin and an enzyme that causes epithelial cell pore formation, which are also upregulated during bacterial vaginosis [20,21]. Consequently, it is more accurate to conclude that vaginal microbiota dominated by *L. crispatus*, *L. gasseri*, and *L. jensenii* are typical of healthy women with lower risk of infection development. Overall, all these three species were demonstrated to be equipped with different weapons for the inhibition of bacteria, fungi, and virus. Moreover, they also prevent the overgrowth of pathogens that are usually present in the vaginal microbiota (i.e. *Gardnerella vaginalis*), demonstrating their crucial role for women's health [16,22].

1.3 Beneficial properties of vaginal lactobacilli

The main health promoting effect of *Lactobacillus* spp. is exerted by the homolactic fermentation of sugars and the production of lactic acid that is correlated with the acidic environment in the female lower genital tract. Several studies highlight that lactic acid in combination with low pH values directly inhibits the colonization of *Chlamydia trachomatis*, *Candida albicans*, as well as Herpes Simplex Virus 2 (HSV-2), Human Immunodeficiency Virus 1 (HIV-1) and a wide range of microorganisms associated to bacterial vaginosis [23–26]. Moreover, lactic acid could exert an indirect antimicrobial effect inducing immunomodulation on vaginal epithelial cells [27,28]. In addition to the *Lactobacillus* fermentation, lactic acid could be also produced by host cells, which release mainly L-lactate while D-isoform is produced in low quantity as methylglyoxal pathway results [29]. In contrast, *L. crispatus* and *L. gasseri* produce high amount of both the lactic acid isomers, while *L. jensenii* secrete only the D-isomer. *L. iners* differs between the other 3 species for its incapability to produce D-lactate and to secrete low amount of L-isoform [30]. High levels of L- and D-lactate were demonstrated to protect from bacterial genital infections and also from HIV-1 acquisition. D- isoform increase HIV-1 virions entrapment in vaginal mucus, while in microbiota dominated by *L. iners* and with low levels of D-lactic acid, HIV virions diffuse faster [23,30,31]. Additionally, the absence of D-lactic acid was observed in vaginal fluids of women affected by bacterial vaginosis and vulvovaginal

candidiasis [32]. These reports suggest that microbiota dominated by *L. crispatus*, *L. gasseri* and *L. jensenii*, but not *L. iners* community group, generally possess high levels of D-lactate with a consequent protection from pathogens infections.

Another important metabolite produced by almost all vaginal lactobacilli (96-98%) is hydrogen peroxide, however its antibacterial and antifungal properties were only demonstrated *in vitro* [33–36]. In this regard, Martin and Suarez questioned if H_2O_2 exerts a real protection against pathogens *in vivo* [37]. The main point about their objections is that lactobacilli produce H_2O_2 only if cultures are well oxygenated and that iron (or iron containing compounds) degrades peroxide hydrogen. Considering these aspects, it is difficult to believe that *Lactobacillus* spp. could produce high amount of H_2O_2 in a microaerophilic environment such as vaginal niche. Moreover, this environment is enriched in iron ions and iron-containing proteins and molecules which could degrade H_2O_2 [37].

Metabolites are not the only protective molecules secreted by vaginal lactobacilli; indeed, it was demonstrated that they also produce proteins and glycoproteic molecules (i.e. bacteriocins and biosurfactants) capable to inhibit the colonisation and overgrowth of undesirable microorganisms. Bacteriocins are small antimicrobial peptides encoded by toxin-antitoxin gene clusters that exert their function through pore formation on bacterial membrane and/or cell wall permeabilization [38]. It has been shown that several strains of vaginal lactobacilli, in particular *L. crispatus* and *L. gasseri*, secrete different bacteriocins (i.e. lactococcin, acidocin, gassericin and others bacteriocins of class IIa and IIc) with inhibitory activity against different opportunistic and commensals pathogens such as, *G. vaginalis*, *C. albicans*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Klebsiella* spp [39–42]. Biosurfactants are amphipathic compounds extracellularly secreted that play a major role in the microbial physiology of the producer strains, such as biofilm formation and development, adhesion, quorum sensing, and competition with other organisms [43]. This latter aspect was usually achieved by inhibiting the adhesion of pathogens, therefore, decreasing their biofilm formation [44,45]. For instance, strains of *L. jensenii* and *L. gasseri* produce biosurfactants that can inhibit *Neisseria gonorrhoeae* and *C. albicans* adhesion to host cells or inhibit planktonic and biofilm growth of *E. coli*, *Staphylococcus saprophyticus*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* [46–48]. In addition to the reduction of pathogen adhesion on host cell exerted by biosurfactants, vaginal lactobacilli cells strongly compete with pathogens for adhesion sites on host-cell surface [49]. *Lactobacillus* adhesion to epithelial cells is mediated by the recognition of host cell-receptors and the production of different molecules (including biosurfactants). Overall, the affinity of lactobacilli to adhere for host receptors was demonstrated to be higher than pathogens in the vaginal niche and they are also capable to displace pathogens already bonded to epithelial cells [49–52]. Several *in vitro* experiments demonstrate that vaginal lactobacilli exert competitive exclusion inhibiting the adhesion

of several pathogens (i.e. *Trichomonas vaginalis*, *Pseudomonas aeruginosa*, *G. vaginalis*, *S. aureus*, *K. pneumoniae* and *E. coli*) to epithelial cells [49,53–55]. Furthermore, the adhesion inhibition and killing of undesirable microorganisms is also mediated by co- aggregation mechanisms [49,56]. Indeed, some strains of *Lactobacillus* such as, *L. crispatus*, *L. gasseri*, and *L. jensenii*, co-aggregate with *E. coli* cells and inhibit their proliferation [56]. Through these mechanisms, *L. gasseri* and *L. brevis* also affect the growth and adhesion of *G. vaginalis* and *C. albicans* [49].

In the last years it was also proved that lactobacilli can exert beneficial properties to the host via the secretion of extracellular vesicles (EVs). EVs are bilayer-enclosed particles secreted by both Gram negative and Gram positive bacteria in the extracellular environment, which recently gain attention for their multifunctionality in the bacterial physiology [57,58]. In terms of beneficial effects exerted by lactobacilli EVs it was proved that some strains of *Lactobacillus* secrete EVs with immunogenic and antiviral properties [59,60]. Indeed, *L. plantarum* EVs were demonstrated to enhance host-immune response against antibiotic resistant *E. faecium* strains [60]. In addition, Ñahui Palomino et al. demonstrated that EVs derived from *L. crispatus* and *L. gasseri* inhibited HIV-1 infection in human cell lines and human cervico-vaginal and tonsillar *ex-vivo* tissues [59].

1.4 Main gynaecologic infections

Different gynaecologic infections could affect women across their life spam causing numerous symptomatic or asymptomatic diseases that may lead to severe consequences depending to several factors such as, the type of pathogen, infection duration and so on (Fig. 2). Bacterial vaginosis is the most common vaginal disease and can cause severe effects on women health, such as infertility, endometritis and abortion [61,62]. Moreover, women affected by bacterial vaginosis have a higher risk to be infected by both bacterial (*N. gonorrhoeae*, *C. trachomatis*) and viral (HIV, HSV2) sexually transmitted pathogens [63,64]. The onset of this disease is still a mystery but it has been shown that it is characterised by the reduction of *Lactobacillus* spp. population and increase abundance of anaerobic bacteria [65,66]. A similar condition is observed also during aerobic vaginitis, but in this case, there is an overgrowth of aerobic bacteria mainly belonging to the genera *Staphylococcus*, *Streptococcus*, *Escherichia* and *Enterococcus* [67].

Another common gynaecological infection is the vulvovaginal candidiasis (VVC), which was estimated to have onset on 75% of women at least one time during their life. Overall, VVC can be classified as uncomplicated, which cause rare infections and moderate effects, or complicated [63]. This latter class affects 10-20% of women and is usually associated with severe symptoms and recurrence of the infection [68]. Almost all the uncomplicated VVC cases and at least the 70% of complicated VVC are caused by *C. albicans*, while only a minor percentage of cases is associated to different *Candida*

species [63,69]. *Candida* species are generally present as commensals in the intestinal and vaginal niche in more than 50% of people and under this condition it usually grows as ovoid yeast cells [70,71]. However, when its environment changes due to endogenous or exogenous factors (i.e use of antibiotics, estrogen based drugs, contraception or glycosuria induced by diabetes), *Candida* can grow as mycelium and this type of growth is associated with VVC [70–73]. In the vaginal niche *Lactobacillus* spp. can compete with *Candida* through different mechanisms described above; however, whether a *Lactobacillus* dominated niche prevents *Candida* colonization is still under analysis due to contrasting results from different studies [9,74,75].

C. trachomatis and *N. gonorrhoeae* cause the two most common bacterial sexually transmitted infections, with an estimation of 100 million and 78 million of new cases every year worldwide, respectively [76,77]. Remarkably, it has been observed that women with *C. trachomatis* infections are frequently co-infected with *N. gonorrhoeae,* making the eradication of both infections more difficult and with higher risk for the health [78]. In women, *C. trachomatis* and *N. gonorrhoeae* infections are frequently asymptomatic and can cause cervicitis, infertility, ectopic pregnancy, and pelvic inflammatory disease if they are not promptly and adequately treated [76,79]. Untreated *N. gonorrhoeae* infections can also lead to gonococcal dissemination with consequent bacteraemia and, occasionally, sepsis. Moreover, it was demonstrated that patients infected with either *C. trachomatis* or *N. gonorrhoeae* are more prone to HIV-1 seroconversion [79,80].

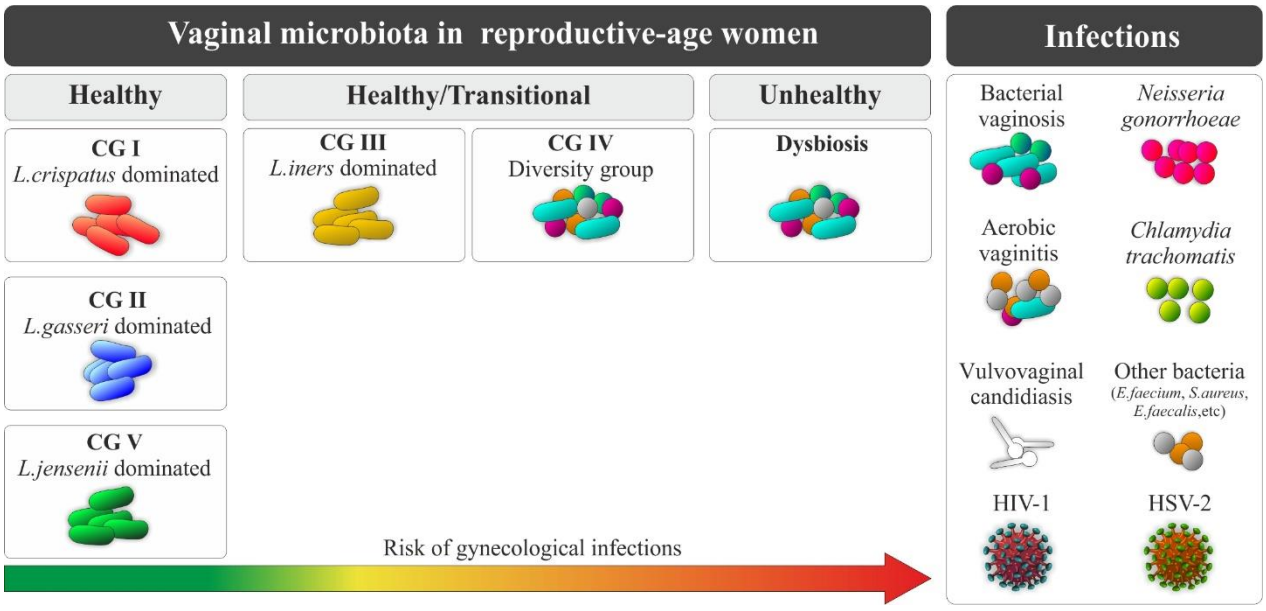

Fig. 2. Main community groups (CG) present in the vaginal niche in correlation with risk of human pathogen infections. CG I, II, and V are considered more stable and are normally associated with lower risk of infections. CG III easily shifts to CG IV or dysbiosis conditions, which have both higher risk of gynaecological infections development.

Vaginal microbiota dominated by *Lactobacillus* spp. was indicated by different studies as protective against both *C. trachomatis* and *N. gonorrhoeae* infections [81].

HIV (Human immunodeficiency virus) is a retrovirus belonging to the family of *Retroviridae*, genus lentivirus. HIV is the etiologic agent of acquired immunodeficiency syndrome (AIDS). AIDS patients present serious immunodeficiency, which could led to central nervous system and degeneration, organ degeneration, cancer and several type of infections. Two different HIV viruses exist: HIV-1, and HIV-2. Among the two groups, HIV-1 is the pandemic species and have higher virulence and infectivity than HIV-2, which is most prevalent in the west Africa [82]. Both species are associated to the sexually transmitted infections and epidemiological studies suggest that women are more infected than men. Sexual transmission of HIV-1 in women happens through the exposure of genital mucosa to HIV-1 virions, which could bind, through gp120 protein, cells expressing CD4 receptor on their surface. This initial binding induces a conformational change in the gp120 proteins with exposure of a second binding site specific for CXCR4 (CXC-chemokine receptor 4) or CCR5 (CC-chemokine receptor 5) [83]. This second binding allows the virus to release virion-content inside the target cell and the two copies of viral ssRNA are retrotranscribed in dsDNA. Finally, viral integrase allots the integration of HIV dsDNA into host genome, where it can remain silent for years or reactivate with consequent virion production [84]. Interestingly, it was observed a correlation between HIV-1 infections and another sexually transmitted virus, HSV-2 (Herpes Simplex virus II). HSV-2, has infected 25% of women in the USA and its infection in immunodeficient AIDS patient induces a reactivation of HIV-1 silent virus [63]. Overall, both HIV-1 and HSV-2 have higher sexual transmissibility and infectivity in women without *Lactobacillus*-dominated vaginal microbiota [85].

2. Genotypic and phenotypic characterization of *Lactobacillus crispatus* and *Lactobacillus gasseri*

This chapther is based on: Costantini PE, Firrincieli A, Fedi S, Parolin C, Viti C, Vitali B, Cappelletti M. Insight into phenotypic and genotypic differences between vaginal Lactobacillus crispatus BC5 and Lactobacillus gasseri BC12 to unravel nutritional and stress factors influencing their metabolic activity. Under review to Microbial Genomics

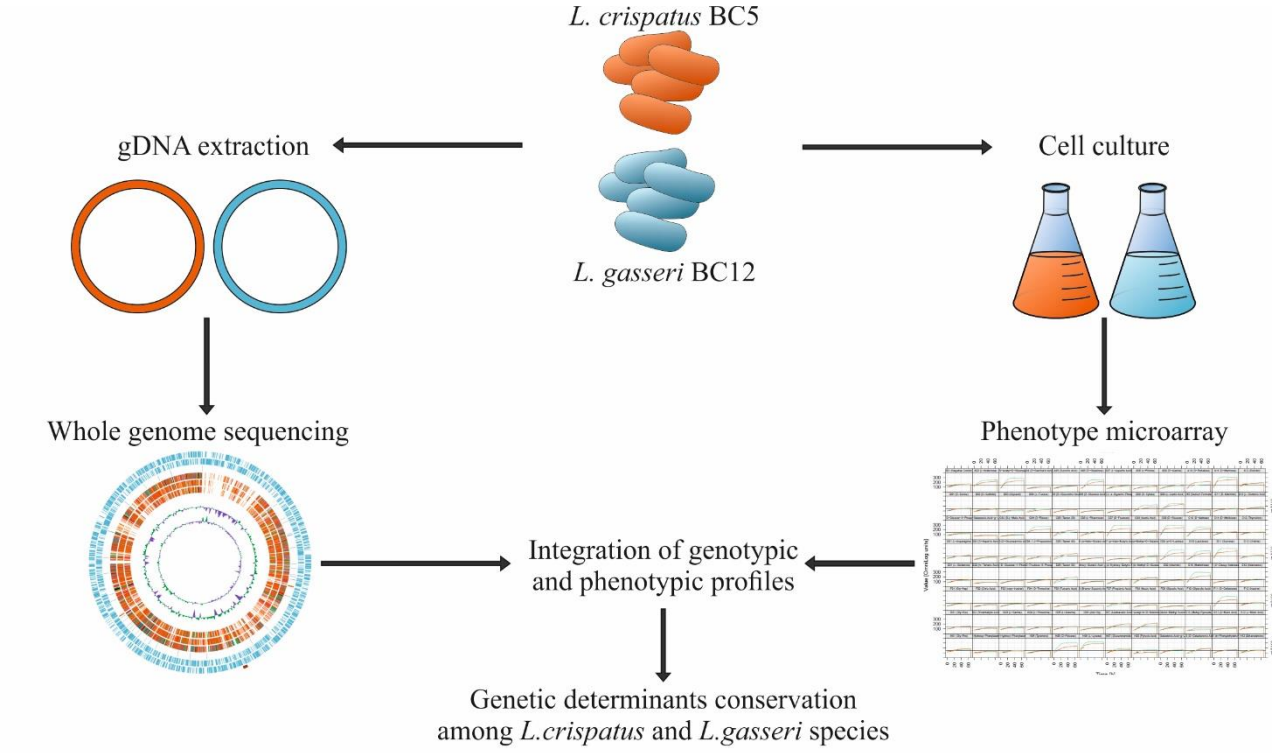

Fig. 3. Graphical abstract showing the approach used in the genotypic and phenotypic characterization of *L. gasseri* BC12 and *L. crispatus* BC5.

2.1 Introduction

The vaginal microbial community is composed of several microorganisms which establish complex linkage with the host and each other. In healthy reproductive-aged women, the vaginal environment is generally dominated by *Lactobacillus* genus and most women display the prevalence of one species among *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* [4]. Lactobacilli stimulate the preservation of vaginal homeostasis and protect their environment from both colonization and overgrowth of

different pathogens. Numerous studies demonstrated that several *Lactobacillus* species exert fungistatic and fungicidal activities against *C. albicans*, protection against numerous bacterial pathogens and inhibition of HIV-1 replication [51,86,87]. The heath promoting effect of lactobacilli has been associated with numerous activities for example, host immunomodulation, production of antimicrobials compounds, reduction of pathogens adhesion, maintaining the vaginal acidic environment, production of extracellular vesicles that can shuttle numerous bioactive molecules, etc. [59,88,89]. Consequently, the decrease of lactobacilli abundance is usually coupled with microbial community instability (drift from an eubiosis to a dysbiosis condition), and onset of different gynaecological pathologies [90].

The microbial community composition in the vaginal niche is highly dynamic and can be influenced by several external and internal factors such as, hormones levels, pregnancy, diet, geographical localization, urogenital infections, etc. [6]. As a result, the community remodelling could involve a change in the *Lactobacillus* pre-dominant species or the arise of pathogens and non-*Lactobacillus* genus. A better understanding of factors that lead to colonization and stability of *Lactobacillus* species in cervicovaginal tract is needed to developed strategies for woman health maintenance. In particular, despite several studies analysed and compared lactobacilli genomics [4,91,92], a full comprehension of efficiency in available nutrients utilisation and resistance to stressors is lacking. In this context, Phenotype Microarray (PM) is a high-throughput technology that allows the investigation of microbial growth in presence of several substrates, drugs, and chemical stressors [93]. The potential of this technology together with whole genome sequencing was previously applied for the characterization of bacterial species clinically and environmentally relevant [94–96]. In the present study, we applied the Phenotype Microarray technology and whole genome sequencing of two strains of vaginal lactobacilli, *L. crispatus* BC5 and *L. gasseri* BC12, that were previously characterized for their antifungal, antibacterial, and antiviral activity [86,87,97]. In particular, we aimed to compare their genomes to detect genetic/genomic traits supporting the metabolic activities revealed by Phenotype Microarray assays. The investigation of the genetic features associated with the PM results in BC5 and BC12 was also performed using an extended dataset including the genomes of any other strains of *L. gasseri* and *L. crispatus* available in the database. These types of investigations provide insights into the genotypic and phenotypic features supporting *L. crispatus* and *L. gasseri* growth in the vaginal niche, also giving indications on the nutritional and stress factors that might influence their beneficial colonization.

2.2 Methods

2.2.1 Bacterial strains use in this chapter

Table 1. List of bacterial strains used in this chapter.

Bacterial strain	Reference
Lactobacillus crispatus BC5	51
Lactobacillus gasseri BC12	51

2.2.2 Genomic DNA extraction

A singles colonies of *L. crispatus* BC5 and *L. gasseri* BC12 from pure culture plates were inoculated in 10 mL of de Man, Rogosa, and Sharpe (MRS) broth and incubated overnight at 37 °C, in anaerobic jar with Gaspak EZ (BD, Franklin Lakes, NJ). Two mL overnight cultures were used for genomic DNA isolation using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol "Pre-treatment for Gram-positive bacteria". DNA quantification, integrity and purity were checked by Nanodrop spectrometer 1000-ND and on agarose gel

2.2.3 Whole genome sequencing and annotation

The genome sequencing was achieved using Illumina MiSeq technology, kit V3, 2X300 PE run and sequencing reads were assembled using SPAdes (16). *L. crispatus* BC5 genome was assembled in 50 contigs with an N50 of 104,423 and genome coverage of 227x whereas, *L. gasseri* BC12 genome was assembled in 48 contigs with N50 of 160,908 and overall coverage of 358x. The assembly of *L. gasseri* BC12 sequencing reads produced, while proteins were functionally annotated using KOfamKOALA in combination with eggnog (17). Additionally, proteins not annotated with KOfamKOALA were further annotated in eggnog (17).

The identification of genes involved in antibiotic resistance and genetic variants associated to antibiotic resistance phenotypes were perfomed using the Comprehensive Antibiotic Resistance Database (CARD) in strict mode (18). Further, identification of protein family domains associated to antibiotic resistance genes was further investigated using the stand-alone version of InterProScan v5.45-80.0 (19). Manual curation was performed ad hoc based on literature information.

The Whole Genome Shotgun projects of Lactobacillus crispatus BC5 and L. gasseri BC12 have been deposited at DDBJ/ENA/GenBank under the accession WUBT00000000.1 and WUBU00000000.1, respectively.

2.2.4 Phylogenomic analysis

Phylogenetic relatedness of *L. crispatus* BC5 and *L. gasseri* BC12 strains to *L. crispatus* and *L. gasseri* species was determined via multilocus sequence analysis (MLSA) and average nucleotide identity (ANI).

MLSA was performed using the bcgTree pipeline on 107 single-copy core genes shared by *L. crispatus* BC5, *L. gasseri* BC12, and all the *Lactobacillus* type strains genomes currently available in the NCBI Genome RefSeq database (20). A maximum likelihood phylogenetic tree based on concatenated single-copy core genes was then constructed in MEGA X (21) applying bootstrap procedure n = 100. The strain *Pediococcus pentosaceus* ATCC 25745 was used as outgroup. Relatedness of BC5 and BC12 to the most closely related *Lactobacillus* type strains was also confirmed via ANI using the python module pyANI (https://github.com/widdowquinn/pyani) implemented in Anvi'o (22).

2.2.5 Comparative genome analysis

Comparative genome analysis was performed using all *L. gasseri* and *L. crispatus* strains currently available in the NCBI Assembly RefSeq database on May 2020, and a pan-genome database was built using the Anvi'o pipeline (22). Briefly, for each strain used in the analysis 'anvi-script-process-genbank' was used to generate genome sequence FASTA file and a TAB-delimited file containing 'gene calls'. The script 'anvi-gen-contigs-database' was used to generate a contigs database using the FASTA and 'gene call' files as input. Each contigs database was used to generate a database of genomes of each species using the script 'anvi-gen-genomes-storage'. Finally, the genome databases were used in 'anvi-pan-genome' to compute sequence similarities and identify clusters of homologous proteins. BLASTP was used to calculate the similarity of each amino acid sequence between all genomes in the database. The MCL cluster algorithm implemented in Anvi'o was used with an inflation parameter (--mcl-inflation) of 10 to identify protein clusters based on sequence similarity. Based on gene clustering, the relationship between genomes were visualized using the script 'anvi-display-pan'. In this respect, a softcore genome was defined by clusters present in at least the 90% of genomes. Functional annotation of the proteins included in each cluster was finally performed using eggNOG (17).

2.2.6 Phenotype Microarray

Phenotype Microarray assay was performed on *L. crispatus* BC5 and *L. gasseri* BC12 using Phenotype Microrray (PM) Technology (Biolog Inc., Hayward, CA) in anaerobic conditions. PM assay is based on the principle that metabolic active cells produce NADH, which could reduce tetrazolium dye. The patented tetrazolium dye turns from colourless to purple when it is reduced and this reaction is incontrovertible. The progressive turn of the colour in each well of a 96 multiwell is recorded every 15 minutes through a camera situated in the Omnilog instrument (Biolog Inc.), which acts also as incubator, allowing the definition of a metabolic kinetic curve of the bacterial strains in presence of specific compounds [98]. Both strains were tested on Biolog microplates for their specific metabolic activity in presence of 190 different carbon sources (PM1 and PM2A), 94 nitrogen sources (PM3B) and for their chemical sensitivity to 250 different drugs and chemical compounds (PM9 to 20) including heavy metals, antibiotics, osmolytes, antiseptics, pH stress and other inhibitors. *L. crispatus* and *L. gasseri* were grown anaerobically on MRS agar plates and then inoculated in PM plates following provider's protocol. Briefly, cells were removed from MRS agar plates using a cotton swab and inoculated in a solution composed of Inoculation Fluid (Biolog Inc.), DyeG (Biolog, Inc.) and additive solution, at a cell density of T=65% (Complete mix were listed in Table1, Table2 and Table3).

Table 2. Composition of PM mix for PM1 and PM2A (carbon sources)

	Chemicals	Final concentration
Additive solution	$MgCl_2$, $6H_2O$	2 mM
	$CaCl_2$, $2H_2O$	1 mM
	L-glutamate, Na	50 μM
	L-cystine, pH8.5	12,5 μM
	5'-UMP, 2Na	25 μM
	L-arginine, HCl	25 μM
	Hypoxanthine	25 μM
	β-NAD, hydrate	5 μM
	Riboflavin	0,25 μM
	Yeast extract	0,005 %
	Tween 80	0,005 %
Inoculation fluid	Inoculation fluid IF-0a GN/GP (1.2x)	1 x

Dye	Dye G (100x)	1 x
Bacterial cells		T=65%

Table 3. Composition of PM mix for PM3 (nitrogen sources)

	Chemicals	Final concentration
	Tricarballylic acid, pH 5.0	20 mM
	$MgCl_2$, $6H_2O$	2 mM
	$CaCl_2$, $2H_2O$	1 mM
	L-cystine, pH8.5	12,5 µM
	5'-UMP, 2Na	25 µM
	L-arginine, HCl	25 µM
	Hypoxanthine	25 µM
	β-NAD, hydrate	5 µM
	Riboflavin	0,25 µM
	Yeast extract	0,005 %
Additive solution	Tween 80	0,005 %
	Na_2-PPi, pH6.0	2 mM
	L-methionine	25 µM
	Lipoamide	10 µM
	Spermine	25 µM
	Cysteamine sodium phosphate	25 µM
	$FeCl_3$	1 µM
	D-glucose	1,25 mM
	Sodium pyruvate	2,5 mM
	Uridine	1,25 mM
	Sodium acetate	2,5 mM
Inoculation fluid	Inoculation fluid IF-0a GN/GP (1.2x)	1 x
Dye	Dye G (100x)	1 x
Bacterial cells		T=65%

Table 4. Composition of PM mix for PM9-20 (Drug and chemical sensitivities)

	Chemicals	Final concentration
Additive solution	$MgCl_2$, $6H_2O$	2 mM
	$CaCl_2$, $2H_2O$	1 mM
	L-glutamate, Na	50 µM
	L-cystine, pH8.5	12,5 µM
	5'-UMP, 2Na	25 µM
	L-arginine, HCl	25 µM
	Hypoxanthine	25 µM
	β-NAD, hydrate	5 µM
	Riboflavin	0,25 µM
	Yeast extract	0,005 %
	Tween 80	0,005 %
	D-glucose	1,25 mM
	Sodium pyruvate	2,5 mM
	Uridine	1,25 mM
	Sodium acetate	2,5 mM
	Na_2HPO_4	5 mM
	KH_2PO_4	25 mM
	$(NH_4)_2SO_4$	2,5 mM
	Bacto peptone (20 x)	1 x
Inoculation fluid	Inoculation fluid IF-10b GN/GP (1.2x)	1 x
Dye	Dye G (100x)	1 x
Bacterial cells		T*=65%

All plates were inoculated with 100 µL per well and the anaerobic atmosphere was created with an Anaerocult P sachet (Sigma-Aldrich) placed together with each plate in a special incubation bag sealed with tape [96]. Lastly, plates were incubated for 72 h at 37 °C in an Omnilog automated incubator/reader (Biolog Inc.). Metabolic activity was correlated with reduction of tetrazolium dye in each well and was measured by the Omnilog system every 15 minutes.

2.2.7 Phenotype Microarray data analysis

Metabolic data were analysed with Omnilog software (O_PM_FM/Kin 1.20.02 and OL_PM_Par 1.20.02) and OPM package on R studio (23). For PM1-2A (Carbon sources), PM3B (nitrogen sources), PM9-10 (Osmolytes and pH), PM data were referred to the value of Area Under the Curve (AUC) obtained per each compound during omnilog data analysis. In PM11C-20B (Drug and chemical sensitivities), each compound was present in 4 different wells at increasing concentration. Consequently, for each compound we assigned a value that ranges from 0 to 4, in which 0 corresponds to no detection of metabolic activity and 4 means metabolic activity detection at the highest concentration tested.

2.3 Results

2.3.1 Comparative genomics, phylogenomic analyses and genome annotation of *Lactobacillus crispatus* BC5 and *Lactobacillus gasseri* BC12

The whole genome sequencing indicated a dimension of 2,064,185 bp with a GC% of 36.7% for *L. crispatus* BC5 and 2,020,322 bp, GC% of 34.7 % for *L. gasseri* BC12. These data were in line with the mean values that we calculated on the basis of 75 genomes of *L. crispatus* strains and 42 genomes of *L. gasseri* strains (those available in the database), i.e. size of 2.1 ± 0.18 Mbp for *L. crispatus* and 1.9 ± 0.26 Mbp for *L. gasseri*, % GC within a range of 32.1% and 36.2%.

MLSA analysis confirmed the phylogenetic affiliation of BC12 and BC5 to *L. gasseri* and *L. crispatus* species, respectively. Phylogenetic relatedness of BC12 and BC5 was also supported via average nucleotide identity (ANI) analysis, resulting for both strains > 96.5 % (i.e. ANI of 97.6% for BC5 with *L. crispatus* ATCC33820, ANI of 97.2% BC12 with *L. gasseri* ATCC33323) with the type strain of the corresponding species. On the other hand, in accordance with their belonging to a different species, the two strains shared an ANI of 75%. By using the same genome dataset of *L. gasseri* and *L. crispatus* strains, we assessed the BC5 and BC12 gene products included in the core-genome of each corresponding species. We observed that *L. crispatus* core genome was represented by 1334 clusters of homolog proteins and that a total number of 1350 BC5 proteins were included in clusters of the core genome. Further, 38 BC5 gene products were identified as singletons. The core genome of *L. gasseri* contained 1259 clusters of homolog proteins, which included 1273 BC12 gene products. The singletons in BC12 resulted to be 34.

Genome annotation analysis identified a total of 2015 coding sequences (CDS) and 81 RNAs genes (16 rRNAs, 62 tRNAs and 3 ncRNAs) in BC5 and 1934 CDS and 76 RNA (10 rRNAs, 63 tRNAs and 3 ncRNA) genes in BC12. According to functional annotation, 1022 and 955 BC5 and BC12 proteins were respectively assigned to known functional orthologues, i.e. KEGG Orthologs (KO). Among those belonging to the major metabolic pathways, by directly comparing the two strains, BC5 and BC12 shared 174 functional orthologues (Table 5). On the other hand, unique KO in each strain were 129 and 99 in BC5 and BC12, respectively (Table 5).

Table 5. Number of shared and unique functional orthologues (indicated as KO) involved in the major metabolic pathways of *L. crispatus* BC5 and *L. gasseri* BC12

KEGG Metabolism	KEGG pathway name	Shared KO	LC BC5 unique KO	LG BC12 unique KO
Carbohydrate metabolism	Glycolysis / Gluconeogenesis	13	2	3
	Citrate cycle	3	-	-
	Pentose phosphate pathway	10	-	1
	Pentose and glucuronate interconversions	1	-	-
	Fructose and mannose metabolism	9	-	1
	Galactose metabolism	7	3	13
	Starch and sucrose metabolism	13	-	1
	Amino sugar and nucleotide sugar metabolism	21	1	3
	Pyruvate metabolism	11	3	1
	Glyoxylate and dicarboxylate metabolism	4	1	2
	Propanoate metabolism	4	1	-
	Butanoate metabolism	2	2	1
	C5-Branched dibasic acid metabolism	-	1	1
	Inositol phosphate metabolism	2	-	-
Amino acids metabolism	Alanine, aspartate and glutamate metabolism	12	-	-
	Glycine, serine and threonine metabolism	7	2	-
	Cysteine and methionine metabolism	9	5	-
	Valine, leucine and isoleucine degradation	1	1	-
	Lysine biosynthesis	4	7	-
	Lysine degradation	1	-	-
	Arginine biosynthesis	2	1	-
	Arginine and proline metabolism	1	1	-
	Histidine metabolism	1	-	-
	Phenylalanine metabolism	1	-	-
	Tryptophan metabolism	2	-	-
	Taurine and hypotaurine metabolism	2	-	-
	Selenocompound metabolism	4	1	1
	Cyanoamino acid metabolism	3	1	-
	D-Glutamine and D-glutamate metabolism	4	-	-
	D-Alanine metabolism	4	-	-
	Glutathione metabolism	2		-
Lipid metabolism	Fatty acid degradation	1	1	-
	Synthesis and degradation of ketone bodies	1	1	-
	Primary bile acid biosynthesis	-	1	
	Glycerolipid metabolism	7	-	1
	Glycerophospholipid metabolism	8	-	1
	Sphingolipid metabolism	1	-	-
Nucleotide metabolism	Purine metabolism	24	-	2
	Pyrimidine metabolism	24	-	1
Metabolism of cofactors and vitamins	Thiamine metabolism	5	-	1
	Riboflavin metabolism	4	5	-
	Vitamin B6 metabolism	1	1	-
	Nicotinate and nicotinamide metabolism	5	1	-
	Pantothenate and CoA biosynthesis	4	1	1

	Folate biosynthesis	3	1	-
	One carbon pool by folate	6	-	-
	Porphyrin and chlorophyll metabolism	2	-	-
	Ubiquinone and other terpenoid-quinone biosynthesis	1	1	2
Membrane transport	ABC transporters	37	8	11
	Phosphotransferase system (PTS)	17	4	13
	Bacterial secretion system	9	-	-
Replication and repair	DNA replication	13	-	-
	Base excision repair	10	-	-
	Nucleotide excision repair	7	-	-
	Mismatch repair	15	1	-
	Homologous recombination	18	-	-
Transcription and translation	RNA polymerase	5	-	-
	Ribosome	53	-	-
	Aminoacyl-tRNA biosynthesis	26	-	-
	RNA transport	2	-	-
Total number of shared and unique KO		174	129	99

2.3.2 Phenotype Microarray of BC5 and BC12 and genetic traits associated to their phenotypic differences

The phenotype profiles of *L. crispatus* BC5 and *L. gasseri* BC12 were defined through Phenotype Microarray analysis under anaerobic conditions in order to mimic the typical oxygen condition to which the microbial community is exposed in the vaginal niche. The metabolic activities were measured in the presence of 190 different carbon sources, 95 nitrogen sources and more than 250 stressors such as ions, osmolytes, pH, a wide range of antibiotic, antiseptics, metals and other inhibitors. We further identified the genetic traits that could be related with the different phenotypes/metabolic activities shown by the two strains in PM assay within each substrate/compound category.

2.3.2.1 Carbon sources

Among the 192 carbon sources tested in PM assays, BC5 and BC12 showed metabolic activity in the presence of 61 and 54 compounds, respectively (Fig. 4A and 4C). In particular, BC5 and BC12 could utilize half of the carbohydrates under analysis, of which around 80% was metabolized by both the strains (hereafter named shared compounds). Overall, on this class of compounds, BC5 generally had higher performance by showing higher metabolic activities on shared carbohydrates and the capabilities to exclusively utilize eight compounds (i.e. inosine, D-melibiose, α-methyl-D-mannoside, 3-0-b-D-galactopyranosil-D-arabinose, uridine, L-rhamnose, thymidine, L-sorbose). In some cases, it was possible to link the higher performance of BC5 towards carbohydrates to the

presence in its genome of specific genetic determinants involved in carbohydrate uptake and metabolic reactions. In the case of melibiose and maltotriose that resulted to be a BC5-specific

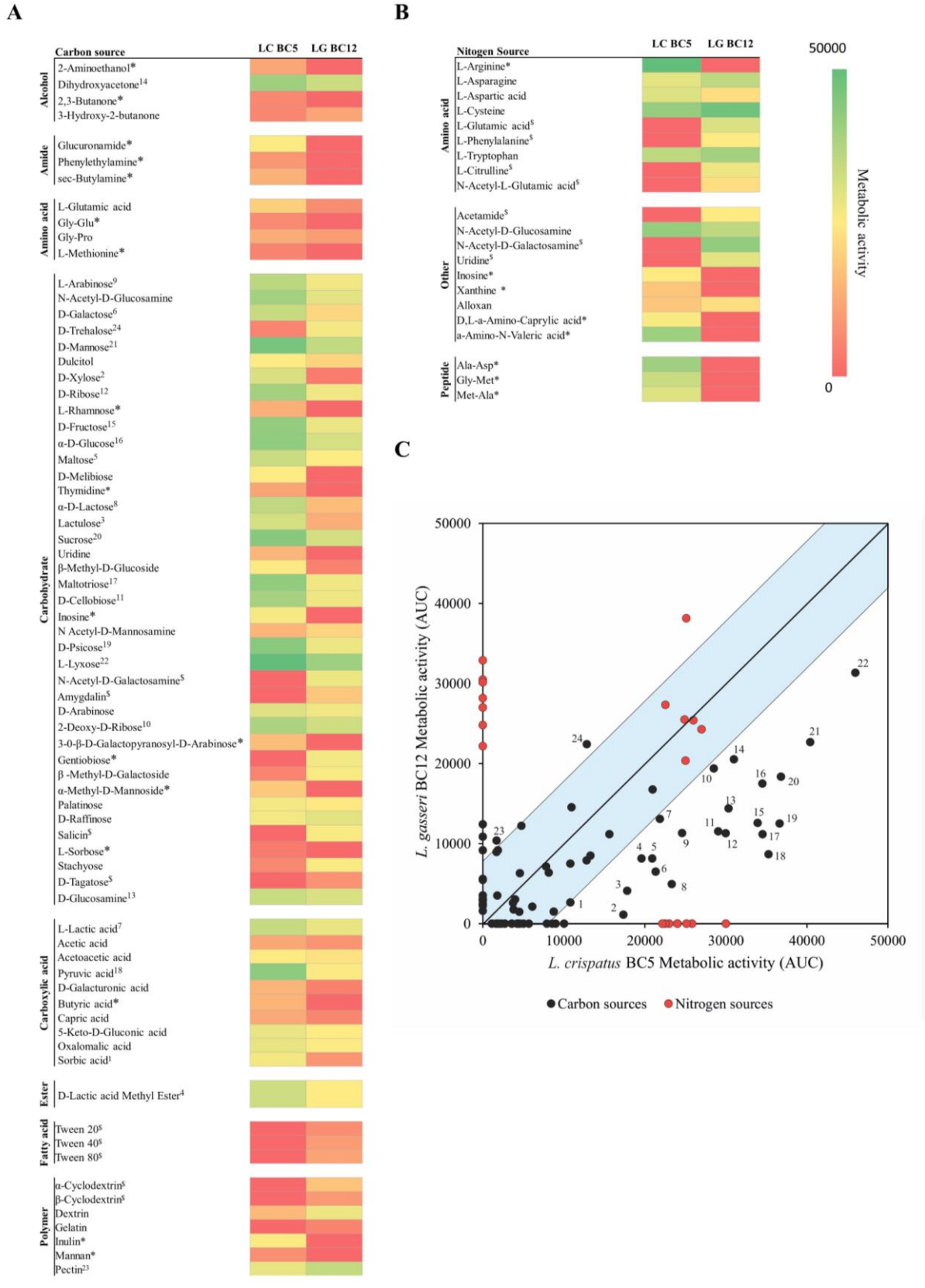

Fig. 4. Carbon and nitrogen source metabolized by *L. crispatus* BC5 and/or *L. gasseri* BC12 in Phenotype Microarray experiments. Heatmaps represents Area Under the Curve (AUC) of respiration kinetic in presence of **A)** carbon sources or **B)** nitrogen sources. **C)** Correlation between *L. crispatus* BC5 and *L. gasseri* BC12 metabolic activities in presence of each carbon and nitrogen sources used at least by one strain. Light blue region represents standard deviation from the average. Numbers in (A) and (C) refer to common carbon source with higher metabolic activity of one strain despite to the other. * indicates carbon and nitrogen sources metabolized only by BC5, $ indicates carbon and nitrogen source metabolized only by BC12.

substrate (i.e. only metabolized by BC5 strain), both the strains possessed the genes predicted to be involved in these two sugars metabolism, while only the *L. crispatus* strain possessed genes coding for systems involved in their import (Table 6, Fig. 5).

Table 6. Genetic features of *L. crispatus* BC5 and *L. gasseri* BC12 involved in differentially metabolized carbon and nitrogen sources[a]

Metabolism	KO	LC BC5 hits (no.)	LG BC12 hits (no.)	KO Name	Frequency (%) in *L. crispatus*/*L. gasseri* species[b]
Galactose/ Lactose/ Tagatose	K00849	1	1	*galK,;* galactokinase	100/100
	K00850	1	1	*pfkA;* 6-phosphofructokinase	100/100
	K00965	3	2	*galT;* UDPglucose--hexose-1-phosphate uridylyltransferase	99.1/95.1-97.5
	K01182	1	3	*malL;* oligo-1,6-glucosidase	94.0-98.3/80.4-100
	K01190	3	0	*lacZ;* beta-galactosidase	1.7-100/NA
	K01220	0	3	*lacG;* 6-phospho-beta-galactosidase	NA/95.1-100
	K01635	0	1	*lacD;* tagatose 1,6-diphosphate aldolase	NA/100
	K01784	1	1	*galE;* UDP-glucose 4-epimerase	100/70.7
	K01785	2	2	*galM;* aldose 1-epimerase	96.5-98.3/92.6
	K01819	0	2	*lacA; lacB*; galactose-6-phosphate isomerase	NA/92.6
	K01835	1	1	*pgm;* phosphoglucomutase	100/95.1
	K02786	0	3	*lacF;* lactose PTS system EIIA	NA/100
	K02788	0	3	*lacE;* lactose PTS system EIICB	NA/4.8-97.5
	K20112	1	2	*gatA;* galactose PTS system EIIA	100/97.5-100
	K20113	0	1	PTS-Gal-EIIB; galactose-specific IIB	NA/100
	K20114	0	1	PTS-Gal-EIIC; galactose-specific IIC	NA/100
	K16209	1	0	*lacS*, *galP*, *rafP*; lactose/raffinose/galactose permease	68.3/NA
Maltose	K02777	2	1	*crr;* sugar PTS system EIIA component	94.0-100/80.4-9.7
	K02750	2	0	*glvC;* alpha-glucoside PTS system EIICB	46.1-100/NA
	K01232	1	0	*glvA*; maltose-6'-phosphate glucosidase	97.4/NA
Sucrose	K02810	2	1	*scrA*; sucrose PTS system EIIBCA or EIIBC	46.1-99.1/97.5
	K01193	3	1	*sacA;* beta-fructofuranosidase	6.8-99.1/95.1
	K00847	1	1	*scrK;* fructokinase	99.1/100
	K01810	1	1	*pgi;* glucose-6-phosphate isomerase	100/100
	K01835	1	1	*pgm*; phosphoglucomutase	100/95.1
	K00692	1	0	*sacB*; levansucrase	35.9/NA

	K00690	2	1	*sucP;* sucrose phosphorylase	63.2/29.2
N-acetyl-galactosamine	K02744	0	2	*agaF*; N-acetylgalactosamine PTS system EIIA	NA/26.6-51.2
	K02745	0	1	*agaV*; N-acetylgalactosamine PTS system EIIB	NA/51.2
Fructose	K02770	1	2	*fruA*; fructose PTS system EIIBC or EIIC	97.4/92.6
	K00882	1	3	*fruK*; 1-phosphofructokinase	98.3/96.6-100
	K01624	1	1	*fbaA*; fructose-bisphosphate aldolase	99.1/97.5
	K01803	1	2	*tpiA*; triosephosphate isomerase (TIM)	99.1/100
Melibiose	K10117	1	0	*msmE*; raffinose/stachyose/melibiose transport system substrate-binding protein	60.6/NA
	K10118	1	0	*msmF*; raffinose/stachyose/melibiose transport system permease protein	61.5/NA
	K10119	1	0	*msmG*; raffinose/stachyose/melibiose transport system permease protein	60.6/NA
	K10112	4	2	*msmX*, *msmK*, *malK*, *sugC*, *ggtA*, *msiK*; multiple sugar transport system ATP-binding protein	98.3-99.1/100
	K07407	1	1	*galA*, *rafA*; alpha-galactosidase	58.9/92.6
	K02777	2	1	*crr*; sugar PTS system EIIA component	94-100/80.4-9.7
	K01232	1	0	*glvA*; maltose-6'-phosphate glucosidase	97.4/NA
	K01226	0	1	*treC*; trehalose-6-phosphate hydrolase	NA/87.8
Arginine	K01478	1	0	*arcA*; arginine deiminase	81.2/NA
Dipeptides	K03305	1	0	Di-/tripeptide transporter	98.3/NA
Dipeptides/ oligopeptides	K15580	7	0	*oppA*, *mppA*; oligopeptide transport system substrate-binding protein	0.85-98.2
	K15581	1	0	*oppB*; oligopeptide transport system permease protein	100/NA
	K15582	1	0	*oppC*; oligopeptide transport system permease protein	100/NA
	K15583	1	0	*oppD*; oligopeptide transport system ATP-binding protein	99.1/NA
	K10823	1	0	*oppF*; oligopeptide transport system ATP-binding protein	99.1/NA

[b] The range of frequency of each gene product (with the same KO) is indicated within the pangenome of *L. crispatus* and *L. gasseri*, respectively

In the case of α-glycosydes such as α-methyl-D-mannoside, BC5 possessed multiple copies of the genes encoding the multiple-sugar-metabolism (Msm) transport systems which are involved in the uptake of these sugars. BC5 possesses a beta-galactosidase gene (*lacZ*) which catalyses the conversion of lactose into D-galactose that can be further converted into alpha-D-glucose-6P (via Leloir pathway), while BC12 can degrade lactose and galactose via the tagatose-6P pathway, in which the two sugars are converted into glyceraldeyde-3P that can enter glycolysis. In respect to the higher metabolic activity on maltose, a maltose 6-phosphate glucosidase gene was present in BC5 genome but not in BC12 (Table 6, Fig. 5).

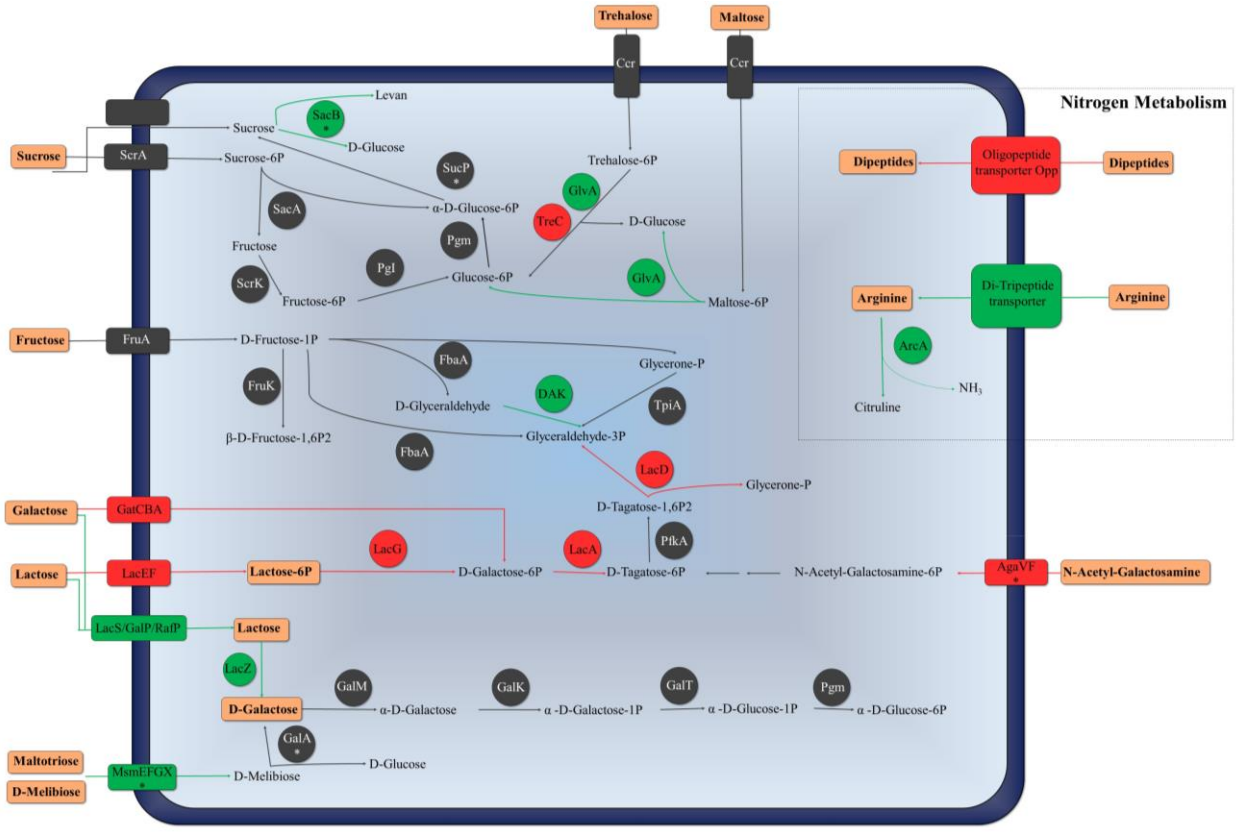

Fig. 5. Metabolic pathways of carbon and nitrogen sources differentiating BC5 and BC12 based on Phenotype Microarray and functional annotation. Metabolic reactions are indicated with coloured arrows: green for BC5 specific reactions, red for BC12 specific reactions, dark grey for reactions predicted to occur in both the strains under analysis. Most of the functions displayed are included in the core-genome of each species with a few exceptions (indicated with *).

In consideration of sucrose, both BC5 and BC12 genomes possessed the genes involved in the conversion of sucrose into alpha-D-glucose-1P via D-fructose, but only BC5 genome carries a levansucrase/invertase gene that converts sucrose into D-glucose and levan. Both the strains BC5 and BC12 showed the capacity to metabolize fructose (Fig. 5). The additional presence of gene encoding the triose/dihydroxyacetone kinase (DhaK) in the only BC5 might support the higher performance shown by this strain as compared to BC12, since this enzyme catalyses the direct conversion of fructose-1P into glyceraldehyde-3P via D-glyceraldehyde that might improve the sugar utilization kinetics. Despite the general lower performance of BC12 as compared to BC5 in the carbon source utilization, this strain showed higher performance on some compounds. In particular, the *L. gasseri* strain showed higher metabolic activity than BC5 on D-trehalose and showed the exclusive capability to utilize the carbohydrates N-acetyl-D-galactosamine, D-tagatose, gentiobiose, amygdalin and salicin (Fig. 4A and 4C). From a genotypic point of view, the presence in BC12 of a tagatose 1,6-diphosphate aldolase gene could be associated to the capacity of this strain to utilize D-tagatose (Table

6). In the same way, the presence of genes encoding the N-acetylgalactosamine PTS system EIIA component and the enzymes involved in tagatose-6P pathway can be associated to the capability of the only BC12 to efficiently metabolize N-acetylgalactosamine. The higher metabolic activity of BC12 on trehalose is probably due to the presence of a *treC* gene in BC12 that encodes the trehalose-6-phosphate hydrolase involved in the conversion of trehalose-6P into D-glucose and D-glucose-6P. Conversely, BC5 possesses the maltose 6-phosphate glucosidase gene that can recognize the trehalose-6-phosphate as substrate but with a different specificity as compared to TreC, therefore possibly inducing a different utilization efficiency. The phenotypic divergence observed in PM experiments between BC12 and BC5 in the presence of salicin and amygdalin, and in the presence of the product of the first amygdalin cleavage, i.e. gentiobiose, can be due to possible differences in the specificity of PTS EII transporters and beta-glucosidase systems as previously reported [99].

Despite the overall carboxylic acids utilization profile was similar in the two strains (Fig. 4A and 4C), BC5 showed higher metabolic activity on lactic acid and pyruvic acid that in association with the higher respiration rate on glucose indicates that BC5 has an improved homolactic fermentation efficiency as compared to BC12. Despite the low number of alcohols and amino acids that were generally metabolized by the two strains, BC5 also showed a higher metabolic performance with these compounds (Fig. 4A and 4C).

BC5 and BC12 were distinct in the capacity to utilize the amines, fatty acids and polymers. In particular, only BC5 could metabolize amines (i.e. phenylethylamine and sec-butylamine) and only BC12 could metabolize fatty acids (i.e. Tween 20, 40 and 80). While the first two compounds can be associated to amine oxidase activity [100–102], the three fatty acids utilized by BC12 are derivatives of poly-oxyethylenesorbitan and can be used by some *Lactobacillus* spp. strains as a source of unsaturated fatty acids [103]. With regard to the polymers tested in PM, both the strains could utilize dextrin and pectin, although BC12 showed higher metabolic activities on these substrates. Furthermore, only BC12 utilizes cyclodextrins and gelatin and only BC5 can metabolize inulin and mannan (Fig. 4A and 4C).

2.3.2.2 Nitrogen sources

BC5 and BC12 strains could metabolize 13 and 14 out of the 97 nitrogen sources tested in PM assay. They shared the capacity to utilize six of them, i.e. four amino acids (L- tryptophan, L- cysteine, L-aspartic acid and L- asparagine), one amino sugar (N-acetyl-D-glucosamine) and the alloxan (Fig. 4B and 4C). On the other hand, more than half of the nitrogen sources utilized were strain-specific (i.e. metabolized by only one of the two strains). In particular, *L. crispatus* BC5 showed the capacity to metabolize arginine, inosine, xanthine, D,L- α-Amino-Caprylic acid, α-Amino-N-Valeric acid, and

the di-peptides Ala-Asp, Gly-Met, Met-Ala, whereas *L. gasseri* BC12 could utilize acetamide, L-glutamic acid, L-phenylalanine, citrulline and N-acetyl-D-galactosamine. From a genotypic point of view, the capacity of *L. crispatus* BC5 to utilize arginine could be related to the presence of a gene (*arcA*) in its genome encoding an arginine deiminase, which is able to convert arginine into citrulline and NH_3 (Table 6). However, BC5 did not possess the additional genes involved in the subsequent conversion of citrulline into ornithine [ornithine transcarbamoylase (encoded by arcB), carbamate kinase (encoded by arcC), membrane transport protein (encoded by arcD)] [104,105], which explains the inability of BC5 to also utilize arginine as carbon source. The different dipeptides utilization capability of BC5 and BC12 is associated to the presence in each strain of a distinct dipeptides/oligopeptides uptake system, i.e. the Opp in BC5 and the DtpT in BC12 (Table 6), which might be involved in the intake of a specific range of dipeptides due to affinity issues [106]. Regarding the nitrogen sources utilized only by BC12, no specific genetic determinants could be detected on the basis of the genome annotation. For instance, genes predicted to be involved in the import and utilization of BC12-specific nitrogen sources L-glutamic acid and L-phenylalanine were equally present in both BC5 and BC12 genomes.

2.3.2.3 Osmotic and ionic stressors

The pH range at which *L. crispatus* BC5 and *L. gasseri* BC12 were metabolically active was similar (i.e. pH 4 - 8.5); furthermore, these two strains were equally resistant to several osmolytic stressors (i.e. NaCl, sodium sulfate, ethylene glycol, sodium phosphate, sodium nitrate and sodium nitrite). On the other hand, the osmotic stress induced by potassium chloride, sodium lactate and sodium benzoate differently affected the two strains, indeed *L. gasseri* BC12 was metabolically active at higher concentrations of these 3 stressors as compared to *L. crispatus* BC5. Furthermore, *L. gasseri* tolerated urea up to 4%, while the minimum concentration tested (2% urea) was enough to completely inhibit the metabolic activity of *L. crispatus* (Fig. 6). The presence of the glycerol and trehalose sustained the growth of both BC5 and BC12 in the presence of high osmolarity (NaCl 6%), while the ability to utilize betaine, proline and trigonelline as osmoprotectants distinguished the two strains (Fig. 6).

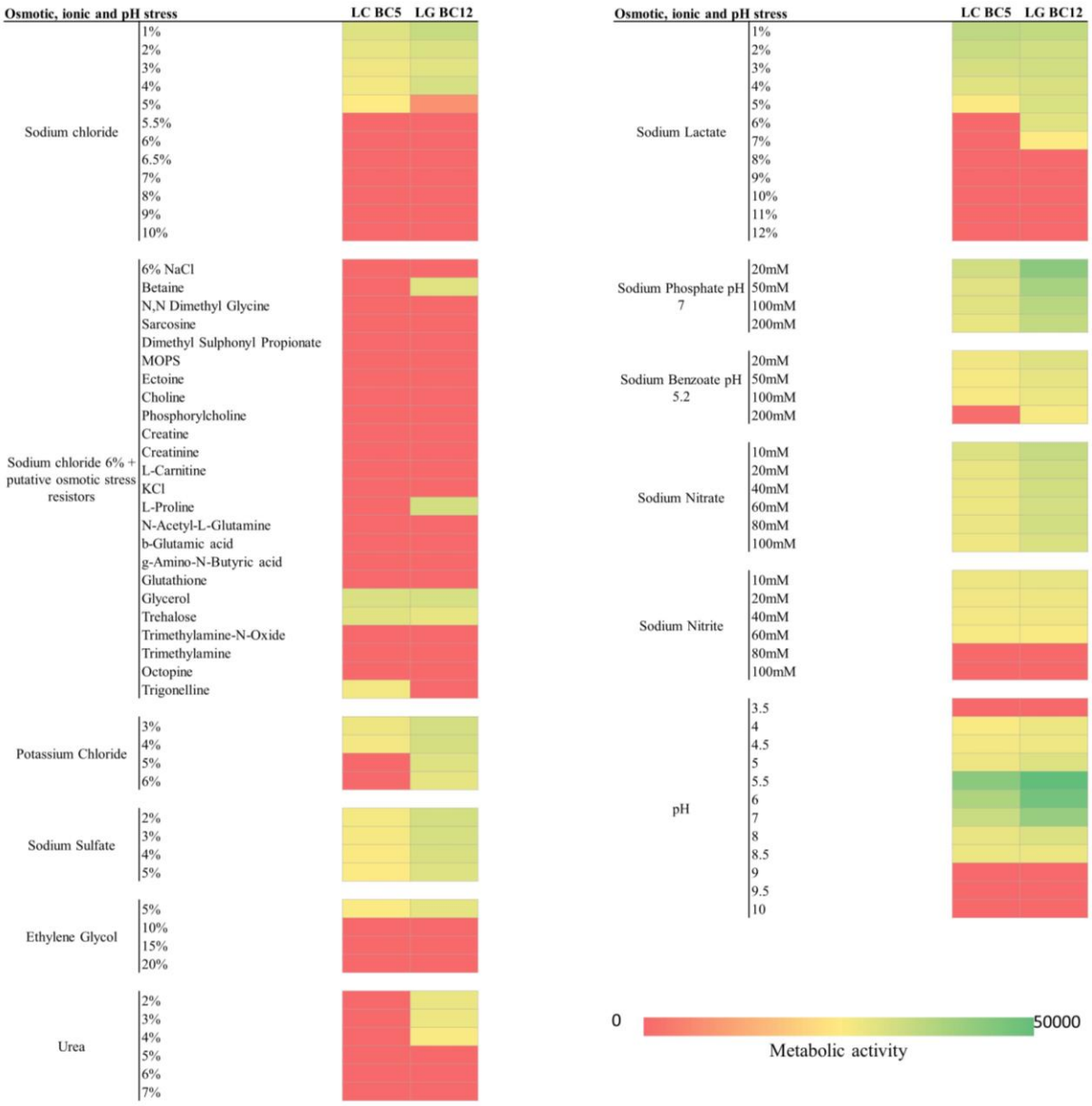

Fig. 6. *L. crispatus* BC5 and *L. gasseri* BC12 osmotic and ionic stress sensitivities detected in Phenotype Microarray. Metabolic activities, expressed as Area Under the Curve (AUC) of the respiration kinetic, are shown as heatmap in which values range from 0 (red) to 50000 (green).

Some genetic traits could be associated to the overall improved resistance and/or tolerance capacities of BC12 as compared to BC5 towards osmotic stressors. For instance, the presence of two copies of potassium uptake permease (KUP) gene in BC12 genome might relate to the capacity of this strain to stand higher concentrations of KCl (Table 7). Regarding the utilization of compatible solutes as osmoprotectants, genes encoding all the components of an osmoprotectant transport system (Opu) were detected in BC12 genome but not in BC5 (Table 7). This genetic trait can be associated to the exclusive capacity of *L. gasseri* strain to utilize the osmotic protectants L-proline and betaine. On the

other hand, choline could not be used by BC12 to counteract osmotic stress, this might be due to a possible lower affinity of the Opu system toward this compound [107]. Genes encoding glycerol ABC transporter (GRH99_01765, GRH99_04495, GRH99_08730, GRI01_08425) and trehalose specific IIB and IIC components were detected in the two genomes under analysis; these genes are possibly involved in the cell import of glycerol and trehalose cell as osmoprotectants to sustain the growth of the two strains in medium with the highest NaCl concentration tested, i.e. 6%.

2.3.2.4 Tolerance/Resistance to chemical stressors including antibiotics and metals

Among the drugs and chemicals tested, BC12 was metabolically active in the presence of 86 chemicals/drugs, while *L. crispatus* BC5 showed resistance to 48 stressors (Fig. 7). In particular, both strains were resistant to 12 antibiotics out of the 91 antibiotics tested (2-nitroimidazole, amoxicillin, ampicillin, cefsulodin, cephalothin, ciprofloxacin, dihydrostreptomycin, gentamicin, penicillin G, polymyxin B, tetracycline and vancomycin) and both the strains resulted sensitive to aminocoumarin, lincomycin, nitrofurans, rifamycin and sulfonamides antibiotic classes. As compared to BC5, BC12 showed resistance to additional 20 antibiotics belonging to aminoglycosides, β-lactams, cephalosporines, fluoroquinolones, glycopeptides, macrolides, nitroimidazole and quinolones classes (Fig. 7A and 7B). At genetic level, the higher metabolic activity of BC12 in the presence of the macrolides erythromycin, josamycin and tylosin could be associated to the presence in its genome of genes encoding both a macrolide ABC transporter (*macB*, GRI01_09250) and a permease (*macA*, GRI01_09255) *(*Table 7). BC12 was also found to be slightly more resistant than BC5 to some β-lactams i.e. moxalactam, aztreonam, amikacin and ampicillin. This property could be correlated to the presence in BC12 genome of a higher number of different genes encoding β-lactamases of class A and C (three and four in BC12, one and two in BC5, respectively) and an ABC efflux transporter AbcA which are involved in the beta-lactams degradation and extrusion, respectively. Additionally, on the basis of previous findings, AbcA can be associated to BC12 resistance towards polymyxin B [108] and quinolones (i.e. pipemidic acid). Additionally, *L. gasseri* BC12 showed higher resistance than BC5 to fluoroquinolones, except for ciprofloxacin, in which both the strains showed the same metabolic activity. In addition to possible non-specific multidrug efflux pumps that might be involved in these antibiotics resistance, we detected in BC12 genome an ABC transporter (GRI01_02970) showing 53% similarity with the multidrug efflux ABC transporter LmrA that is responsible of the quinolones efflux in *Lactococcus lactis* [109]. In regard to genetic determinants associated to antibiotic

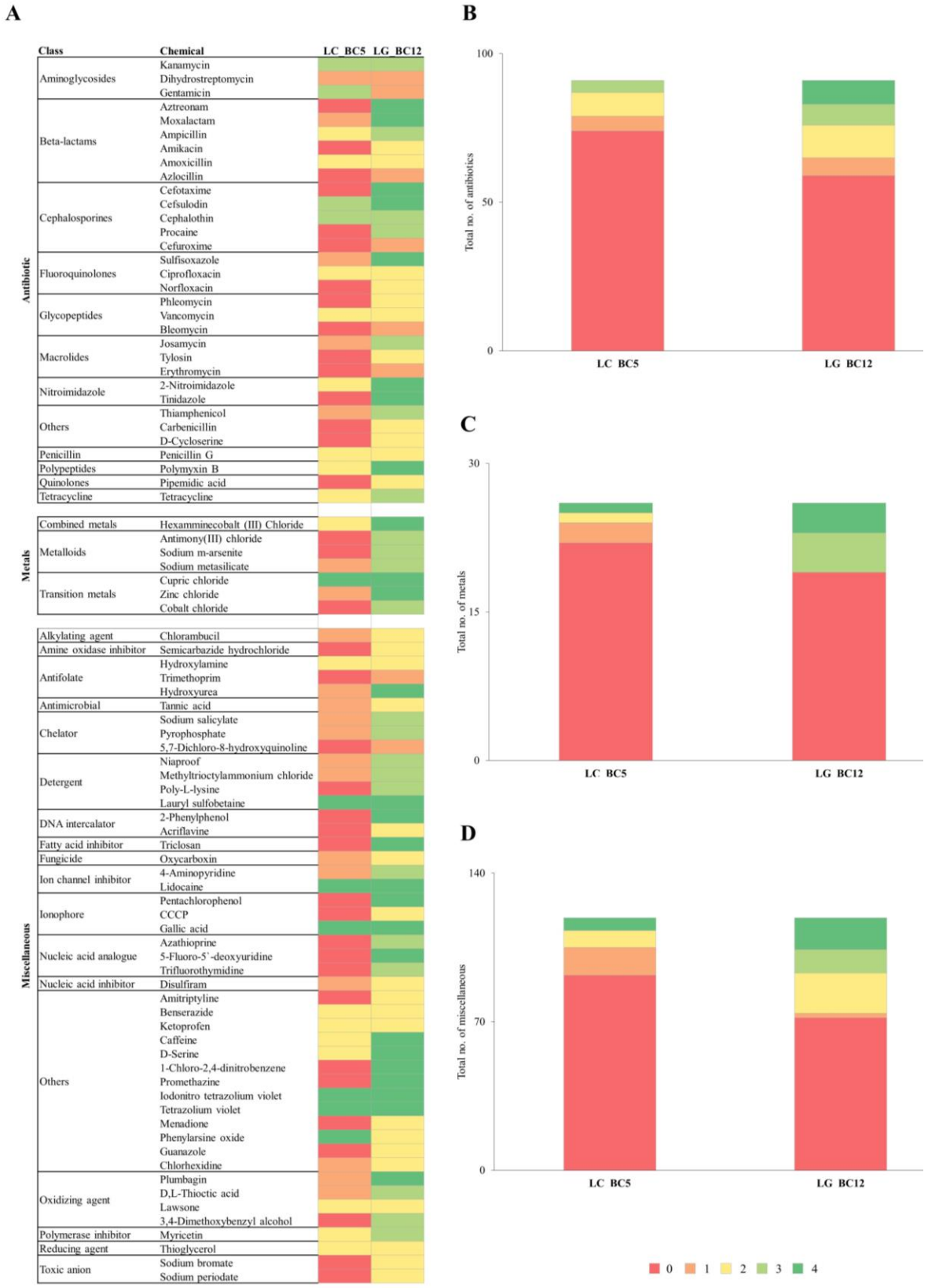

Fig. 7. Drug and chemical resistance of *L. crispatus* BC5 and *L. gasseri* BC12 detected in Phenotype Microarray. Each compound was tested at 4 increasing concentration and a value ranging from 0 (absence of metabolic activity at lowest concentration tested) to 4 (resistant at the highest concentration tested) was assigned for both strains to each compound. Chemical

compounds for which resistance was detected at least of one strain were reported as heatmap **(A)**. All the drugs and chemical compounds tested were classified in 3 main groups (antibiotics, metals and miscellaneous) and the total number of resistances identified within each group was shown in **B)** antibiotics, **C)** metals and **D)** miscellaneous.

resistance in *L. crispatus* BC5, this strain possessed two *mep*A genes encoding MATE family multidrug efflux pumps possibly involved in the resistance to ciprofloxacin, gentamicin, streptomycin, and kanamycin. Both strains were resistant to antibiotics belonging to aminoglycosides class, i.e. kanamycin and dihydrostreptomycin at the same levels, while *L. crispatus* BC5 could cope with higher concentrations of gentamycin than *L. gasseri* BC12. From a genetic point of view, both the strains carried the genes *aaaC3* encoding the aminoglycoside N3'-acetyltransferase (GRH99_07855) mainly involved in gentamycin resistance, while the only BC12 possessed the *aph* gene encoding the aminoglycoside 3'-phosphotransferase (GRI01_08780), which phosphorylates the kanamycin through an ATP dependent mechanism. Both these genes were previously associated to aminoglycoside resistance in other *Lactobacillus* spp. [110].

The results of PM experiments testing the resistance/tolerance to 26 metals and metalloids showed that BC12 and BC5 were both metabolically active in the presence of 4 metals, although BC12 generally showed higher metabolic activity values. Further, the *L. gasseri* strain was resistant to additional 3 metals, to which BC5 was sensitive i.e. antimony (III), cobalt, arsenic (III) (Fig. 7A and 7C). Both the strains were equally resistant to copper (cupric chloride), while BC12 was more resistant to zinc as compared to BC5. From a genetic point of view, the resistance to copper could be associated to the presence of an operon *cop*YACB in the genomes of both the strains (Table 7). CopY acts as negative regulator of the *cop* genes expression while *copA* and *copC* encode soluble periplasmic Cu+-binding proteins which assist CopB in copper extrusion. The *copB* encodes a Copper-translocating P-type ATPase that shows similarity with Lead, cadmium, zinc and mercury transporting ATPase, therefore being probably involved in other metal resistance phenotypes. Interestingly, in BC5 genome, one of the two cop operons (GRH99_09225- 09235) is located within a 13 Kbp-long genomic island which also includes the gene encoding the MDR efflux pump of the MATE family. A *czcD* gene encoding a cation transporter involved in multi-metal (cobalt, zinc and cadmium) resistance was detected in both BC5 and BC12 genomes. However, only in BC12 this gene was flanked by a *czrA* gene encoding the metal regulator ArsR/SmtB which might contribute to an efficient metal stress response [111]. This might explain the resistance to cobalt and the higher resistance to zinc shown by BC12 as compared to BC5. On the other hand, the higher susceptibility to arsenite and antimonites shown by BC5 could not be explained at genetic level as BC5 but not BC12 genome carries *ars* genes (i.e. *arsRCB* operon) which are the genetic traits typically associated to the bacterial

resistance to arsenic and antimony [112]. Possible additional cationic antiporter (one of these annotated as arsenic efflux pump) might be involved in arsenite resistance, although further analyses are needed. Multidrug efflux proteins, possible multidrug ABC transporters, as well as proteins involved in oxidative stress response and other detoxification processes (Table 7) can support the ability of the two strains to resist to some of the toxic molecules included within the 119 stressors classified as miscellaneous. In this regard, BC12 and BC5 shared the resistance to 16 of these stressors; further, the *L. gasseri* strain showed tolerance to additional 31 compounds (Fig. 7A and 7D). From a genotypic point of view, both strains possess a thiol peroxidase, Tpx-type (EC 1.11.1.15) that might be involved in oxidative stress resistance, while only the *L. gasseri* strain possesses also a glutathione reductase which might be involved in protection mechanisms from different physiological stressors, including toxic concentrations of metal ions, as well as osmotic and acid stress [113]. Lastly, a bile salt hydrolase gene was found in both the strains which are related to still unclear tolerance mechanisms to these detergent-like biological molecules, and possibly to other toxicant (e.g. detergent and antibiotic) that were tested in PM assay (Table 7). Two tandem genes encoding putative bile transporter genes (one partial and one complete) are organized in operon with one of the two bile salt hydrolase genes in BC12, this type of organization being typical of lactobacilli strains isolated from the human environment (Ruiz et al. 2013).

Table 7. Genetic features of *L. crispatus* BC5 and *L. gasseri* BC12 involved in stress response

Stress	**KO**	**BC5 hits (no.)**	**BC12 hits (no.)**	**KO Name**	**Frequency (%) in *L. crispatus*/*L. gasseri* species[b]**
Osmotic	K05845	0	1	*opuC*; osmoprotectant transport system substrate-binding protein	NA/95.1
	K05846	0	1	*opuBD*; osmoprotectant transport system permease protein	NA/95.1
	K05847	0	1	*opuA*; osmoprotectant transport system ATP-binding protein	NA/100
	K03549	1	2	KUP; system potassium uptake protein	99.1/100
	K03455/ K03315	2	3	Monovalent cation / H(+) antiporter	99.1-100/95.1-100
Beta-lactam/ quinolones	K18104	0	1	*abcA*; Multidrug resistance, efflux pump AbcA	NA/63.4
Beta-lactams	-	3	7	beta-lactamase/penicillin binding proteins*	97.4-99.1/53.6-100
Macrolide	K19350	2	1	lincosamide and streptogramin A transport system ATP-binding/permease protein	84.6-91.4/92.6
	K05685	0	1	*macB*, macrolide transport system ATP-binding/permease protein	NA/85.3
Aminoglycosides	K00662	1	1	*aacC*; Aminoglycoside 3-N-acetyltransferase	88.0/85.36
	K19272	0	1	*aph*; aminoglycoside 3'-phosphotransferase	NA/75.60
Fluoroquinolones	K18908	2	0	*mepA*; Multidrug resistance, efflux pump MepA	99.1/NA
Tetracyclin	K18220	1	1	Ribosome protection-type tetracycline resistance related proteins	97.4/97.5
Arsenic	K03892	1	0	*arsR*; ArsR family transcriptional regulator	2.5/NA
	K00537	1	0	*arsC*; arsenate reductase	95.7/NA

	K03325	1	0	*arsB*; arsenite transporter	2.5/NA
Copper	-	1	1	*copY*; negative regulator of copper transport operon	100/95.12
	K17686	3	3	*copA*; P-type Cu+ transporter	97.4-100/97.5-100
	K07213	1	0	*copZ*; copper chaperon	59.8/NA
	K01533	1	1	*copB*; P-type Cu2+ transporter	97.4/97.5
Cobalt, zinc and cadmium	K16264	1	1	*czcD*; cobalt-zinc-cadmium efflux system	98.3/100
	-	0	1	*czrA*; ArsR family transcriptional regulator, zinc-responsive transcriptional repressor	NA/97.5
Bile resistance	K01442	1	2	Choloylglycine hydrolase/bile salt hydrolase	97.4/92.6-90.2
	-	2	0	Putative Bile Salt Transporter[¥]	NA[§]
Miscellaneous stresses	K04078	1	1	chaperonin GroES	100/100
	K03687	1	1	molecular chaperone GrpE	100/100
	K04043	1	1	molecular chaperone DnaK	100/100
	K03686	1	1	molecular chaperone DnaJ	100/97.5
	K04077	1	1	chaperonin GroEL	99.1/100
	K04083	1	1	molecular chaperone Hsp33	97.4/95.2
	K01358	1	1	ATP-dependent Clp protease, protease subunit	100/97.5
	K03544	1	1	ATP-dependent Clp protease ATP-binding subunit ClpX	100/92.6
	K06149	2	1	universal stress protein A	100/97.5-100
	K11065	1	1	thioredoxin-dependent peroxiredoxin	88.0/9.7
	K08161	2	1	MFS transporter, DHA1 family, multidrug resistance protein	47.8-58.1/95.1
	K18926	2	4	MFS transporter, DHA2 family, lincomycin resistance protein	95.7-98.2/9.7-87.1
	K18936	1	1	MFS transporter, DHA2 family, multidrug resistance protein	95.7/87.8
	K00383	0	1	Glutaredoxin reductase	NA/95%

[b] The range of frequency of each gene product (with the same KO) is indicated within the pangenome of *L. crispatus* and *L. gasseri*, respectively. NA = not applicable

*Predicted via manual curation. Ref *L. crispatus* BC5 GRH99_00740-07030-08995-06145; Ref *L. gasseri* BC12: GRI01_00605, 06430,05880,07430,08775,05675,05870

[¥] Predicted via manual curation. GRI01_06085

§ Predicted as pseudogene by NCBI annotation pipeline and therefore not included in pan-genome analysis

2.3.3 *Lactobacillus gasseri* and *Lactobacillus crispatus* species-wide analysis of the genetic traits associated with the phenotypic differences between BC5 and BC12

Overall, most of the strain specific features linked to the metabolism of sucrose, N-acetyl-galactosamine, galactose and lactose, maltose, fructose, melbiose, maltotriose, trehalose and D-tagatose differentiating BC5 and BC12 were well preserved at species-level (Table 6, Fig. 5). The exceptions are the sucrose phosphorylase gene that were present in both BC5 and BC12 and in few *L. crispatus* and *L. gasseri* strains. Similarly, the levansucrase/invertase and triose/dihydroxyacetone kinase (*dhaK*) genes were present in BC5 and only in few other *L. crispatus* strains (Table 6).

In respect to nitrogen metabolism, the BC5 arginine deiminase was identified in a relative few number of *L. crispatus* strains while the BC5 genes encoding for the dipeptide transport system were included in clusters within the *L. crispatus* core genome.

High conservation of genes functionally linked to osmotic stress tolerance, heavy metals and resistance to antibiotics in BC5 and BC12 was observed within *L. crispatus* and *L. gasseri* species, respectively. These results are partially in line with a previous functional and genomic studies reporting similar phenotypes of resistance and susceptibility to different classes of antibiotics in most of the *Lactobacillus* species under analysis [110]. Among the genetic features associated to stressors resistance only the BC5 *ars* operon (*arsRBC*) was not included in the *L. crispatus* core genome, it only occurred in a few *L. crispatus* strains (Table 7)

2.4 Discussion

The microbial community in the vaginal niche is highly dynamic and characterized by the transition from a bacterial community type to another with a consequent change in predominant species. A transition frequency of 0 was observed between communities I (*L. crispatus* dominated) and II (*L. gasseri* dominated), suggesting different environmental specialization and competitive mechanisms of the two species [6]. In this work we performed the complete genome sequencing and phenotypic characterization of *L. crispatus* BC5 and *L. gasseri* BC12, two strains isolated from the vaginal niche of healthy premenopausal women that showed promising antipathogenic activities. The genome features (genome size, % GC) of these two strains resulted to be in line with those we calculated from a dataset of 117 *L. crispatus* and 41 *L. gasseri* genomes available in the database (in May 2020) and with those previously reported by Mendes-Soares et al. (2014). Both these works pointed out that the genomes of strains belonging to *L. crispatus* are generally larger than those of vaginal *Lactobacillus* strains belonging to different species [114]. Genome annotation analysis was combined with the study of phenotypic differences between *L. crispatus* BC5 and *L. gasseri* BC12, in terms of utilization of carbon and nitrogen sources and in terms of resistance/tolerance to different stressors to obtain information on the nutrients and stress conditions that could influence their capacity to colonize a common environmental niche. The possible genetic traits associated with these phenotypic features were further identified in the BC5 and BC12 genome as well as in each species core genome in order to assess their conservation among *L. crispatus* and *L. gasseri* strains. BC5 and BC12 showed a very limited number of singleton features (characterizing each strain from other members of the same species) that were mostly annotated as phage proteins and mobile elements and not associated with metabolic activities, with a few exceptions. Despite strain-level differences are known within *Lactobacillus* species [114], we believe that the identification of the PM-related genetic features preserved across *L. crispatus* or *L. gasseri* strains could provide insights into those factors contributing to selective dominance of each species in the vaginal tract.

One of the most important aspect of microbial colonization of any environmental niche regards the capacity to utilize the available nutrients for growth [115]. In particular, the composition of vaginal fluid in terms of glycoproteins and free carbohydrates is important in vaginal colonization by bacteria [116]. Accordingly, in the PM experiments described in this work, if on one hand, both the strains displayed a preference for sugars (over amino acids, carboxylic acids, amines, amides, esters, polymers, fatty acids), *L. crispatus* BC5 generally showed an improved capacity to utilize carbon sources, in terms of number of metabolized compounds and metabolic activity values. Specifically, some unique capacities were identified within the carbon and nitrogen source utilization pattern of each strain that could contribute to the selective colonization of a common niche. The genome analysis performed in this work allowed to associate the different phenotypes shown by BC5 and BC12 in terms of carbon and nitrogen source utilization to specific genetic traits that were mostly conserved on either *L. crispatus* or *L. gasseri* core genomes, with a few exceptions (Table 6, Fig. 5), thus providing some phenotypic insights at species level. The main phenotypic differences in terms of carbon source utilization included the capacity of BC5 to utilize glycosylamines in the form of nucleosides and the capacity to utilize melibiose, rhamnose, 3-0-b-D-Galactopyranosyl-D-Arabinose, and a-Methyl-D-Mannoside, while BC12 was able to utilize amygdalin, tagatose, gentiobiose, salicin. Both the strains could utilize host derived mannose, galactose and most of the hexamine, except for the N-Acetyl-D-Galactosamine that was only used by BC12. Within the context of vaginal tract niche, the nutrients typically present are free mono- and oligosaccharides, glucosamine, glycoproteins, glycerol, lactic acid, acetic acid, albumin, mucin, urea and ions (e.g. Na^+, K^+ and Cl^-) [117–119]. In particular, glycogen and glucose represent the major carbohydrates of the vaginal fluid, while mannose, maltose and glucosamine were found in much smaller quantities [120]. PM results indicated that BC5 could metabolize glucose, pyruvic acid and lactic acid at higher rate indicating a higher lactic acid fermentation efficiency. BC5 showed also higher metabolic activity in the presence of mannose[121], while acetic acid and glucosamine were equally utilized by the two strains. Glycogen could not be directly utilized by any of the two strains; however, BC5 showed higher metabolic activity in the presence of the glycogen debranching products (maltose, maltotriose and glucose), which might be released in the vaginal tract through depolymerization activity of amylases from the host or other bacterial strains [117,122]. Previous study indicated that glycogen could be utilized as carbon and energy source by *L. crispatus* strains that possessed in their genome an active pullulanase type I presenting an N-terminal signal peptide probably involved in extracellular localization of the enzyme. In line with the inability of BC5 to utilize glycogen in PM, its pullulanase gene presented amino acid deletion in the N-terminal sequence that probably hampers the utilization of extracellular glycogen [123]. Some genes encoding transporters and enzymes involved in the glycogen debranching products

consumption were identified in BC5 and were included in *L. crispatus* core genome, while they were not found in both BC12 and *L. gasseri* core genome. In particular, in the case of maltose, the lower metabolic activity of BC12 seen in PM could be due to possible cellular stress induced by the accumulation of the sugar that can retard the growth and lower the metabolic rate of *L. gasseri* strains cells. This stress condition may not arise in *L. crispatus* strains due to the activity of a maltose 6-phosphate glucosidase, which can protect cells from maltose accumulation in the cytosol through its hydrolyses [124]. Based on these findings therefore we propose that an efficient utilization of glycogen degradation products actively contributes to the dominance of *L. crispatus* strains in a healthy vaginal niche. Furthermore, the higher metabolic activity of BC5 on lactose and galactose as compared to BC12, can be associated to the presence of distinct metabolic pathways which are respectively conserved also in the two species (Fig. 5), i.e. Leloir pathway in *L. crispatus* and Tagatose-6P pathway in *L. gasseri*. BC5-strain specific traits (not included in *L. crispatus* core genome) supporting the higher metabolic performance of BC5 as compared to BC12 include a few genes involved in sucrose and fructose metabolism. In the case of sucrose, the BC5-specific levansucrase/invertase gene can contribute to the formation of exopolysaccharides which are involved in biofilm development and various stress responses, being therefore possibly related to metabolic advantages [125]. The possibility to produce EPS with specific carbohydrates can lead to peculiar host interaction and persistence in different *L. crispatus* strains [116,126].

Unlike BC12, BC5 showed the capacity to utilize amines and amides as carbon sources. These properties might be associated with synergistic relationships that may occur within the vaginal flora, even under bacterial vaginitis conditions in which a series of biogenic amines are produced by pathogenic bacteria [127,128]. On the other hand, BC12 specific phenotypic traits were related to the utilization of i) mucin-derived compound N- acetyl-galactosamine as both nitrogen and carbon source (while both the strains could metabolize N-acetyl-glucosamine), ii) diet acquired compounds amygdalin and tagatose, iii) fatty acids in the form of polysorbates (Tween compounds). Furthermore, BC12 showed higher metabolic activity on trehalose as compared to BC5, despite both the strains possessed the genes involved in this sugar import. A previous study reported the presence of a complete operon possibly involved in trehalose import and metabolism in almost all (65 out of 67) the *L. crispatus* strains isolated from humans (vagina and gut). Conversely, we propose this operon being mainly involved in maltose metabolism, which explains the very low metabolic activity of BC5 in the presence of trehalose and the absence of works describing this carbohydrate as a carbon source typically utilized in the human urogenital tract [129]. Carbohydrates metabolised only by BC12 were always associated to the presence in its genome of specific transport and metabolism genes, which were all included in the *L. gasseri* core genome. The capacity of strains belonging to this species to

utilize Tween compounds was previously related with membrane synthesis needs and with increased survival capacity in acidic environments [130,131]. In other cases, Tween compounds were shown to have a toxic effect on bacterial cell due to the solubility properties and detergent effect [103] and this aspect could be related with the lack of metabolic activity observed with BC5. Furthermore, BC5 and BC12 differently metabolized oligosaccharides that have been described as possible prebiotics e.g. inulin and mannan [132,133], suggesting an effect of these polymers on selective colonization of these probiotics.

Only a few amino acids were metabolized by BC5 and BC12 as carbon source, whereas a higher number of them could be utilized as nitrogen sources. Within hosts, nitrogen is available primarily as free amino acids and proteins [134]. Among the amino acids that are normally present in the vaginal tract, aspartic acid and tryptophan could be utilized by both the strains [135], while only BC12 could metabolize glutamic acid as both carbon and nitrogen source and arginine resulted to be utilized as nitrogen source only by BC5. This latter capacity was associated with the presence in BC5 genome of a gene encoding an arginine deiminase that converts arginine into citrulline and NH_3, also contributing to counteract environmental stresses, such as acidity and starvation [104,136]. BC5 could also utilize di-peptides as nitrogen and carbon sources in association to the presence of a specific peptide transport system that might be involved in these molecules import. The ability to efficiently assimilate complex nitrogen molecules was also described in a *L. plantarum* strain. In this strain it was also proved that the presence of dipeptides in the medium increased sugar utilization [137].

Among those carbon sources that could not be utilized by any of the two strains but are important in the vaginal tract, glycerol seemed not be utilized by any of the two strains despite the presence in their genomes of genes encoding a glycerol-3-phosphate dehydrogenase, a glycerol kinase and a glycerol facilitator protein. Glycerol is usually present at low concentration in vaginal niche [117], however, it was described as an underappreciated carbon source due to upregulation of glycerol pathways in *L. iners* under bacterial vaginosis conditions [20]. Both the *Lactobacillus* strains could not use urea both as carbon and nitrogen source and this is in line with the lack of a urease coding operon in their genomes. However, BC12 showed resistance to urea when added as osmolyte stressor up to 4%, probably due to non-specific resistance mechanisms.

The capacity to resist/tolerate chemical stressors is also an important aspect for the successful colonization and persistence of a bacterial population within a specific environmental niche. In particular, during their life as commensals, vaginal lactobacilli encounter several stresses including pH, osmotic stress and antibacterial compounds such as antibiotics, detergents, and metals [138,139]. In PM assays, we tested the capacity of BC5 and BC12 to cope with a wide range of stressors, not performing viable cell count to define their minimal inhibitory concentration (MIC) of the toxic

compound, as previously reported by various studies [140–142], but revealing the metabolic activity of the cells exposed to the toxicants. This type of analysis includes the activity not only of the viable cells but also of possible phenotypic variants, which arise during severe stresses and are in a quiescent physiological state. These "injured" cells are not able to proliferate but can still contribute to the stress response and the metabolic activity, representing an important aspect of bacterial environmental adaptation and persistence [143–145]. In PM assay, both BC5 and BC12 showed the capacity to cope with acidic pH values that is typical of a healthy vaginal niche. Further, both the strains showed the capacity to resist several osmolytic and ionic stressors, although BC12 generally showed improved metabolic performance in the presence of higher concentration of these osmolytes as compared to BC5. Among these, BC12 showed higher tolerance towards salts and molecules present in the vaginal fluid such as KCl and sodium lactate (that is the dissociated form of lactic acid existing at neutral pH), and urea. These results generally indicated the capacity of BC12 to cope with lower water activity values as compared to BC5 strain; further, the higher tolerance to KCl could be associated to the presence of two copies of the gene encoding a low affinity K^+ uptake system KUP in BC12 possibly involved in a primary or initial phase of osmoadaptation [146]. The two strains also showed the possibility to accumulate distinct compatible solutes (i.e., betaine and proline in BC12 and trigonelline in BC5) to stand high osmolarity stress, and in the case of BC12, this metabolic adaptation could be associated to the presence of a glycine/betaine/carnitine ABC transporter of Opu family possibly involved in both proline and betaine import. Additional osmotic stress response capacity in both the strains could be associated to the presence of cation antiporter systems which catalyze efflux of cytoplasmic monovalent cations such as Na^+, K^+ or Li^+ in exchange for external H^+ [147]. The improved resistance capacity of BC12 is even more evident when chemical stressors are considered, including antibiotics, metals and other miscellaneous chemicals. In particular, the metabolic activity of the *L. gasseri* strain on a broader range of antibiotics than the *L. crispatus* strain suggests a possible advantage during specific antibiotic therapy. Antibiotics greatly influence the human microbiota and the microbial composition of the vaginal niche is known to undergo rapid modification during and after antibiotic treatment [139,148,149]. The evaluation of survival capacity of probiotic strains during antibiotic treatment may allow predicting their persistence in the vaginal niche. On the other hand, some works have pointed out the possibility that *Lactobacillus* spp. strains could act as donors or reservoirs for antibiotic resistance genes, with the potential risk of transferring these genes to pathogenic bacteria cohabiting the same environmental niche, e.g. food matrices and the gastrointestinal tract [110]. In particular, a recent work that described the antibiotic susceptibility pattern of 182 *Lactobacillus* strains (including only one type strain for each *L. gasseri* and *L. crispatus* species) reported that resistances to trimethoprim, vancomycin and kanamycin were common

phenotypes in this genus. In this regard, only BC12 showed the resistance to trimethoprim and only when added at the lowest concentration, while both BC5 and BC12 strains were resistant to vancomycin and kanamycin and at the same level. Generally, *Lactobacillus* spp. strains were found to be susceptible to low concentration of b-lactams [150]; however, in our study, we found BC12 to be resistant to seven out of eleven b-lactams tested (including penicillins) and both BC5 and BC12 were resistant to ampicillin and penicillin. Similar to what previously indicated for other b-lactam resistant *Lactobacillus* strains [110], no mutations of the genes encoding penicillin binding proteins (PBPs) were detected in BC5 and BC12 that could explain this resistance phenotype. However, in these strains, we detected b-lactamase encoding genes that justified the wide resistance capacity towards this antibiotic class. Both the strains were resistant to tetracyclin and polymyxin B, although *L. gasseri* BC12 showed metabolic activity at higher concentration of this latter compound. Further, BC12 was also resistant to low concentration of erythromycin and generally showed a better performance on other clinically important antibiotics such as macrolides, fluoroquinolones and quinolones, while BC5 showed higher resistance to gentamycin. In addition to antibiotics, BC5 and BC12 showed some resistance to toxic metal(loid)s. Human beings around the globe are exposed to increasing amounts of toxic metals due to pollution released by industrial activities [151] and some works have reported the promising capacity of *Lactobacillus* strains to tolerate heavy metals such as cadmium and lead [152], by reducing metal toxicity. In relation to this, both bioaccumulation and biosorption were described to be possible detoxification strategies of *Lactobacillus* that can prevent the exposure of heavy metals to the host body cells and tissues [151]. This implies the possible application of these strains in dietary strategies for people at risk of specific metal exposure. In our study we found that BC5 and BC12 has resistance to copper, cobalt, silica and zinc, although the *L. gasseri* strain showed higher metabolic activity level. Further, only BC12 was also resistant to antimony and arsenite. A few transporters possibly involved in multi-metal (cobalt, zinc and cadmium) resistance were identified in both BC5 and BC12 genomes as well as an operon *copYACB* related to copper resistance. On the other hand, the higher susceptibility to arsenite and antimonites shown by BC5 could not be explained at genetic level as BC5 but not BC12 genome carries ars genes (i.e. *arsRCB* operon) which are the genetic traits typically associated to the bacterial resistance to arsenic and antimony [112]. Possible additional cationic antiporters (one of these annotated in RAST as arsenic efflux pump) might be involved in arsenite resistance, although further analyses are needed.

Additionally, non-specific multidrug transport systems [153], ABC multidrug transporters, major facilitator superfamily members (Table 7) and oxidative stress response components that we identified in BC5 and BC12 genomes might be involved in non-specific detoxification mechanisms of antibiotics, metals and other chemicals classified as miscellaneous, such as oxidizing agents, DNA

synthesis inhibitors, folate antagonists, and detergents. More in general, multifactorial mechanisms might be implied in *Lactobacillus* response to most of the stressors that were tested in PM assay. In this regard, the induction of general stress response, protection against oxidative damages, as well as global glycolytic reorganizations were described to be involved in *Lactobacillus* resistance mechanisms to detergent-like molecules, such as bile [154]. Further, non-specific mechanisms, such as multidrug transporters [153], might be involved in the detoxification of molecules of different categories. For instance, previous studies reported that deletion of one of the five transporters detected in the strain *L. acidophilus* NCFM rendered the mutant strains more sensitive to bile and certain antibiotics, highlighting the possibility of transporters to act on different molecules detoxification [154,155].

Taken together, the PM results indicate that the *L. crispatus* strain possess a wider range of carbon source utilization, while the *L. gasseri* strain is more resistant to different environmental stressors. This aspect is probably related to the multi-niche adaptation of *L. gasseri* species, that is commonly associated to the human intestine, which is known as a competitive and complex niche leading bacterial strain to acquire unique stress coping mechanisms [129]. On the other hand, in human beings, *L. crispatus* is relatively restricted to the female lower genital tract, with few exceptions of human isolates from human eye, human gut and human oral cavity. In several cases these phenotypic features could be associated with genetic traits that are included in each species core genome, and therefore highlighting phenotypic features that are common at species level and justify the selective dominance of strains of either one of the two species.

In conclusion, strains belonging to *L. gasseri* and *L. crispatus* have shown implications in human health in terms of protection against pathogen and in wound healing [116]. The knowledge about the metabolic activity of *L. gasseri* and *L. crispatus* strains in the presence of hundreds of different nutrients and environmental stressors provide information on environmental factors affecting the two species colonization of vaginal niche and probiotic functionality. These findings could provide guidance to be taken into consideration during probiotic formulation for enhanced efficacy.

3. *Lactobacillus gasseri* biosurfactants loaded into liposomes as novel anti-biofilm agent

This chapther is based on: Giordani B & Costantini PE, Fedi S, Cappelletti M, Abruzzo A, Parolin C, Foschi C, Frisco G, Calonghi N, Cerchiara T, Bigucci F, Luppi B, & Vitali B. Liposomes containing biosurfactants isolated from Lactobacillus gasseri exert antibiofilm activity against methicillin resistant Staphylococcus aureus strains. 2019 Eur J Pharm Biopharm. 139:246-252

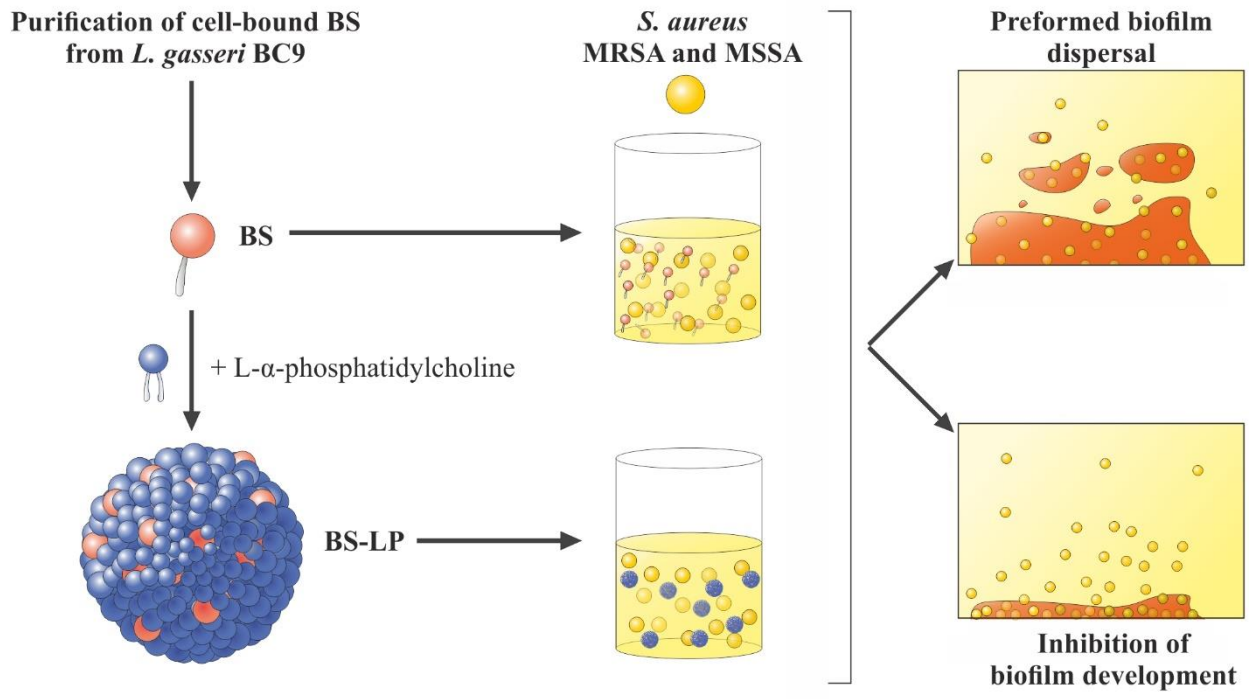

Fig. 8. Graphical abstract showing the overall approach use in the study of *L. gasseri* biosurfactants and biosurfactants loaded into liposomes as antibiofilm-agents. BS=biosurfactant, BS-LP=liposome formulation carrying biosurfactant.

3.1 Introduction

Biosurfactants (BS) are amphipathic compounds produced by several microorganisms and localized on the cell surface (cell-bound BS) or in the extracellular region (cell-free BS) of the producer organism [156]. BS are usually produced by microbial secondary metabolism and are involved in several bacterial cell processes such as nutrient transport, quorum sensing and competition with other microorganisms. This latter function is exerted through different mechanisms including interference in microbe-host interaction, antibacterial, antifungal, antiviral, anti-biofilm, and anti-adhesive

activities [43,44,157–159]. The most commonly isolated BS include glycolipids, rhamnolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids [159]. Unlike synthetic surfactants, BS are "green" compounds due to their natural origin, biodegradability and low toxicity [160]. Other advantages of BS are represented by their highly selective, specialized functions and effectiveness at a wide range of temperatures, pH and salinity [43,161]. Due to these characteristics, BS have been widely studied for possible applications in various industrial fields, includes food, cosmetics and bioremediation industries [44,160]. Moreover, considering their antimicrobial properties, they were studied for possible medical applications such as the treatment of infection disease or the creation of anti-adhesive coating on medical devices [44]. In particular, lactobacilli BS were demonstrated to be excellent candidate due to their specificity against human pathogens and their antimicrobial and antiadhesive properties [162]. However, only few studies report anti-biofilm activity of BS against human pathogens [158,161,163]. Furthermore, the inclusion of active molecule in nanocarriers was demonstrated to improve the molecule activity through its direct released on bacterial cell or biofilm surface [164]. Among the several delivery systems developed in the last years for prevention and treatment of infections, liposomes (LP), were demonstrated to be safety nanocarriers with high penetration of biofilm extracellular matrix [165]. Among the different human pathogens capable of causing chronic infection with biofilm formation, *S. aureus* was reported to be one of the principals and its infections are usually associated with onset of different disease, such as skin infections, infective endocarditis and bacteremia [166]. In addition to the biofilm growth, the high genetic variability and the fast acquisition of antibiotic resistance genes by *S. aureus* have decreased the number of effective therapies to contrast *S. aureus* infections [167–170]. In particular, the presence of multiple antibiotic resistances is an overall feature of MRSA (Methicillin Resistant *S. aureus*) strains, while strains classified as MSSA (Methicillin Sensitive *S. aureus*) usually possessed few or none antibiotic resistances [171,172]. In this study we investigated antibiofilm activity exerted by *L. gasseri* BC9 cell-bound BS on *S. aureus* biofilm belonging to both MRSA and MSSA groups as well as their safety for medical application through cytotoxicity evaluation on human and murine cell line. Moreover, formulation of liposome loading BS (BS-LP) was prepared, chemical characterized and assayed for antibiofilm activity. The coupling of BS with nanocarriers for dispersion of pre-formed biofilm and inhibition of biofilm development represent an innovative approach for prevention and treatment of *S. aureus* MRSA and MSSA infections.

3.2 Methods

3.2.1 Bacterial strains use in this chapter

Table 8. List of bacterial strains used in this chapter.

Bacterial strain	Reference
Lactobacillus gasseri BC9	51
Staphylococcus aureus ATCC29213™	American Type Culture Collection (ATCC)
Staphylococcus aureus 2	173
Staphylococcus aureus 7	173
Staphylococcus aureus 45	173
Staphylococcus aureus 83	173
Staphylococcus aureus 86	173
Staphylococcus aureus 88	173

3.2.2 Biosurfactants isolation

Biosurfactants (BS) were purified from pure culture of *L. gasseri* BC9 following biosurfactant purification protocol previously reported [47]. Overnight culture of *L. gasseri* BC9 was diluted 1:10 in 400 mL of MRS and incubated at 37 °C for 24 hours in anaerobic atmosphere. The obtained cultures were centrifuged for 10 minutes at 10000 g and the pellet-containing cells was wash twice with sterile water. Cells were than resuspended in 150 uL of PBS and stirred for 2 hours in order to allow the cell-bound BS released. BS were then isolated from cells through centrifuge and filtration with 0.22 μm filters. Purification was completed by dialysis in sterile demineralized water for 24 hours and subsequently BS lyophilisation at -45 °C, 0.001 atm using Christ Freeze Dryer ALPHA.

3.2.3 Biosurfactant loaded liposomes preparation and characterization

Liposomes loaded with BS (BS-LP) were prepared following ethanol injection method previously described ([174]). Briefly, BS and phosphatidylcholine (PC) were resuspended together in 5 mL of ethanol 96 % with a weight ratio of 1:3 and then injected, with a constant flow of 1 mL/min, in 10 mL of water stirred at 200 rpm. Control liposomes (control-LP) were prepared without the addition

of BS. Ethanol was removed from each sample using rotary evaporation under vacuum (Rotavapor R-200, Flawil) for 10 minutes at 60 °C. All the liposomes formulations were finally filtered through 0.22 μm filtered in order to ensure sterility and store at 4 °C. Liposomes were characterised for their surface potential (zeta potential) and size distribution. For both analysis samples were diluted 1:1000 v/v in milli-Q water. Polydispersity was analysed using dynamic light scattering through Brookhaven 90-PLUS instrument (Brookhaven Corp) with He-Ne laser beam set with wavelength = 532 nm and scattering angle=90°. Liposomes stability over time was evaluated by measuring polydispersity index (PDI) and size after 0, 7, 14, 21 and 28 days of storage at 4 °C. Liposomes surface potential was measured with Malvern Zetasizer 3000 HS (Malvern Panalytical Ltd) at 25 °C.

3.2.4 *Staphylococcus aureus* biofilm dispersal and inhibition of biofilm development

Biosurfactant (BS), biosurfactant loaded liposomes (BS-LP) and liposomes (control-LP) were resuspended in Nutrient broth (NB) before their use in biofilm assays.

Both inhibition of biofilm development and biofilm dispersal was assessed using previously reported protocol with some modifications [158]. For the inhibition of biofilm development, *S. aureus* cells were collected from NB plate and resuspended, up to an OD_{600} of 0.025, in NB (control), BS, BS-LP or control-LP. Bacterial suspensions were transferred to 96 wells plate (150 uL per well) and incubated at 37 °C, for 24 hours with shaking at 100 rpm.

Biofilm dispersal assay was achieved similarly but with few modifications in comparison to inhibition of biofilm development experiment. Briefly, *S. aureus* biomass collected from NB plate were inoculated in liquid NB to a final OD_{600} of 0.025 and transferred to 96 wells plates (150 uL per well). Plates were incubated at 37 °C, for 24 hours in agitation at 100 rpm to allow biofilm formation on the lateral surface of the well. Liquid suspension was gently removed from each well, without detaching biofilm attached, and 150 μL of NB (control), BS, BS-LP or control-LP were transferred to each well. Plates were finally incubated for other 24 hours at 37 °C in agitation at 100 rpm.

Both inhibition of biofilm development and biofilm dispersal were evaluated using crystal violet assay, while toxic effect on bacterial cells was evaluated through count of CFU on planktonic cells. Attached biofilms to the well were washed twice with 200 μL of PBS, fixed with 200 μL of pure ethanol for 10 minutes and stained with 180 μL of crystal violet (0.41% w/v in ethanol 12% ethanol). Samples were washed 3 times with 200 μL milli-Q water and finally incubated for 5 minutes with 200 μL of pure ethanol to release crystal violet biofilm-associated. Absorbance was measured in each well using EnSpire Multimode Plate Reader (PerkinElmer Inc) with fixed wavelength = 595 nm, no. of reads per well = 20.

Results were calculated on 3 independent biological replicates using the equation:

$$\text{Biofilm dispersal/Inhibition of biofilm development (\%)} = \left[1 - \left(\frac{\text{mean ABS sample}}{\text{mean ABS control}}\right)\right] \text{x}100$$

3.2.5 Biosurfactants cytotoxicity

Biosurfactants cytotoxicity was evaluated on human adult and murine fibroblast (HDFa and NIH/3T3) through MTT assay. Cell cultures were transferred to 96-well culture plates at a final concentration of $1.5\text{x}10^4$ cells per well and growth for 24 hours in RPMI supplemented with FBS 10% and L-glutamine 2 mM. Cytotoxicity was tested by the addition of biosurfactants, resuspended in RPMI, at increasing concentration in the cell culture. After 24 or 48 hours of incubation with BS, cells were treated with MTT (dissolved in PBS at the concentration of 0.2 mg/mL) for 2 hours at 37 °C. Finally, each sample was treated for 20 minutes with 0.1 mg/mL of isopropanol and the absorbance at 570 nm was measured using Victor2 plate reader (PerkinElmer Inc).

3.3 Results

3.3.1 Biosurfactant cytotoxicity

Cytotoxic effect of biosurfactants purified from *L. gasseri* BC9 was evaluated on human (HDFa) and murine (NIH/3T3) fibroblasts through MTT assay. Cell viability was measured after 24 or 48 hours of exposition to increasing concentration of BS. As shown in Fig. 9, HDFa (A) and NIH/3T3 (B) cell viability is not influenced by BS of *L. gasseri* BC9 at all the concentrations tested, suggesting that BS are not toxic for eukaryotic cell. Similar results were obtained with *L. jensenii* and *L. rhamnosus* cell-bound BS on lung epithelial cell lines [158], suggesting that usually BS produce by lactobacilli are safety product for medical application.

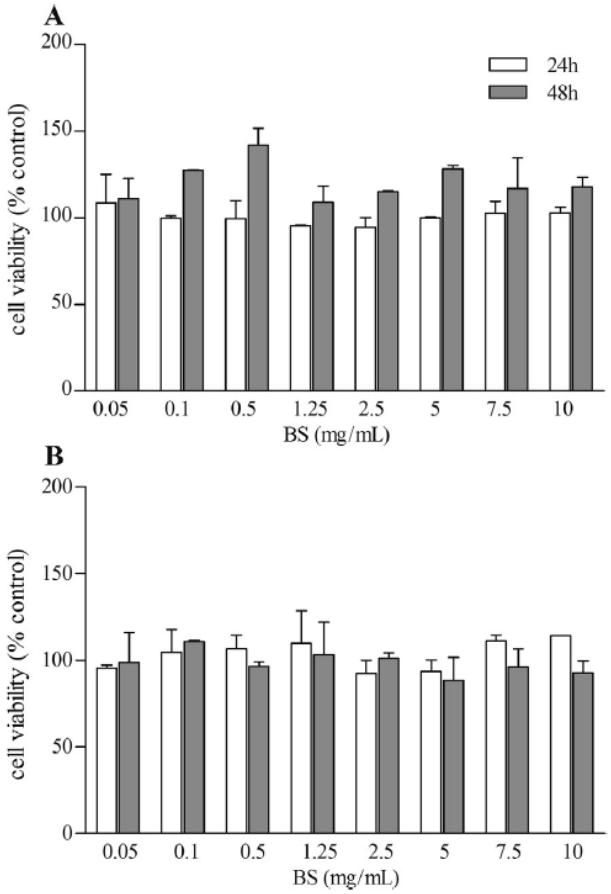

Fig. 9. Cell viability in percentage of control measured through MTT assay of (a) HDFa and (b) NIH/3T3 cells after the treatment with increasing BS concentration for 24 h or 48 h. Data refers to the mean of 3 different replicates and statistical significance was calculated using one-way ANOVA ([173]).

3.3.2 Antibiofilm activity of *Lactobacillus gasseri* BC9 BS against *Staphylococcus aureus*

Antibiofilm activity of BS was assessed by testing the effect of different BS concentration on both inhibition of biofilm development and biofilm dispersal. Experiments were performed on 7 different *S. aureus* strains, of which 1 reference strain without antibiotic resistance (*S. aureus* ATCC 29213) and 6 clinically isolated strains classified as MRSA (*S. aureus* 2, 7, 45, 83, 86) or MSSA (*S. aureus* 88). Considering the critical micellar concentration (CMC) of *L. gasseri* BC9 BS (2 mg/mL) [47] we decided to test antibiofilm activity using BS concentration that are more or less half, equal or double of CMC (1.25, 2.5 and 5 mg/mL). *L. gasseri* cell-bound EVs caused a statistically significant dispersion of preformed biofilm on all the strain tested (Fig. 10).

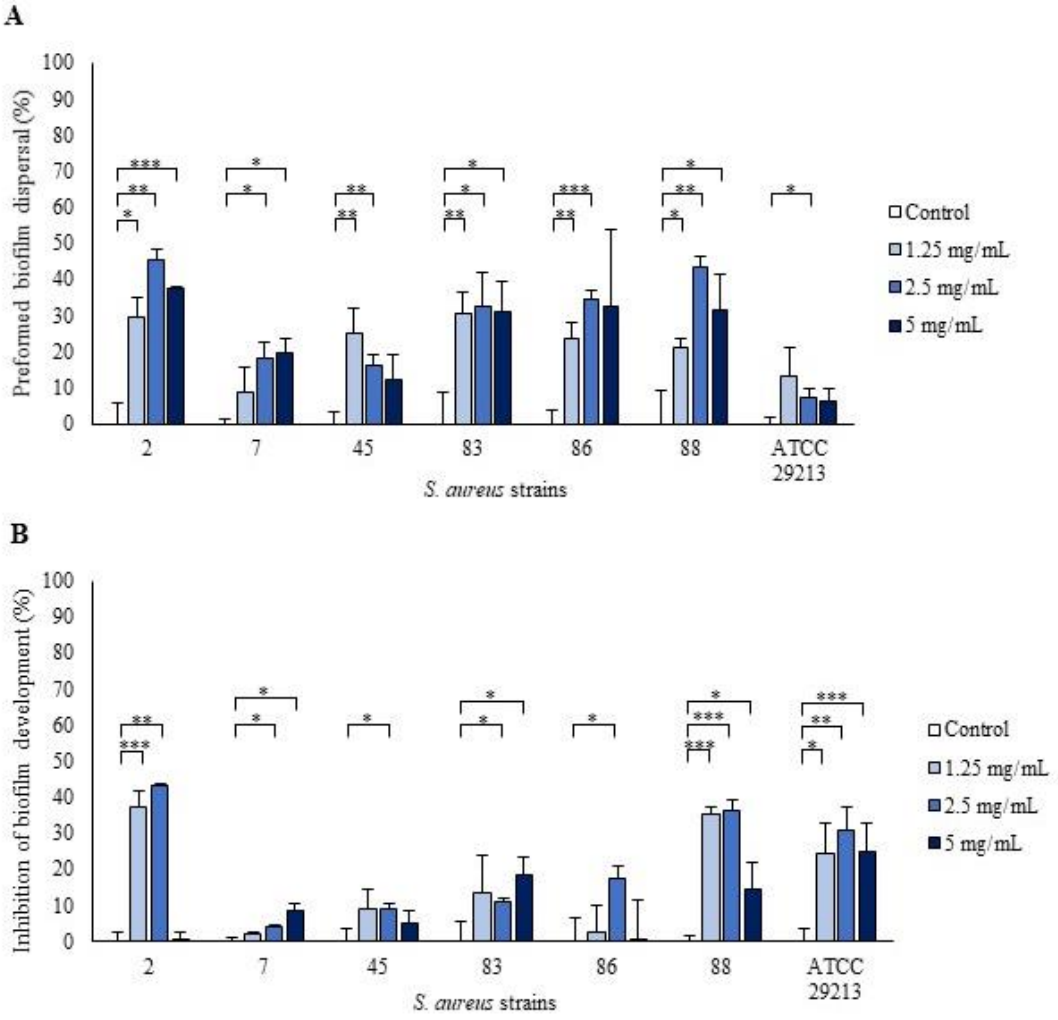

Fig. 10. Antibiofilm activity of BS against different strains of *S. aureus* were analysed in terms of dispersion of preformed biofilm (A) and inhibition of the development (B). Biofilms were treated with NB (white bars) or with increasing concentrations of BS: 1.25 mg/mL (light blue bars), 2.5 mg/mL (blue bars) and 5 mg/mL (dark blue bars). Data represents mean value ± standard deviation (%) calculated on 3 independent replicates. Statistical significance was evaluated using the student's t-test against the control. * refers to p-value<0.05, ** p-value<0.01 and *** p-value<0.001. (Modified from [173])

In particular, the higher biofilm dispersal values were observed for *S. aureus* strains 2,83,86 and 88 using BS at the concentration of 2.5 mg/mL. BS biofilm dispersion is not dose-dependent and higher dispersion values were observed using a concentration of BS 2.5 mg/mL. BS exerts a role also in the inhibition of *S. aureus* biofilm development and the activity is not subject to dose-dependency. Indeed, the higher inhibition was observed on *S. aureus* strains 2, 88 and ATCC29213 at the concentration of 2.5 mg/mL (Fig. 10). To test if those antibiofilm activities were induced by BS toxicity on bacterial cells, count of the CFU were performed on planktonic growth. Results obtained revealed that BS are not toxic for the *S. aureus* strains tested (Fig. 10), suggesting that BS acts only on the biofilm growth and development without killing bacteria cells.

3.3.3 Characterization of liposomes

During liposomes preparation through ethanol injection method [175], the fast dilution of ethanol in water allow the formation of bilayer portions that could aggregate through stirring with the formations of spherical structure [176]. Liposomes formulations were characterized in terms of polydispersity index (PDI) and mean diameter immediately after the preparation (day 0) or after 7, 15, 21 and 28 days of storage at 4 °C. As shown in figure Z, all prepared liposomes present a size lower than 200 nm, which makes them good candidate as anti-biofilm agent [177], and BS-LP possesses a major mean diameter in comparison to control-LP. Both the formulations are characterised by low PDI which demonstrates low heterogeneity among liposomes generated. PDI and mean diameter did not present any significant variations during 28 days of storage, suggesting an excellent liposome stability among time.

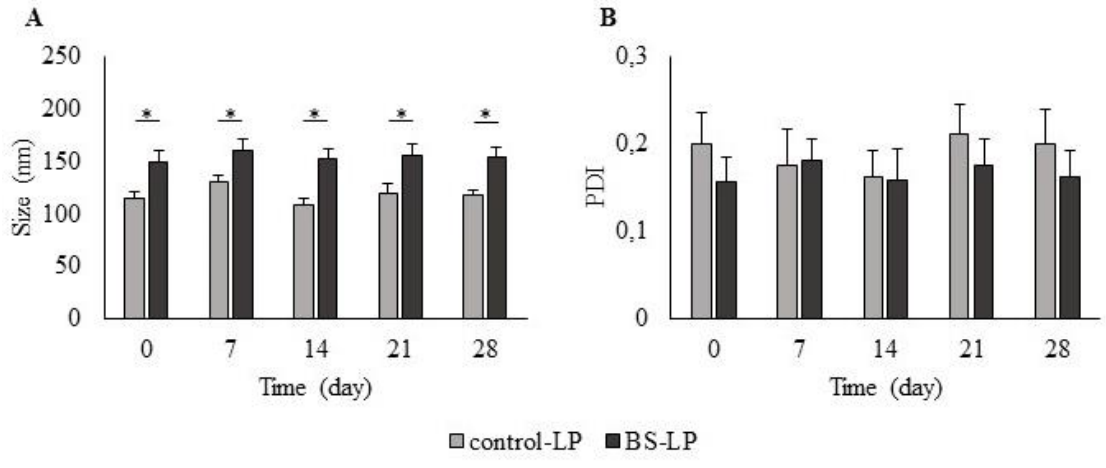

Fig. 11. Liposomes formulation stability over time. Size (A) and PDI (B) were measured every 7 days in a period of 28 days. Data refer to the mean of 3 different replicates and statistical significance was calculated using one-way ANOVA. * refers to p-value<0.05. (Modified from [173])

Zeta potentials measured (BS-LP= -36.14 ± 2.12 mV / control-LP= -38.41 ± 1.11 mV) are all near or over the limit required for stability [178] and did not present significant variations between BS-LP and control-LP.

3.3.4 Antibiofilm activity of liposomes containing BS against *Staphylococcus aureus* strains

Liposomes formulations, include control-LP, were tested for their antibiofilm activity at the final concentration of 2.5 mg/mL. The dispersion assay of preformed biofilm revealed that BS-LP are active against all the strain tested, while liposomes alone (control-LP) did not produce any significant dispersion of *S. aureus* biofilm (Fig. 11). Furthermore, the loading of BS into liposome strongly increases the biofilm dispersion in confront to the activity observed using pure BS. The higher biofilm dispersion value of BS-LP was observed on strain 45 (92.4%) and the overall activity is above the 50% on all the strain tested, except *S. aureus* ATCC29213 (42.3 %). Similar results were obtained using BS-LP for the inhibition of biofilm development. Indeed, control-LP have no effect and BS-LP inhibition is higher in confront to crude-BS, except for *S. aureus* 2 (Fig. 12). This was the only case in which liposome formulation did not enhance antibiofilm activity of BS. In this context, biofilm of *S. aureus* 2 was the most inhibited by BS and this could explain the absence of improvement biofilm inhibition by liposome on this strain.

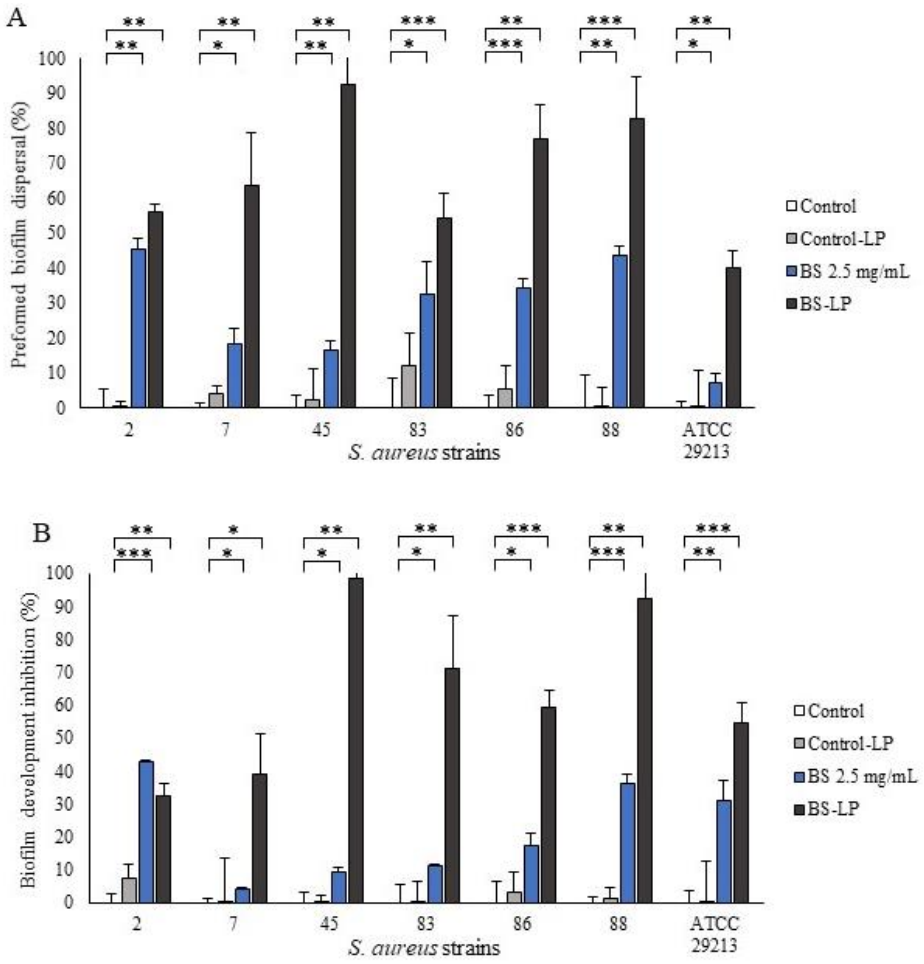

Fig. 12. Antibiofilm activity of liposomes formulations against different strains of *S. aureus* was evaluated in terms of dispersion of preformed biofilm (A) and inhibition of the development (B). Biofilms were treated with nutrient broth (white bars), BS-free liposomes (light grey bars), BS (blue bars) or BS loading liposomes (dark grey bars). Data represent mean value ± standard deviation (%) calculated on 3 independent replicates. Statistical significance was evaluated using the student's t-test against the control. * refers to p-value<0.05, ** p-value<0.01 and *** p-value<0.001. (Modified from [173])

Finally, to understand if the antibiofilm activities detected for BS-LP are partially or totally related to a cytotoxic effect on bacterial cells, bacterial cultures were grown in presence of BS-LP, control-LP and PBS (control). Count of the CFU shows that both liposome alone and liposome loading biosurfactant are not toxic for bacterial cells, demonstrating that antibiofilm activity is not related to *S. aureus* cell death (Fig. 13).

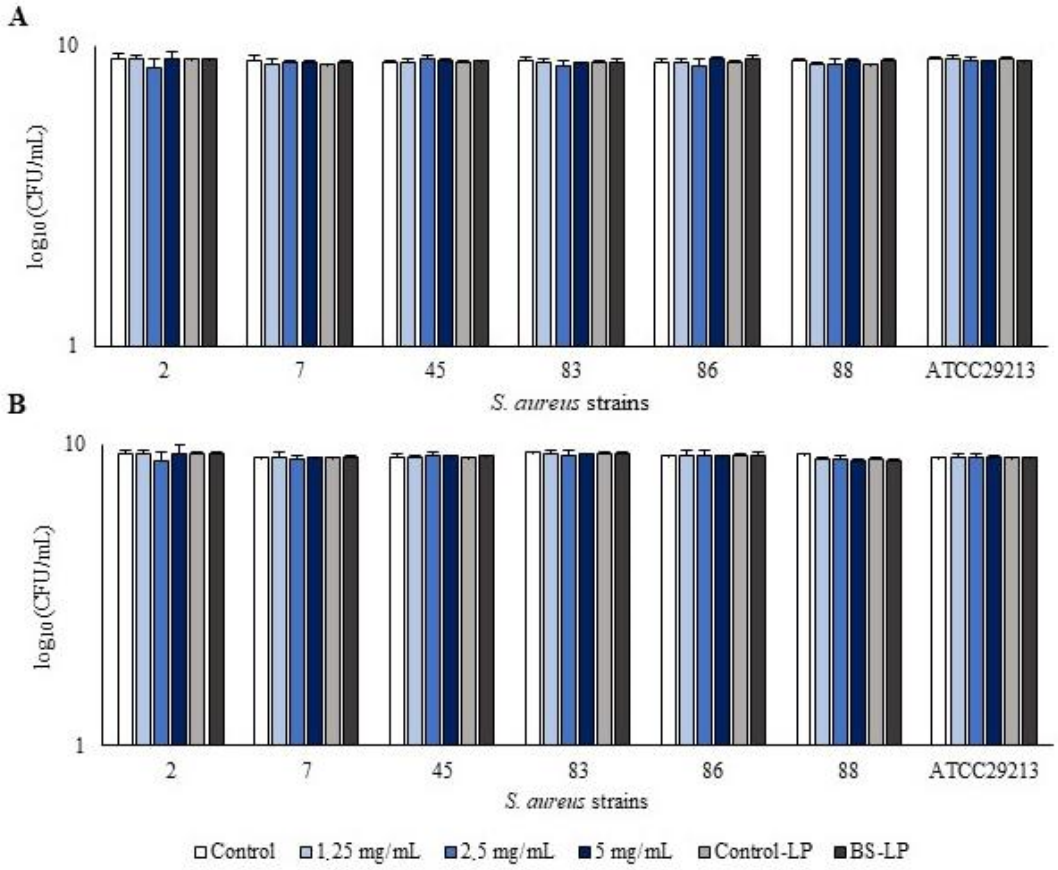

Fig. 13. Evaluation of BS and liposomes formulations cytotoxicity on *S. aureus* strains. Serial dilutions and count of CFU were performed on planktonic growth after preformed biofilm dispersal assay (A) or inhibition of biofilm development assay (B). Data represent mean value ± standard deviation of CFU/mL in logarithmic scale and were calculated on 3 independent replicates. Statistical significance was evaluated using one-way ANOVA multiple comparison.

3.4 Discussion

Biosurfactants, especially those produced by lactobacilli, were demonstrated to possess interesting properties against *Candida* adhesion [162], as well as to affect biofilm growth of human pathogen bacteria [161,163,179]. *S. aureus*, in particular MRSA strains, was frequently reported as infective agent capable of forming biofilm with consequent difficulties in the eradication of its infections [166]. Considering this, as well as the spreading of antibiotic resistance among bacteria, it appears clear that the investigation of novel possible strategies for the treatment of *S. aureus* infections is essential. BS produced by vaginal *L. gasseri* BC9 were purified and tested alone or in formulation with liposomes for their potential application as antibiofilm agent on *S. aureus* MRSA and MSSA strains. BS-derived from *L. gasseri* BC9 resulted as able to inhibit the biofilm development and eradicate pre-formed biofilm of all the *S. aureus* strains tested. The activities were not BS dose-dependent, as demonstrated by overall higher inhibition at the middle concentration assessed (2.5 mg/mL). This observation could be explained by structural modification of BS conformation at concentrations significantly greater than the critical micellar concentration (2 mg/mL), with consequent reduction in the antibiofilm activity. Inhibition values of biofilm development are comparable to those previously reported using BS of *L. gassseri* P65 on *E. coli* and *S. saprophyticus* biofilms [163], suggesting a potential spread antimicrobial activity of *L. gasseri* BS. In addition, the antibiofilm properties of *L. gasseri* BC9 biosurfactants were significantly enhanced by their loading into liposomes. Liposomes, when are formulated in appropriate size and concentration, are able to penetrate biofilm matrix and/or release their cargo directly on biofilm and cell surface, with consequent increase of loaded molecule effect [177]. Liposomes formulations were prepared using ethanol injection method and their characterization points out excellent stability over a period of 28 days and negative zeta-potential. This latter point is in accordance with other reports which indicated negative values in zeta-potential for vesicles derived from L-α-phosphatidylcholine [178]. Considering the potential application of *L. gasseri*-derived BS and BS-LP for prevention and treatment of *S. aureus* infections, it was evaluated their effect on eukaryotic cells. Results obtained proved that BS use in this study did not alter the cell viability of both human and murine fibroblasts. The absence of cytotoxicity on eukaryotic cells is a general characteristic of BS which makes them, together with their antibacterial properties, good candidates for medical application [160]. The antibiofilm mechanisms exerted by BS and BS-LP, which are not associated to bacterial cell death, are under investigation and should be related to anti-adhesive properties of BS [46]. Conversely, BS derived from other species were reported to possess antibacterial activity on planktonic cells[48]. In summary, this study reports that *L. gasseri* BC9 BS included into liposomes are safety compounds with excellent antibiofilm activity against biofilms of clinically relevant *S. aureus* MRSA and MSSA, highlighting BS-LP potential applications in the treatment of *S. aureus* infections.

4. Vaginal lactobacilli and pathogen-derived extracellular vesicles: effect on bacterial growth and anti-HIV-1 activity

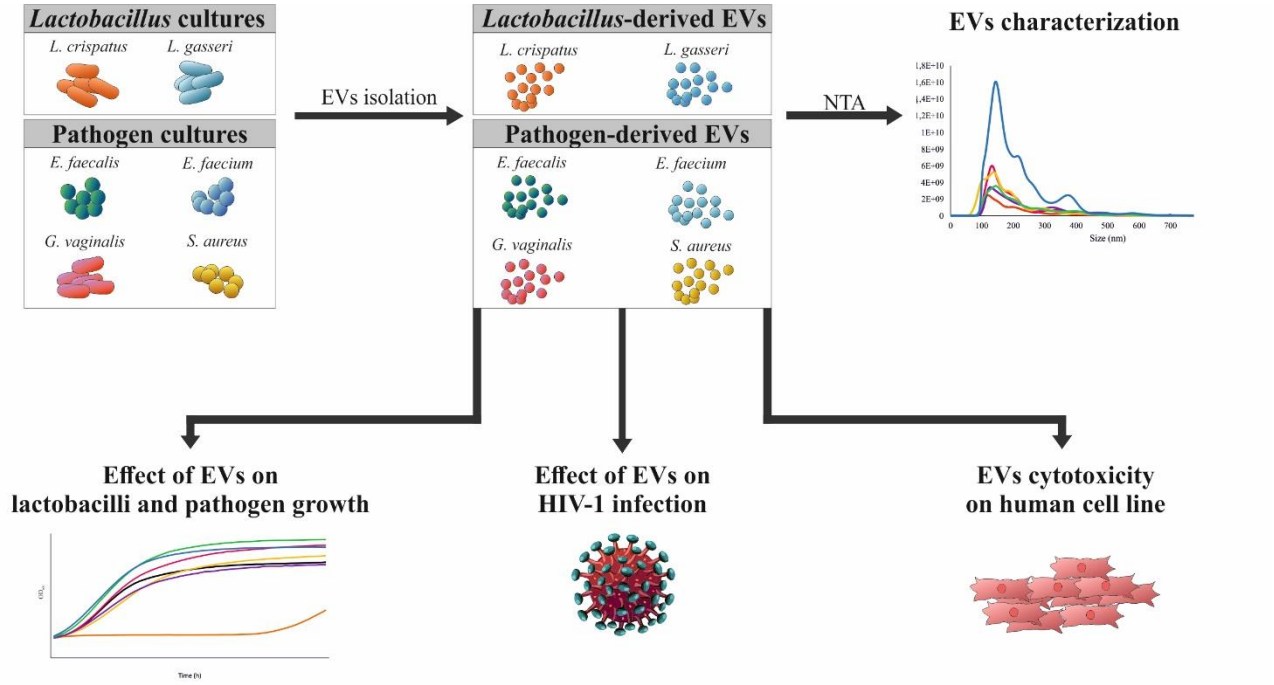

Fig. 14. Graphical abstract showing the approach used in the study about *Lactobacillus* and pathogens derived extracellular vesicles effect on bacterial growth and HIV infection.

5.1 Introduction

Numerous studies reported that Gram-positive and Gram-negative bacteria release extracellular vesicles (EVs) in their environment ([57,180,181]). EVs are considered important mediators for cell-cell communications. EVs are nanosized bilayer spheres of proteolipid nature carrying lipids, proteins, nucleic acids, metabolites, and nutrients [182]. Bacterial EV-complexity reflects their multifunctionality, indeed it was demonstrated that EVs can shuttle numerous bioactive molecules that are actively involved in interactions among bacteria such as nutrient sensing and delivery, stress resistance, horizontal gene transfer, competition with other microorganisms. Bacterial EVs are also involved in bacterial-host interactions, i.e. immune-modulation, virulence, etc.[183].

Antibiotic resistance is a fundamental trait for microbial communities which populate the human body and bacterial EVs mediate this process through different mechanisms, such as horizontal gene transfer (HGT), entrapment of extra- and intra-cellular antimicrobials or secretion of specific enzymes in the environment. This latter mechanism, that was consistently found in different bacteria belonging to both Gram-positive and Gram-negative group, allows the survival of the producer strain and the

protection of other bacteria not equipped with antibiotic resistance enzyme. Indeed, *S. aureus* EVs carrying β-Lactamase were found to confer ampicillin resistance to *S. enterica*, *E. coli* and *S. epidermis* [184]. Similarly, *Haemophilus influenzae* outer membrane vesicles equipped with β-Lactamase protected group A streptococci by amoxicillin [185]. Bacteroides, which is the predominant genus in the microbial community gut, protects pathogens and commensal bacteria against β-Lactam through the secretion of EVS-carrying cephalosporinases [186]. In addition to these mechanisms, bacterial EVs can provide antibiotic resistance to microbial communities via HGT by transferring antibiotic resistance genes [187]. In both these latter cases, inclusion of antibiotic-degrading enzymes or DNA inside EVs guarantees the protection of the cargo by possible inactivation or degradation [188–190]. Furthermore, bacterial EVs can capture antibiotic molecules in the extracellular compartment with a consequent reduction in their concentration and a protection of the microbial community. This was demonstrated in a hyper-vesiculating mutant strain of *E.coli* which better survived than wild type strain to polymyxin B and colistin exposure [191]. An analogous mechanism of action allowed bacteria to prevent bacteriophage adsorption using EVs as phage decoys. In *E. coli* and *V. cholerae* the direct interaction between phage and EVs was observed through electron microscopy resulting in a significant reduction of phage killing activity [191,192]. Besides the cooperative mechanisms described above, EVs can be also used for competition between bacteria. Indeed, during EVs biogenesis, intracellular antibiotic molecules can be encapsulated in EVs allowing at the same time either a cell detoxification effect or a bacteriolytic activity on other bacteria [193,194]. *P. aeruginosa* produced EVs packaged antimicrobial quinolones that could be used to efficiently compete with other bacteria. In particular, the quorum sensing signalling molecule PQS (*Pseudomonas* quinolone signal) positively regulated its own packaging and the inclusion of quinolones inside EVs, demonstrating a correlation between quorum sensing communication, vesicles biogenesis and competitive mechanisms [195]. Furthermore, *Pseudomonas aeruginosa* (as well as other Gram-negative bacteria) could compete with other microorganisms by secreting EVs carrying autolysin enzymes that can hydrolase the peptidoglycan of other bacteria [193,196].

Bacterial EVs have also been demonstrated to interact with host cells by three mechanisms: (i) EVs release enzymes which directly interact with host-cell; (ii) EVs bind directly the host-receptors which activate intracellular signal cascades; (iii) EVs fuse with the host cell and release their content into the cell cytoplasm [197–199]. All these mechanisms can lead to both positive and negative effects on the host cells. Indeed, it was widely demonstrated that EVs derived from different human pathogens deliver virulence factors or possess a cytotoxic effect. On the other hand, EVs-derived from mutualistic lactobacilli could improve host immune-responses against pathogens or positively stimulate immune and nervous systems [200–202]. Furthermore, EVs derived from some vaginal

Lactobacillus strains (*L. crispatus* BC3 and *L. gasseri* BC12) inhibited HIV-1 infections in both human cell lines and *ex-vivo* tissues, demonstrating that bacterial EVs can protect host from viral infections [59]. This effect was demonstrated to correlate with the binding of *Lactobacillus*-derived EVs to HIV-1 Env protein, resulting in a reduction of HIV-1 entry and binding to the target cells [59].

In this study, we isolated EVs from two vaginal lactobacilli, *L. crispatus* BC5 and *L. gasseri* BC12 as well as from four opportunistic human pathogens: *S. aureus*, *G. vaginalis*, *E. faecium* and *E. faecalis*. The possible competition mechanisms between *Lactobacillus* strains and pathogens via EVs secretion were investigated by growing either lactobacilli or pathogens in the presence of isolated EVs. Next, to complete the understanding of the interactions mediated by EVs, we investigated the effect of EVs purified from the four pathogens on their growth.

The previously reported antiviral effect of *L. gasseri* BC12 derived-EVs on HIV-1 infections was deepen through the combination of different approaches. Starting from the results of HIV-1 replication inhibition and proteomic characterization of *L. gasseri* BC12 derived-EVs, we investigated whether the protein component associated to EVs derived from lactobacilli is involved in their anti-HIV-1 activities. Also, we examined the effect on HIV-1 replication of two (Enolase 2 and Elongation Factor TU) of the 15 proteins previously identified in the vesicles isolated from *L. gasseri* BC12.

Moreover, considering that lactobacilli regulate HIV-1 infections through the secretion of EVs, we also tested whether other bacterial species, in particular opportunistic pathogens, influence HIV-1infection through the release of vesicles. This latter point is particularly interesting considering the correlations reported between increase of pathogen species in the vaginal niche and onset of sexually transmitted infections (including HIV-1).

4.2 Methods

4.2.1 Bacterial and viral strains used in this chapter

Table 9. List of bacterial and viral strains used in this chapter.

Bacterial strain	**Reference**
Lactobacillus crispatus BC5	[51]
Lactobacillus gasseri BC12	[51]
Staphylococcus aureus ATCC29213™	American Type Culture Collection (ATCC)
Gardnerella vaginalis ATCC14018 ™	American Type Culture Collection (ATCC)
Enterococcus faecium ATCC19434 ™	American Type Culture Collection (ATCC)
Enterococcus faecalis ATCC19433 ™	American Type Culture Collection (ATCC)
Viral strain	American Type Culture Collection (ATCC)
$HIV_{LAI.04}$	Rush University Virology Quality Assurance Lab
HIV_{BAL}	Rush University Virology Quality Assurance Lab

4.2.2 Extracellular vesicles isolation and characterization

L. crispatus BC5, *L. gasseri* BC12, *G. vaginalis* ATCC14018, *E. faecium* ATCC19434, *E. faecalis* ATCC19433 and *S. aureus* ATCC12600 were inoculated 1:100 in 50 mL of MRS broth pre-filtered through 0.1-μm filters to decrease the particles that are present in the medium. All bacterial cultures were incubated overnight at 37 °C, in anaerobic (*L. crispatus, L. gasseri, G. vaginalis*) or aerobic (*E. facium, E. faecalis, S. aureus*) conditions. The optical density of the overnight cultures were measured using spectrophotometer (Biophotometer, Eppendorf) and the EVs were extracted by ultracentrifugation [203]. Briefly, 50 mL of bacterial culture was centrifuged for 15 minutes at 2800 g, 4 °C and the obtained supernatants were filtered through 0.22 μm filters to remove any remaining bacteria. Then, EVs were isolated by ultracentrifugation at $100{,}000 \times g$ for 70 min at 4 °C (Ultracentrifuge WX ultra 80, Thermo Fisher Scientific) and the pellet, comprising EVs, was resuspended in 150 μL of PBS.

Bacterial EVs were characterized in terms of size and concentration through nanoparticle tracking analysis (NTA). NTA utilizes the properties of both light scattering and Brownian motion to obtain the particle size distribution of samples in liquid suspension. Bacterial EVs extracted were diluted

1:100 in PBS and loaded in 1 mL syringe placed in a syringe pump controller. This latter allowed to flow samples with a constant pressure inside NanoSight NS300 (Malvern instruments Ltd) which measured particles of the liquid suspension in a setting interval of time. Samples were analysed 60 second for 3 times with camera level = 13, detect threshold = 6 and syringe pump speed = 15. NTA results were analysed using NTA software, version 3.1.54 (Malvern instruments Ltd).

4.2.3 Determination of the bacterial growth

L. crispatus BC5, *L. gasseri* BC12, *G. vaginalis* ATCC14018, *E. faecium* ATCC19434, *E. faecalis* ATCC19433 and *S. aureus* ATCC12600 were inoculated 1:100 in 50 mL of MRS broth and incubated overnight at 37 °C, in anaerobic (*L. crispatus, L. gasseri, G. vaginalis*) or aerobic (*E. facium, E. faecalis, S. aureus*) atmosphere. The overnight cultures were diluted in MRS up to an OD_{600} equal to 0.15 and 100 µL were inoculated in a 96-well culture plate with 50 µL of controls or bacterial EVs. As controls PBS and Gentamycin (5 µg/mL) were used. Plates were incubated at 37 °C with orbital agitation using the hybrid multi-mode reader Synergy H1 (Biotek) which automatically recorded OD_{600} every 30 minutes for 24 hours. Data were used to generate bacterial growth curve and to calculate growth rate and generation time during exponential phase using the following equation:

$$\text{Growth rate (Gr)} = \frac{\ln(\text{OD600t2} - \text{OD600t1})}{(\text{t2} - \text{t1})}$$

$$\textbf{Generation time (G)} = \frac{\ln(\text{t2} - \text{t1})}{\text{Gr}}$$

4.2.4 Cell viability of human cell line treated with bacterial EVs and supernatants

Cell viability was evaluated on MT4 cell line using the Cellometer Auto 2000 Cell Viability Counter (Nexcelom Bioscience). Cell cultures were transferred to 24-well plates at the final concentration of $3x10^5$ cells per well and treated with 10^{10} EVs, 0.5 % of bacterial supernatants or controls (PBS and MRS). After day 3 of cell cultures, 20 µL of cell suspensions were mixed with 20 µL of ViaStain™ Acridine Orange and Propidium Iodide (AOPI) staining solution (Nexcelom Bioscience). AOPI staining is use for live/dead mammalian nucleated cells. Total number of live and dead cell were measured with Cellometer Auto 2000 Cell Viability Counter (Nexcelom Bioscience) to calculate the cell viability.

4.2.5 Anti-HIV-1 assay on human cell line

Anti-HIV-1 properties of EVs, supernatant and recombinant proteins (ENO2 and EFTU) were evaluated on MT4 ($CXCR4^+$) cell line infected with $HIV\text{-}1_{LAI.04}$ as previously described [59,204].

Recombinant proteins expression in *E. coli* and purification were performed by R&D System (USA). For anti-HIV-1 assay, 50 µL of HIV-1 viral stock (350 ng $p24_{gag}$ per mL) were preincubated with $1x10^{10}$ EVs, bacterial supernatants, recombinant proteins or PBS (negative control) for 1h at 37 °C. Preincubated mixtures were then used to infect 3 x 10^5 MT4 cells for 1h, at 37 °C under constant agitation at 400 rpm. After the infections, cells were washed with 10 mL of PBS, centrifuged for 5 minutes at 400 g and resuspended in 3 mL of RPMI medium supplemented with $1x10^{10}$ EVs, bacterial supernatants, recombinant proteins or PBS. Cells were transferred to a 24-well plate (1 mL, $1x10^5$ cells per well) and incubated for 3 days at 37 °C. After the incubation, samples were store at -80 °C. HIV-1 replication was measured as described below.

4.2.6 Measurement of HIV-1 replication by p24 Luminex capture assay

HIV-1 replication were evaluated by measuring the HIV-1 capsid protein, $p24_{gag}$, on cell culture medium (Anti-HIV-1 assay on human cell line) or solution (HIV-1 binding assay and HIV-1 capture assay) as described previously [204]. Briefly, 15 µL of each sample were initially transferred to a 96-well plate and lysed with 135 uL of Luminex lysis buffer (PBS containing 1% Triton X-100, 0.02% Tween20, 0.02% BSA, 20 mM Tris-HCl pH 6) for 30 minutes at 37 °C. The lysed samples were mixed 1:1 (50 µL:50 µL) with a solution containing p24 antibodies coupled to magnetic beads at the concentration of $1x10^3$ beads/mL and incubated for 1h at room temperature, in agitation at 400 rpm. Magnetic beads were previously coupled with anti-p24 antibody following protocol previously described [204]. Then, each plate was washed twice with 200 µL of Luminex wash buffer (PBS containing 0.02% Tween20, 20 mM Tris-HCl pH 6) using ELx405 magnetic microplate washer (BioTek). 100 µL of the detection antibody RD1-anti-p24 (Beckman Coulter) were added to each well (final concentration 0.5 µg/mL), followed by 1h of incubation at room temperature, in agitation at 400 rpm. The plates were washed twice, as described above. To measure the p24 concentration, 100 µL of Luminex buffer was added (PBS containing 0.02% Tween20, 0.02% BSA, 20 mM Tris-HCl pH 6). The latter was performed on a Luminex 200 (BioRad) using Bioplex manager software version 6.0.

4.2.7 HIV-1 binding assay

Nunc-maxisorp flat-bottom 96 well immune plates (Biostad Analytical) were coated with 100 µL of ELISA coating buffer (BioRad) containing 1 µg of recombinant proteins (rENO2 or rEFTU), rCD4 (positive control) or BSA (negative control) per well and incubated overnight at room temperature. The coating solution was removed, 200 µL of blocking buffer (PBS containing 1% BSA, 0.02% sodium azide) were added per well and the plate was incubated 1h at room temperature. Each well

was then washed with 200 µL of washing buffer 1 (PBS containing 0.02% sodium azide). Then, 50 µL of HIV-1_{Bal} (120 ng $p24_{gag}$ per mL) were added and incubated for 1h at room temperature. The assay was completed by 3 washes with the washing buffer 2 (PBS containing 0.05% Tween20) followed by resuspension with 200 µL of Luminex lysis buffer. The p24 measurement was conducted as described above (chapter 4.2.6).

4.2.8 HIV-1 capture assay

HIV-1 capture assay was performed to study the interactions of bacterial EVs with HIV-1 envelope protein, gp120, using protocol previously reported [59]. 50 µL of $HIV_{LAI.04}$ was treated with 50 µL of bacterial EVs at the concentration of $1x10^{10}$ particles per mL or with PBS (control) at 37 °C, for 1h under constant agitation at 400 rpm. HIV-1 virion capture was performed by adding 50 µL of magnetic iron oxide nanoparticles (MNPs) coupled with monoclonal antibody PG9 and incubating for 1h at 37 °C, under constant agitation at 400 rpm. The coupling of PG9 antibody, which preferentially recognises the trimeric form of gp120, was previously performed following manufacturer's instructions (Ocean NanoTech). To evaluate the amount of virus captured, the virions captured with PG9-magnetic beads were separated from free virions using magnetic columns inserted in a high field MACS magnet (Miltenyi Biotech). Columns were then washed 3 times with 600 µL of washing buffer (0.5% BSA, 1 mM EDTA), demagnetized for 5 minutes, and eluted with 600 µL of Luminex lysis buffer. The $p24_{gag}$ antigen concentration in the eluate was measured with p24 Luminex capture assay (chapter 4.2.6).

4.3 Results

4.3.1 *Lactobacillus* strains and pathogens produce extracellular vesicles

After isolation from pure overnight bacterial cultures by ultracentrifugation, EVs were characterized in terms of size and concentration using Nanoparticle Tracking Analysis (NTA). The analyses were performed using extracts obtained from at least a biological triplicate. As a result, EVs were isolated from two vaginal *Lactobacillus* strains (*L. crispatus* BC5, *L. gasseri* BC12) and four main opportunistic human pathogens (*S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*). All the tested strains were found to release extracellular vesicles with comparable size in terms of average diameter. Indeed, bacterial EVs mean diameter ranged from 182.65 ± 20.15 nm (EVs from *G. vaginalis*) to 239.7 ± 3.2 nm (EVs from *S. aureus*) (Fig. 15A). No statistically significant EV-size variations were observed between the different strains.

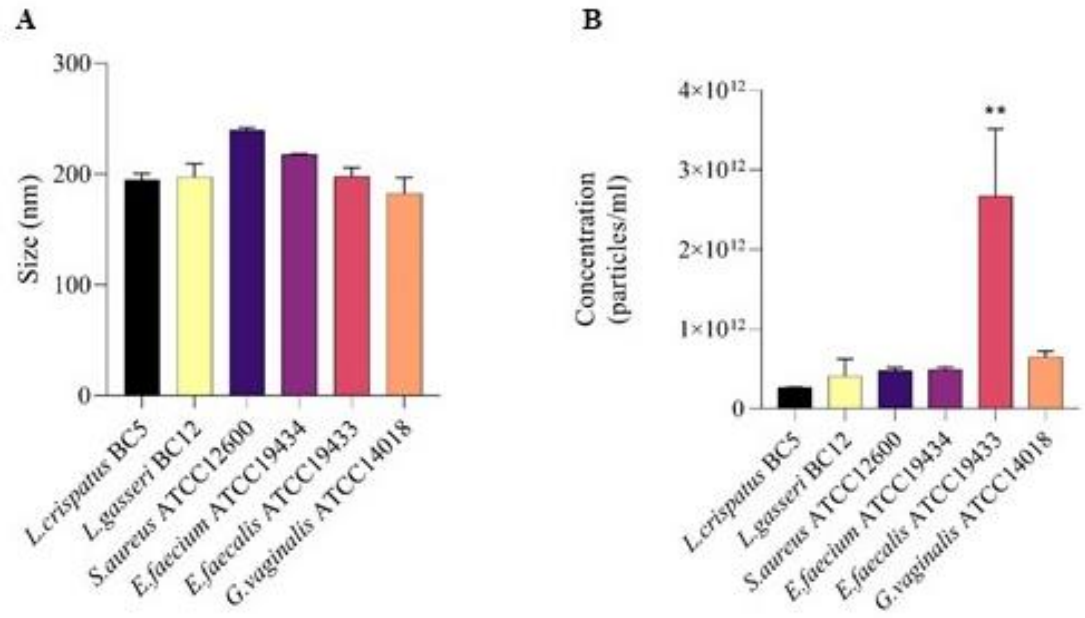

Fig. 15. Nanoparticle tracking analysis of bacterial EVs derived from *L. crispatus* BC5, *L.gasseri* BC12, *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*. **A)** EV-sizes expressed as mean ± standard deviation of particle diameter (nm). **B)** Mean ± standard deviations of the EVs concentration (particles/mL). Statistical analysis was performed with one-way ANOVA multiple comparison (** = $p < 0.01$).

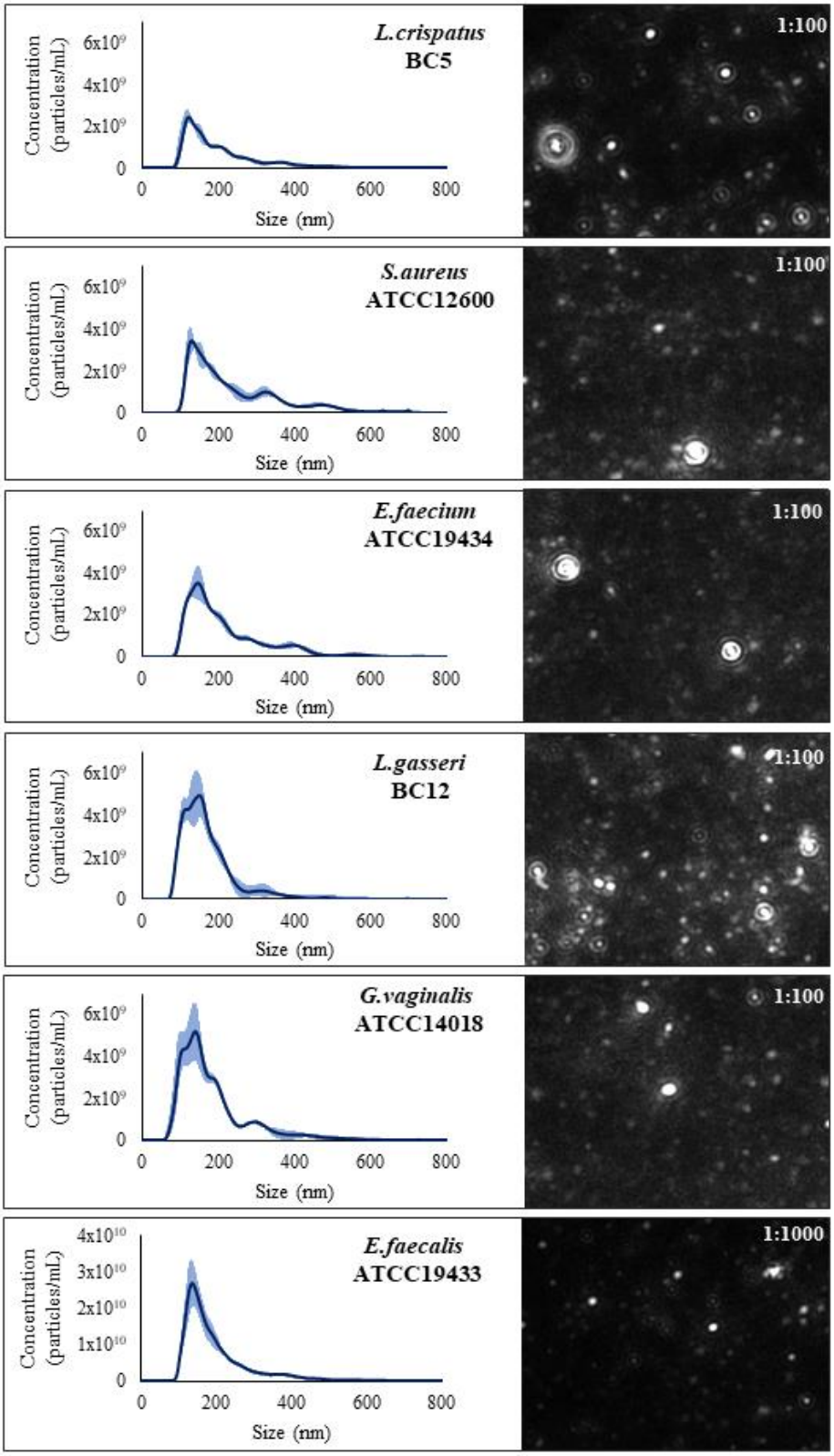

Fig. 16. Nanoparticle tracking analysis of EVs derived from *L. crispatus* BC5, *L.gasseri* BC12, *S. aureus*, *E. faecium*, *E. faecalis*, and *G. vaginalis*. Graphs on the left show the distribution of EV-concentrations (Y axis) in relation with EV-sizes (X axis). Dark blue lines represent means of the concentrations detected while light blue areas show the standard deviation. Panel on the right are screenshots of the video of the EVs preparation recorded by Nanosight.

In terms of quantity of vesicles released, *E. faecalis* produced a significant higher amount of EVs as compared to the other five strains under analysis, with a total amount of $2.6x10^{12}$ particles/mL. Conversely, among the other strains, no significant divergences were observed in terms of concentration of EVs which ranged between $2.6x10^{11}$ particles/mL (EVs from *L. crispatus*) and $6.5x10^{11}$ particles/mL (EVs from *G. vaginalis*) (Fig. 15B). The distribution of concentration in relation with size demonstrated that in each bacterial strain the majority of particles had diameter inferior to 200 nm (Fig. 16), in accordance with previous reports [59,205].

4.3.2 The effect of EVs derived from lactobacilli and pathogens on bacterial growth

The effect of extracellular vesicles on bacterial growth was assessed by the addition of EVs, extracted from overnight bacterial cultures, to bacterial cells. The OD_{600} was used to monitor the bacterial growth over time and was automatically recorded every 30 minutes for 24 hours. The bacterial EVs were added at the final concentration of $1x10^{10}$ particles/mL. As controls were used: PBS, EVs extracted from MRS and Gentamycin (5 μg/mL). The bacterial strains used in these experiments were mutualistic microorganisms (*L. crispatus* BC5 and *L. gasseri* BC12) and opportunistic pathogens (*S. aureus, E. faecium, E. faecalis* and *G. vaginalis*). The data obtained were used to graph bacterial growth curve and to calculate the generation time. In particular, the latter was calculated over an interval time that was the same for all the treatments and corresponded to the exponential phase of control culture treated with PBS.

As a result, growth curve and generation time clearly showed that *L. crispatus* and *L. gasseri*-derived EVs did not alter the growth of pathogen strains, suggesting that EVs of lactobacilli do not directly slow down or enhance the proliferation of the tested pathogens (Fig. 17C, 17D, 17E and 17F). Similarly, pathogen EVs tested did not influence lactobacilli growth (Fig. 17A and 17B). These results suggested the absence of direct competition among the strains under analysis mediated by the release of EVs.

On the other hand, the investigation of EV mediated interactions on growth between pathogens revealed that *E. faecalis* growth was decrease significantly when the strain was co-cultured with $1x10^{10}$ *E. faeciums*-derived EVs. Indeed, an increase in the generation time of 4.25 hours was observed in comparison to EVs-free control culture (Fig. 17E and 18). In contrast, the addition of $1x10^{10}$ EVs/mL secreted by *E. faecalis* had a positive effect on the growth of *E. faecalis* itself resulting in a decrease of the generation time, which passed from 1.94 to 1.27 hours (Fig. 17E and 18). To test if these two-opposite effects on *E. faecalis* growth were dose-dependent, we added $1x10^{10}$, $1x10^{9}$ and $1x10^{8}$ EVs/mL isolated either from *E. faecalis* or *E. faecium* to the *E. faecalis* cultures. As shown in Fig. 18, the *E. faecalis* growth reduction exerted by *E. faecium*-derived EVs

was dose dependent. The only concentration of $1x10^8$ EVs/mL (the lowest EVs concentration tested) did not negatively influence the generation time of *E. faecalis*.

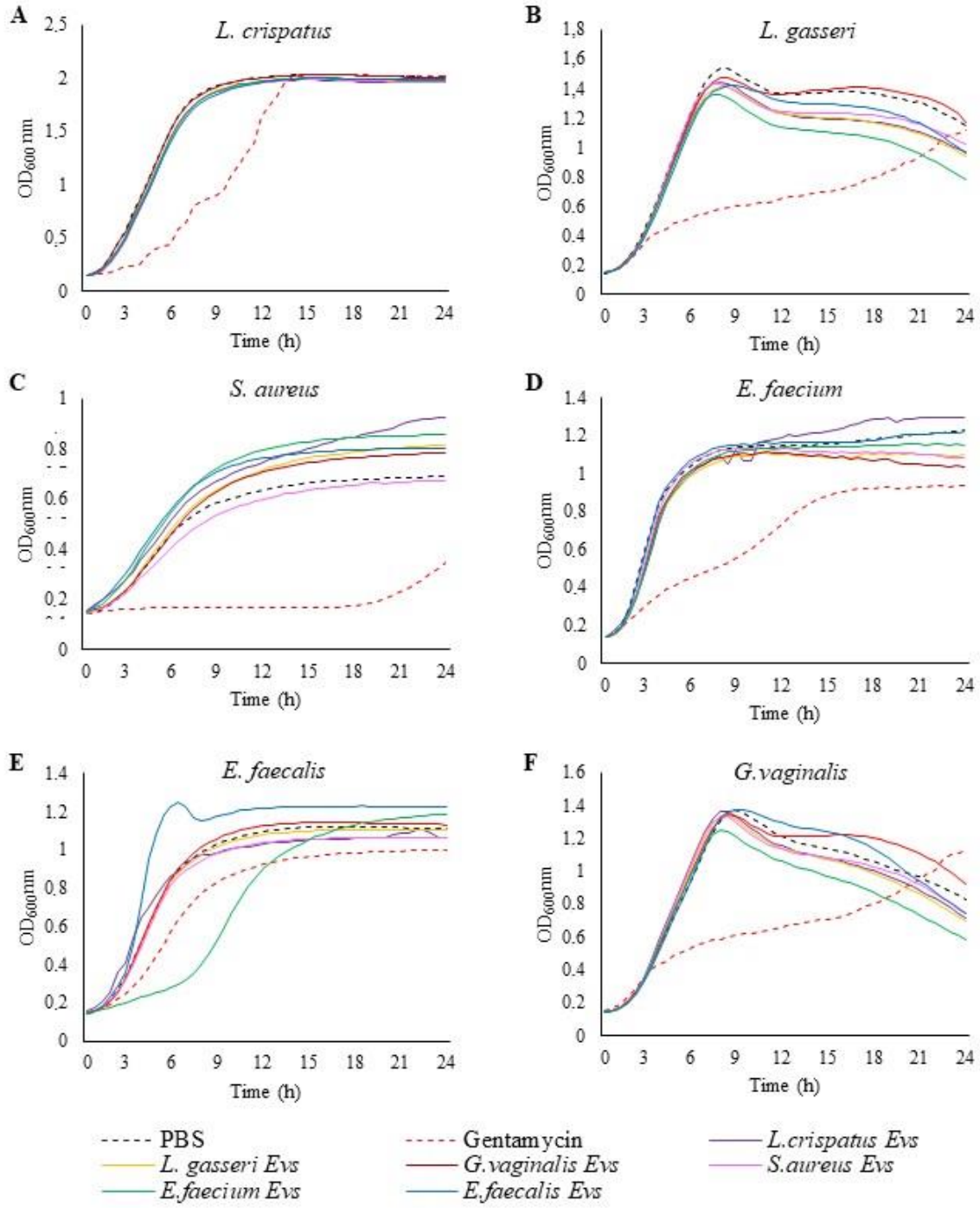

Fig. 17. Bacterial growth curves in the presence of bacterial EVs. Optical density (OD) at 600 nm was recorded every 30 minutes for 24 hours. **A)** *L. crispatus* BC5, **B)** *L. gasseri* BC12, **C)** *S. aureus*, **D)** *E. faecium*, **E)** *E. faecalis*, **F)** *G. vaginalis*. Each bacterial strain was grown in presence of $1x10^{10}$ EVs/mL derived from *L. crispatus* (purple line), *L. gasseri* (yellow line), *S. aureus* (pink line), *E. faecium* (green line), *E. faecalis* (blue line) and *G. vaginalis* (dark red line). PBS (dashed black line) and gentamycin (dashed red line) were used as controls.

The positive effect of *E. faecalis*-derived EVs on *E. faecalis* growth was also dose dependent and was not detectable at the lowest concentration of EVs tested ($1x10^8$ EVs/mL). In order to investigate more deeply on the opposite effects detected of the EVs derived from the different pathogens, we tested if the addition, at the same time, of both *E. faecalis*-derived EVs and EVs derived from *E. faecium* could suppress the inhibitory activity exerted by EVs-derived from *E. faecium* on *E. faecalis*. The results obtained showed that in presence of the EVs derived from both the strains, *E. faecalis* growth was still retarded and perfectly comparable with the growth curve obtained in presence of *E. faecium*-derived EVs alone (Fig. 18). These results suggested that *E. faecium* efficiently compete with *E. faecalis* by releasing of *E. faecalis* growth inhibiting EVs and this latter cannot counteract this effect with its own EVs.

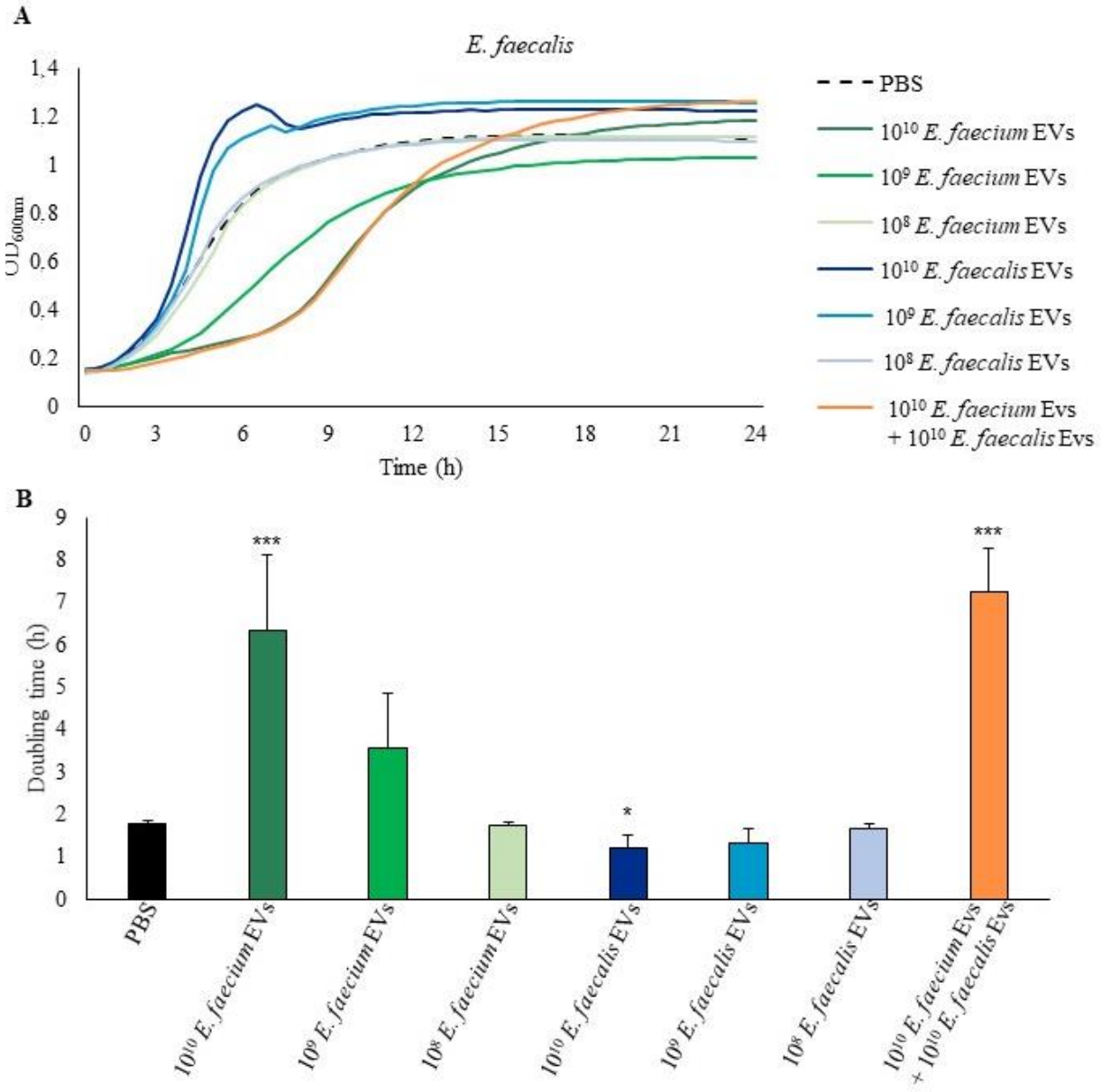

Fig. 18. Growth curves of *E. faecalis* in the presence of different concentrations of EVs derived from *E. faecium* and *E. faecalis*. **A)** Optical density at 600 nm measured during the *E. faecalis*

growth in the presence of PBS (as a control) (dashed black line); $1x10^{10}$ (dark green), $1x10^{9}$ (green), $1x10^{8}$ (light green) *E.faecium* derived EVs; $1x10^{10}$ (dark blue), $1x10^{9}$ (blue), $1x10^{8}$ (light blue) *E. faecalis* derived EVs or both EVs derived from *E. faecalis* and *E. faecium* at the concentration of $1x10^{10}$ (orange). **B)** Doubling time expressed in hours, calculated during the time interval 3-5 hours corresponding to the exponential growth phase of the control culture. (Statistical analysis was performed with one-way ANOVA multiple comparison (* = $p < 0.05$, *** = $p < 0.001$).

4.3.3 *Lactobacillus gasseri*-derived EVs protein component is responsible of their anti-HIV-1 effect

The previously reported anti-HIV properties of EVs derived from *L. gasseri* BC12, opened new questions regarding the mechanisms of action of these EVs. The main antiviral effect exerted by *Lactobacillus*-derived EVs was associated to the reduction of the viral entry/attachment to the target cells and this can be related to their ability to interact with the HIV-1 glycoprotein gp120 [59]. Considering these promising results, we decided to investigate the role exerted by proteins present in *L. gasseri*-derived EVs during HIV-1 infections. For this purpose, EVs isolated from *L. gasseri* BC12 were treated with proteinase K, re-isolated through ultracentrifugation, and then added to MT4 cells infected with $HIV_{LAI.04}$.

Proteinase K treatment, which degrades all the proteins exposed on the external surface of bacterial EVs [206], leaded to the complete loss of antiviral properties exerted by *L. gasseri*-derived EVs (Fig. 19A). This observation suggested that the HIV-1 inhibition exerted by *L. gasseri*-derived EVs could be associated to the proteins exposed on their external surface. Additionally, we did not observe variations in the viability of MT4 cells grown for 3 days in presence of EVs either treated or untreated with proteinase k (both at the final concentration of $1x10^{10}$ EVs/mL). This result indicated that the difference in the antiviral properties observed between treated and untreated vesicles could not be associated with cytotoxic effects on human lymphoid cells (Fig. 19B).

Nevertheless, the characterization through NTA analysis revealed that proteinase K treatment led to an increase of 257.7 nm in *L. gasseri* EVs size, which switched from 178.4 ± 16.8 nm of the untreated EVs to 436.1 ± 3.1 nm of the treated ones (Fig. 19C).

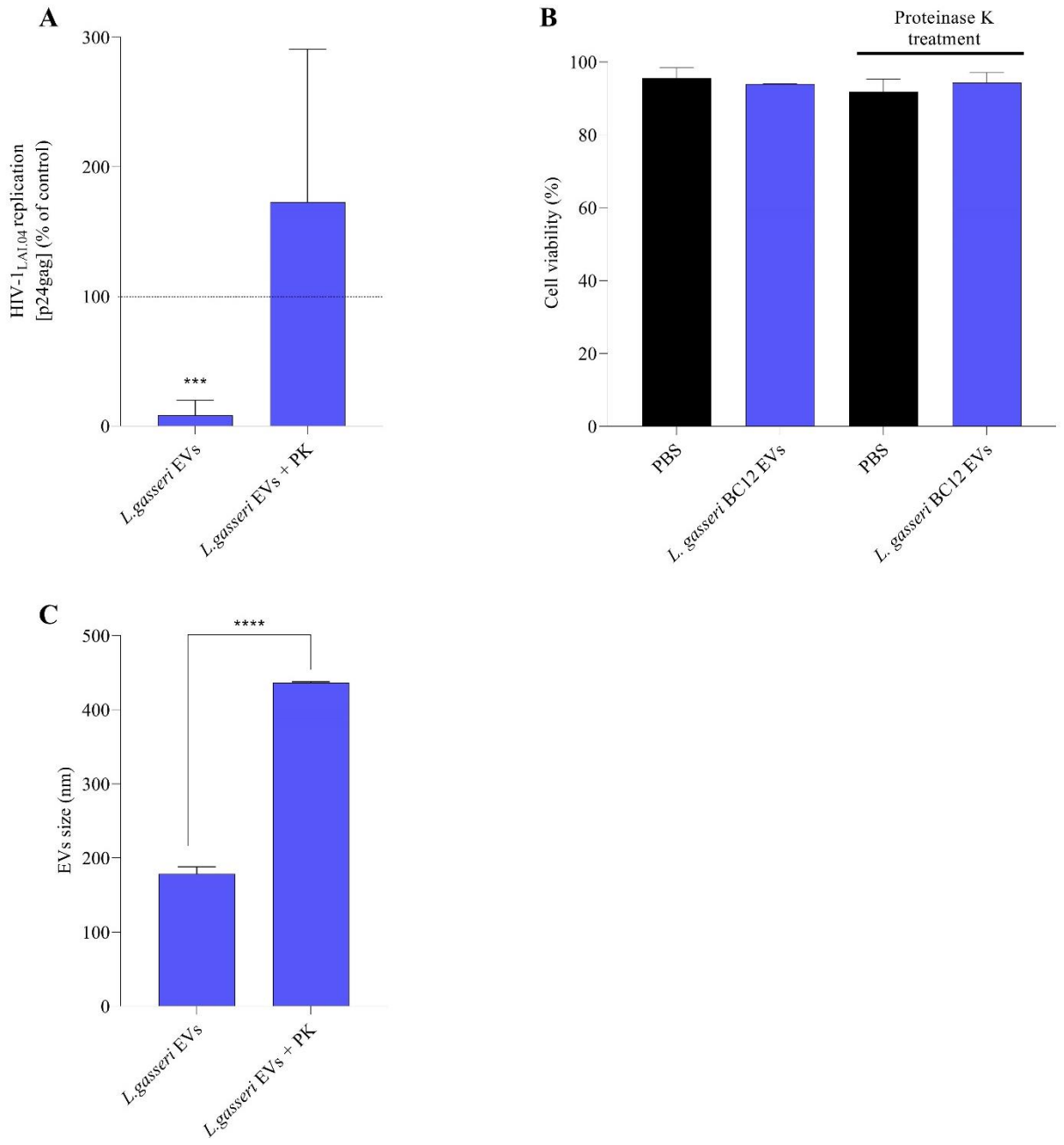

Fig. 19. *L. gasseri*-derived EV protein component is involved in its anti-HIV-1 activity. **A)** Antiviral assay of *L. gasseri* BC12-derived EVs on MT-4 cells infected with $HIV_{LAI.04}$. Replication of the virus was quantified through p24 Luminex capture assay performed on cell culture medium. Data are represented as percentage of $p24_{gag}$ concentration compared to the control (PBS). **B)** Cell viability of MT-4 cells after 3 days in the presence of *L. gasseri* BC12-derived EVs either treated or untreated with proteinase K ($1x10^{10}$ EVs/mL). PBS and PBS treated with proteinase K were used as controls. **C)** Conformational change induced by proteinase K treatment (PK) on EVs derived from *L.gasseri* BC12. Analyses were performed using NTA and results showing EVs size are expressed as mean ± standard deviation of particle diameter (nm). Statistical analysis was performed with one-way ANOVA multiple comparison (*** = $p < 0.001$, **** = $p < 0.0001$).

4.3.4 Elongation factor TU of *Lactobacillus gasseri* EVs inhibit HIV-1 infections

The proteomic analysis of EVs derived from *L. gasseri* BC12 previously reported by Ñahui Palomino et al, allowed the identification of 8 proteins (ENO2, CH60, EFTU, ATPG, PRSA1, ATPD, KPYK, TPIS) that might be responsible of these anti-HIV-1 effect [59]. Thus, it is possible to assume that these proteins, alone or in combination, could be involved in the HIV-1 inhibition. In order to assess this hypothesis, we initially investigated the effect of two of them, Enolase 2 (ENO2) and Elongation Factor TU (EFTU). The choice of these proteins was driven by the multifunctionality of these proteins, indeed both ENO2 and EFTU were described as moonlighting proteins, being involved in different bacterial physiological processes [207,208]. Thus, ENO2 and EFTU were expressed in *E. coli* BL21, purified and then added at different concentrations to MT-4 cells infected with $HIV_{LAI.04}$. The addition of recombinant EFTU (rEFTU) inhibited HIV-$1_{LAI.04}$ in a dose-dependent manner. Indeed, the addition of 5 and 10 µg/mL of rEFTU significantly reduced HIV-1 infection by 78.3 and 91.0 %, respectively (Fig. 20A).

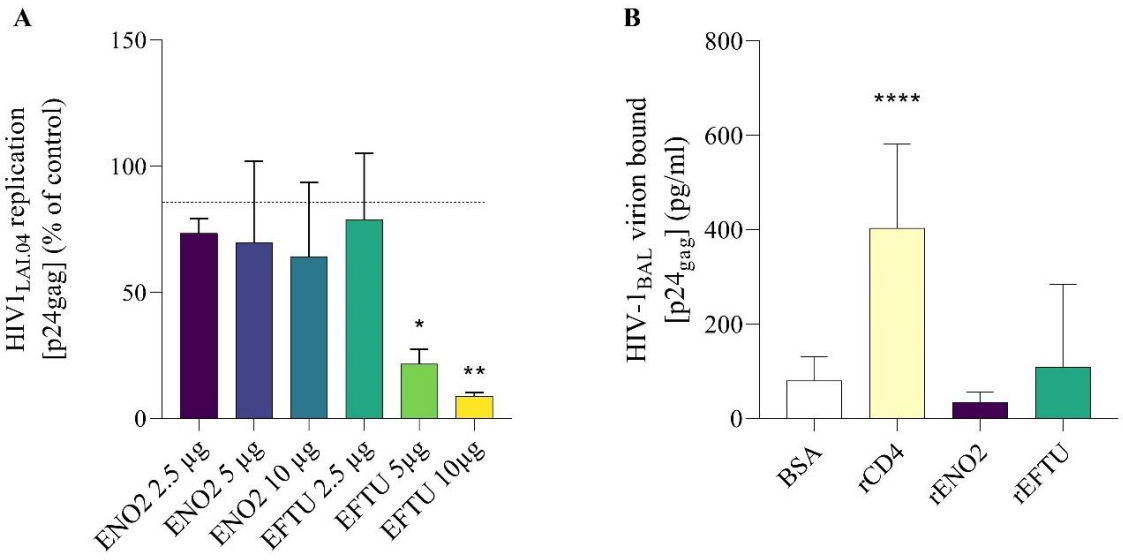

Fig. 20. Antiviral effect and interactions with HIV-1 envelope of the recombinant proteins Enolase 2 (ENO2) and Elongation Factor TU (EFTU). **A)** Antiviral assay performed on MT-4 cells infected with HIV-$1_{LAI.04}$ in the presence of different concentrations (2.5, 5 and 10 µg/mL) of rENO2 and rEFTU. Replication of the virus was quantified through p24 Luminex capture assay performed on cell culture medium. Data are represented as the percentage of $p24_{gag}$ concentration compared to the control (PBS). **B)** HIV binding assay performed by the addition of HIV_{BAL} aliquots to 96 multiwell coated with 1 µg of rENO2 and rEFTU. BSA and rCD4 were also coated on the plate (1 µg) and used as negative and positive control, respectively. The amount of HIV_{BAL} virions bounded to the coating was evaluated through measurement of $p24_{gag}$ concentration. Statistical analysis was performed on both experiments with one-way ANOVA multiple comparison (* = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$).

Conversely, rENO2 did not produce any significant reduction of HIV-1 infection on MT4 cells, even at the highest concentration tested (10 µg/mL). It was tested the capacity of these two proteins to bind HIV-virion through HIV binding assay. In this case, both proteins displayed virion binding comparable to the negative control (BSA), suggesting that they were not capable to efficiently attach HIV-1_{BAL} virions (Fig. 20B). Nevertheless, this did not exclude possible interactions of rEFTU with essential viral Env proteins. In this regard, HIV-1 capture-assay using PG9 antibody in presence of both rEFTU and rENO2 are currently in progress.

4.3.5 Conditioned medium and EVs derived from pathogens inhibit HIV-1 infections

Considering the promising results obtained with EVs derived from *L. gasseri* we decided to investigate whether EVs secreted by human pathogens can positively or negatively influence HIV-1 infections *in vitro*. We started assessing the effect of conditioned mediums (CMs) derived from overnight cultures of four main opportunistic human pathogens (*S. aureus, E. faecium, E. faecalis* and *G. vaginalis*) during HIV-1 infection on human lymphoid cell line. Bacterial CMs were added, at the concentration of 0.5%, to MT4 cells infected with HIV-1. The unexpected results obtained, showed that all CMs derived from *S. aureus, E. faecium, E. faecalis,* and *G. vaginalis* significantly reduced HIV-$1_{LAI.04}$ replication (Fig. 21A). The highest inhibition was observed with *G. vaginalis* CM (91.7% of inhibition) while *E. faecalis* CM exerted the lowest inhibition (71.14% of inhibition) (Fig. 21A). The unconditioned medium (UCM), MRS medium, did not alter HIV-1 replication levels even at highest concentration tested, as also previously reported on human *ex-vivo* tissues [87] (Fig. 21A). This result suggested that the antiviral effects observed with *S. aureus, E. faecium, E. faecalis* and *G. vaginalis* CMs were related to antiviral factors that were extracellularly secreted by these bacterial cells. These antiviral factors can be associated to extracellular vesicles (EVs) secreted by these bacteria. Thus, pathogen-derived EVs present in the conditioned mediums were then isolated, as previously described, and tested for their effect on HIV-1 infections. Our results showed that under the presence of pathogen-derived EVs, HIV-$1_{LAI.04}$ replication was significantly reduced as compared to the control. In particular, HIV-$1_{LAI.04}$ replication was reduced by 93.9, 77.5, 87.8, and 87.4 % when cells were treated with $1x10^{10}$ EVs derived from *S. aureus, E. faecium, E. faecalis,* and *G. vaginalis,* respectively (Fig. 21B). On the contrary, as expected, particles isolated from MRS medium did not reduced HIV-1 replication, demonstrating that the antiviral effect was also, in part, related to EVs released by these bacteria.

To discriminate whether these anti-HIV-1 effects were merely due to cell cytotoxicity, cell viability was evaluated on MT4 cell incubated for 3 days in the presence of PBS (negative control), CMs (0.5%), UCM, as well as with pathogen-derived EVs ($1x10^{10}$ particles/mL) or MRS-derived EVs

($1x10^{10}$ particles/mL). All bacterial conditioned mediums showed no cytotoxic effect on human lymphoid MT-4 cells at the concentration of 0.5%, on the basis of viability values of MT-4 cells in the presence and absence of bacterial CM (Fig. 21C).

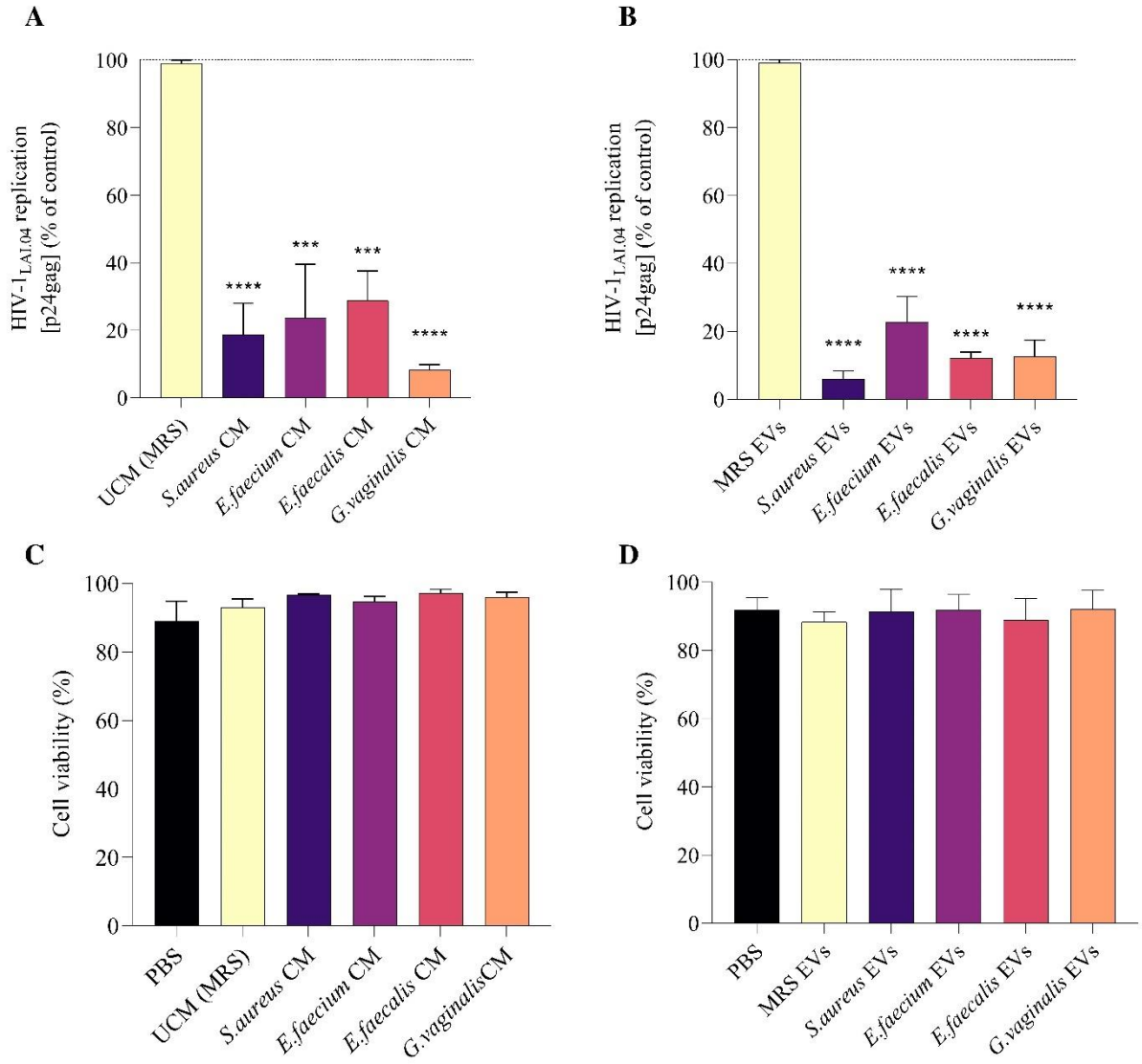

Fig. 21. Antiviral effect of conditioned medium and EVs derived from opportunistic human pathogens. Antiviral assay of CMs **(A)** and EVs **(B)** derived from *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*. MT-4 cells were infected with $HIV_{LAI.04}$ in the presence of 0.5% CMs or $1x10^{10}$ EVs/mL. Replication of the virus was quantified through p24 Luminex capture assay performed on cell culture medium. Data are represented as percentage of p24gag concentration as compared to the control (PBS). Cell viability of MT-4 cells treated for 3 days with pathogen-derived CMs at the concentration of 0.5% **(C)** or with $1x10^{10}$ EVs/mL **(D)**. PBS, the unconditioned medium (UCM), which is the bacterial medium MRS, and EVs derived from MRS were used as controls. Statistical analysis was performed with one-way ANOVA multiple comparison (*** = $p < 0.001$, **** = $p < 0.0001$).

Similarly, cell viability of MT-4 cells was not affected by EVs isolated from the four pathogens, as demonstrated by cell viability values which ranged from 88.37 % (MRS EVs) to 92.13 % (*G. vaginalis* EVs) (Fig. 21D). This latter result was in line with data obtained from similar experiments performed with vaginal lactobacilli-derived EVs, suggesting that also EVs derived from the pathogenic bacteria tested are not toxic for human cell line [59].

It was also assessed the dependency of HIV-1 inhibition of bacterial EVs at different EV concentrations ($1x10^{10}$, $1x10^{9}$, $1x10^{8}$, $1x10^{7}$) (Fig. 22).

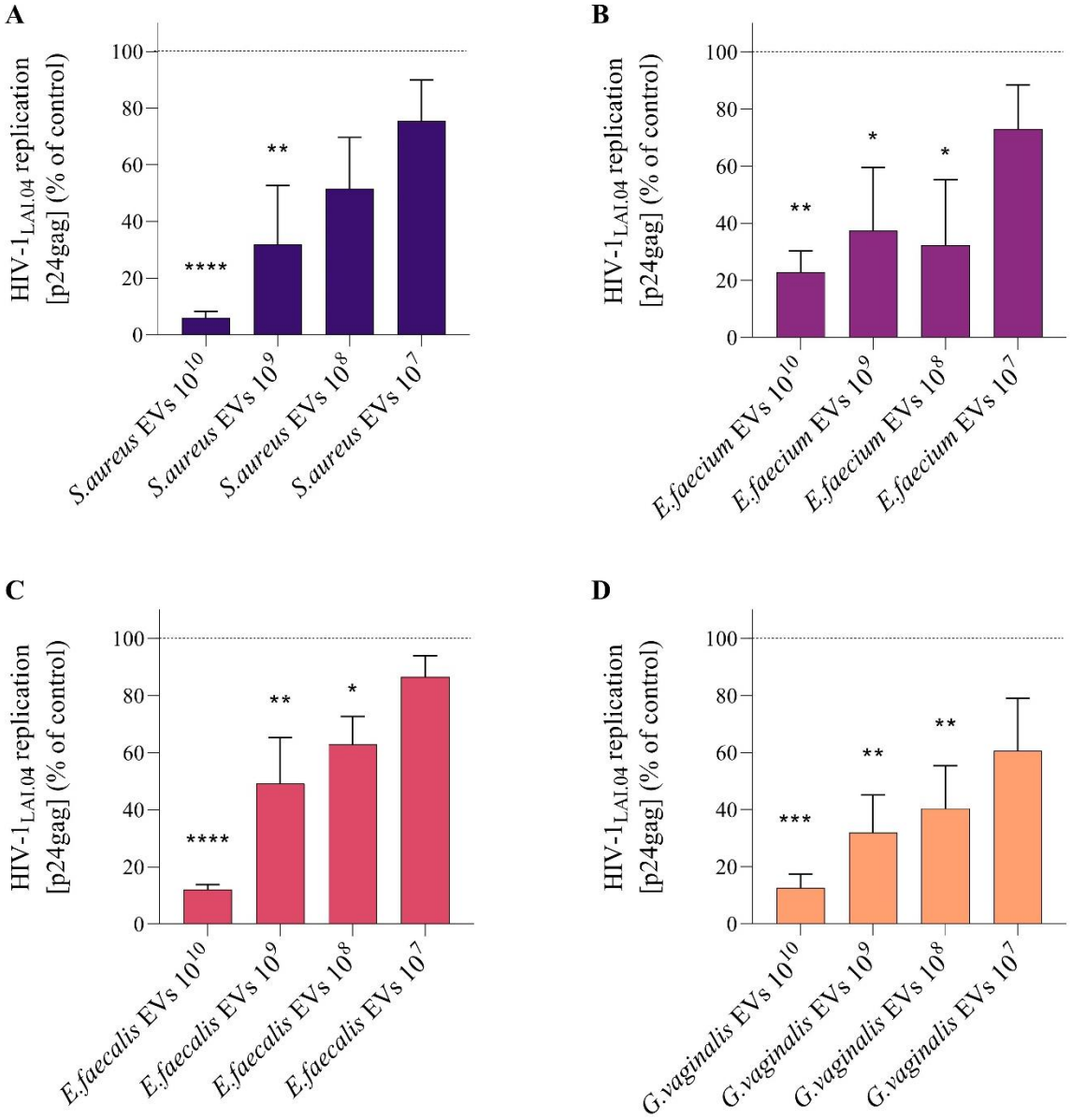

Fig. 22. Concentration dependency of the bacterial-derived EVs antiviral activity. MT-4 cells were infected with $HIV_{LAI.04}$ in presence or not of $1x10^{10}$, $1x10^{9}$, $1x10^{8}$, and $1x10^{7}$ EVs derived from **A)** *S. aureus*, **B)** *E. faecium*, **C)** *E. faecalis* and **D)** *G. vaginalis.* Replication of the virus was quantified through p24 Luminex capture assay performed on cell culture medium. Data are represented as percentage of $p24_{gag}$ concentration as compared to the control (PBS). Statistical analysis was performed with one-way ANOVA multiple comparison (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

In all the cases, the decrease in numbers of bacterial EVs lead to a reduction or loss in the anti-HIV-1 activity, demonstrating that pathogen-derived EVs antiviral properties are dose-dependent (Fig. 22). EVs isolated from *E. faecium*, *E. faecalis* and *G. vaginalis* significantly inhibited HIV-1 replication when they were added in the total amount of $1x10^{10}$, $1x10^{9}$, and $1x10^{8}$, while a thousand-fold dilution (10^{7} EVs) suppressed their antiviral effect. For *S. aureus*-derived EVs, HIV-1 inhibition resulted significant only at the concentration of $1x10^{10}$ and $1x10^{9}$ particles/mL.

4.3.6 Pathogen-derived EVs protein component is responsible of their anti-HIV-1 effect

Similarly to the experiments previously performed with *L. gasseri* BC12 and *L. crispatus* BC3 [59], the demonstration of anti-HIV properties of EVs derived from *S. aureus*, *E. faecium*, *E. faecalis,* and *G. vaginalis* opened new questions about the nature of the component of EVs that was responsible of this anti-HIV effect. In order to test the possible protein nature of the component, bacterial EVs were treated with proteinase K, re-isolated by ultracentrifugation, characterized with NTA, and then added to MT4 cells infected with $HIV_{LAI.04}$.

As shown in figure 23A, the anti-HIV-1 activities of all bacterial EVs were completely lost under proteinase K treatment. Indeed, MT-4 cells infected with $HIV_{LAI.04}$ in the presence of EVs treated with proteinase K displayed no significant difference in viral infection as compared to the control (PBS), while EVs free of proteinase K were all able to inhibit HIV-1 infection (Fig. 23A). Our results indicated that the protein component of the EVs is involved in the antiviral effect observed. Moreover, the lost of antiviral properties of EVs treated with proteinase K, was not due to cytotoxic effect for MT-4 cells, as established by the absence of decrease in the cell viability (Fig. 23B). Similar to EVs isolated from *L. gasseri*, the proteinase K treatments led to an overall significant increase of EVs size, with the exception of *G. vaginalis* derived EVs in which there were not significant differences between treated and untreated vesicles (Fig. 23C). The major difference was observed on *E. faecium* derived EVs, with an increase in the vesicle diameter of 173.2 nm after proteinase K treatment (from 216.8 ± 0.8 to 390.0 ± 9.0 nm), while the minimum significant difference was measured on *S. aureus* (+ 100.6 nm). Taken together, these results demonstrate that the digestion of proteins that are localized on the EVs surface changes the vesicles structure with the consequent increase in the EVs size, except for *G. vaginalis*-derived EVs

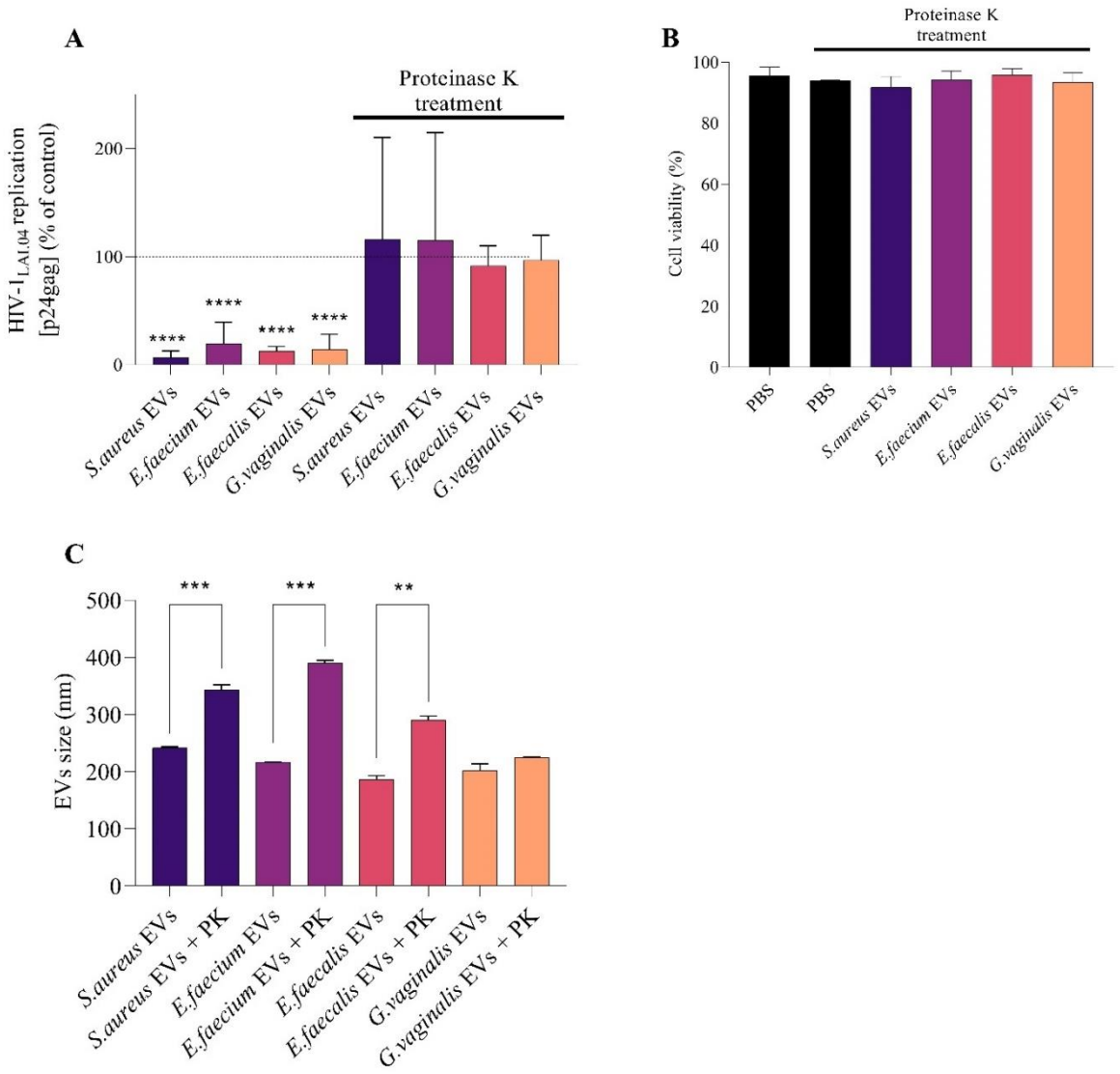

Fig. 23. Antiviral effect, cytotoxicity and characterization of bacterial-derived EVs treated or untreated with proteinase K (PK) on MT-4 cells infected with $HIV_{LAI.04}$. **A)** Antiviral assay of bacterial-derived EVs treated or untreated with PK on MT-4 cells infected with $HIV_{LAI.04}$. Replication of the virus was quantified through p24 Luminex capture assay performed on cell culture medium. Data are represented as percentage of $p24_{gag}$ concentration as compared to the control (PBS). **B)** Cell viability of MT-4 cells grown for 3 days with $1x10^{10}$ EVs of *S. aureus*, *E. faecium*, *E. faecalis,* and *G. vaginalis* treated with PK. PBS and PBS treated with proteinase K were used as control. Statistical analysis was performed with one-way ANOVA multiple comparison (**=p<0.01, ***=p<0.001, **** = p < 0.0001).

4.3.7 EVs released by bacterial pathogens prevent HIV-1 infection affecting viral Env

Considering that the digestion of proteins exposed on the external surface of pathogen-derived EVs led to the loss of their anti-HIV-1 activity, we tested whether these bacterial EVs, in somehow, alter the HIV-1 envelope protein gp120. This latter is an essential viral surface glycoprotein, responsible of the HIV-1 binding to the CD4 receptor, and involved in viral entry into target cells [209]. HIV-1

captured assay were performed, as previously described for *L. gasseri* BC12 [59], by incubating HIV-$1_{LAI.04}$ in presence of $1x10^{10}$ vesicles isolated from each of the four pathogen strains. Next, virions were captured using magnetic beads conjugated with PG9 antibody, which specifically recognise the HIV-1 envelope protein, gp120. Our results showed that the incubation of HIV-$1_{LAI.04}$ with *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* derived EVs significantly reduced the amount of HIV-1 virions captured using PG9 antibody (Fig. 24), demonstrating that all the four pathogens strains released EVs capable to interact with gp120 protein of HIV-1. The exact mechanism of these interactions are currently studied.

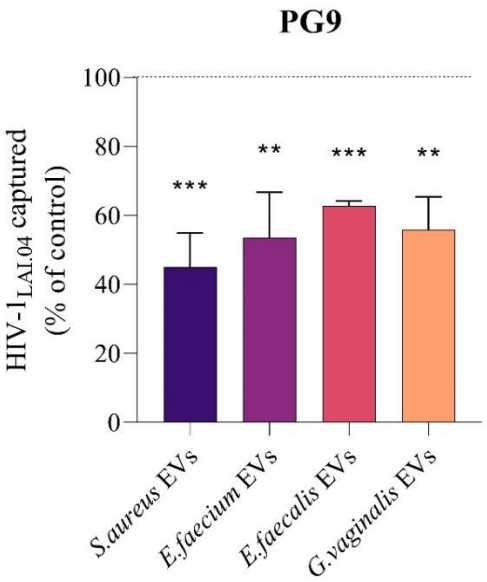

Fig. 24. HIV capture assay. $HIV_{LAI.04}$ was pre-treated with $1x10^{10}$ EVs derived from *S. aureus*, *E. faecium*, *E. faecalis* or *G. vaginalis*, and then incubated with magnetic nanoparticles conjugated to PG9 antibody. Virions captured by the PG9 antibody were isolated using magnetic column and quantified using p24 Luminex capture assay. Data are represented as percentage of $p24_{gag}$ concentration as compared to the control (PBS). Statistical analysis was performed on both experiments with one-way ANOVA multiple comparison (** = $p < 0.01$, *** = $p < 0.001$).

4.4 Discussion

Vaginal microbiota dominated by lactobacilli, especially *L. crispatus*, *L. gasseri* and *L. jensenii* species, reduce the transmissibility and possibility to acquire sexual transmitted infections, such as HIV-1 [210]. Their antiviral activity is exerted by different mechanisms, including lactic acid production, acidification of the niche and release of extracellular vesicles (EVs) [23,59]. On the other hand, the increase of pathogen species abundance typically leads to an increase risk to develop viral and bacterial infections [63]. Here, we investigated whether EVs can be exploited by both vaginal mutualistic (*L. crispatus* and *L. gasseri*) and pathogen bacterial (*S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*) strains to compete among each other. Furthermore, it was investigated the mechanism of action by which *L. gasseri* BC12-derived EVs inhibits HIV-1 and the effect of vesicles released by vaginal pathogen during HIV-1 infections. The vesicles characterization through NTA revealed that EVs produced by both *L. crispatus* and *L. gasseri* had similar size ; further, they were also comparable to vesicles isolated from cultures of *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* in terms of mean diameter (~ 200 nm). Moreover, the mean diameter of EVs from *G. vaginalis*, *L. crispatus* BC5 and *L. gasseri* BC12 were comparable to those previously reported in other studies [59,205], while *S. aureus* and *E. faecium* were greater [211–213]. These latter differences can be related to strain-specific divergences or to change in EVs size induced by the cultivation in different growth medium [214].

The investigation of possible regulations exerted by isolated EVs on bacterial growth was tested by growing *L. crispatus*, *L. gasseri*, *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* in the presence of their own EVs or with EVs produced by the other strains. EVs isolated from lactobacilli did not directly altered vaginal pathogens growths and lactobacilli growths were not retarded or enhanced by pathogen derived EVs, suggesting the absence of competition mechanism EVs-related between beneficial lactobacilli and opportunistic pathogens. Conversely, EVs-dependent growth modulations were observed between pathogen species. In particular, the retarded growth of *E. faecalis* in the presence of *E. faecium* EVs could be associated with bactericidal activity of these EVs on *E. faecalis* cells. Indeed, antibiofilm activities and killing activity on planktonic cultures by bacterial EVs were previously demonstrated and linked with the inclusion of autolysin in the secreted vesicles [193,214,215]. Autolysins, such as AtlA and SagA, were present in vesicles of *E. faecium* strains ST17, ST18, ST78 and ST192 [211]. Furthermore, SagA produced by *E. faecium* was demonstrated to enhance host tolerance to *E. faecalis* and *S. typhimurium* infections through the promotion of epithelial barrier integrity [216]. Interestingly, Rangan et al. observed also that the heterologous expression of *sagA* in *E. coli* and *E. faecalis* caused toxic effect and retarded growth on both species. Considering all these

data, *E. faecium* could protect host by *E. faecalis* infections through promotion of epithelial barrier integrity and bactericidal effect EVs-mediated. Moreover, EVs killing activity could be related to inclusion of autolysin (SagA or AtlA) in *E. faecium* external vesicles. Further experiments, including proteomic characterization of EVs, are required for the validation of this hypothesis and the complete understanding of the mechanism by which *E. faecium* EVs inhibit *E. faecalis* growth. The stimulation assessed by *E. faecalis* EVs on its own growth could be related to promotion of genetic factors that stimulate cell replication or to the delivery of nutrients. In particular, this latter mechanism was observed in some marine bacteria, where EVs carrying nutrients produce a positive effect on the growth of the EVs producer strains [217].

Despite lactobacilli derived EVs use in this study did not possess any antibacterial properties, they were demonstrated to exert antiviral effect on HIV-1. Considering this, we deepen the analysis of the antiviral mechanism of vesicles released by *L. gasseri* BC12. EVs isolated from *L. gasseri* and treated with proteinase lost their antiviral properties, suggesting that protein component, and in particular proteins exposed on the external surface of EVs, are actively involved in the antiviral mechanism against HIV-1 previously described [59]. On the other hand, proteinase treatment of *L. gasseri* EVs did not induce any cytotoxic effect on human cells but caused an increase in the EVs size. This latter point was probably due to vesicles fusion, as demonstrated by Grande et al. on *Lactobacillus reuteri* vesicles [206]. Among the different proteins reported to be present in *L. gasseri*-derived EVs [59], ENO2 and EFTU were considered two of the most promising due to their multifunctionality in bacterial physiology [207,208], and were tested for their anti-HIV-1 properties. It was proved that rEFTU present in *L. gasseri* EVs inhibited HIV-1 replication in a dose-dependent manner, while rENO2 did not exert any antiviral effect. Moreover, HIV-1 binding assay suggested that both the recombinants proteins used were not able to efficiently bind HIV-1 virions. Nevertheless, it is not possible to exclude possible interactions of EFTU with HIV-1 glycoproteins. Indeed, the main function of EFTU is the transfer of aminoacyl-tRNA to the ribosome during translation, however various secondary functions were associated to this protein, such as host-immunomodulation, adhesion to host-cells, cell shape and binding of immune effectors [218]. It is a cytosolic protein that can be localised on cell surface and, for this latter reason, is not unusual to find it in bacterial-derived EVs. Indeed, EFTU was detected in EVs derived from several bacterial species, including *S. aureus*, *G. vaginalis* and *E. faecium* [205,211,219], and in some studies it was also reported as the most abundant protein in the EVs [181,219]. Among the different bacterial species EFTU is highly conserved, resulting in a divergence in the aminoacidic sequence not greater than the 30 % [220]. Moreover, the eukaryotic homologue of EFTU, EF1A, was demonstrated to interact with RNA viruses, includes HIV-1, with different effects on their replication and pathogenesis [221]. The inhibition of HIV-1 by *L. gasseri* BC12 through the secretion of EVs

containing EFTU could be a feature shared with other bacterial strain/species which release vesicles carrying EFTU on their external surface.

The role of pathogen-derived EVs on HIV-1 infections was investigated by using immortalized human T cell in the presence of bacterial culture supernatant (conditioned medium – CM) or purified EVs. CMs derived from pathogens cultures unexpectedly inhibited HIV-1 replication on MT4 cells at the concentration of 0.5% while they were not cytotoxic for human T cell. Comparable inhibition activities were reported for the supernatant of vaginal lactobacilli (*L. crispatus* BC3, *L. crispatus* BC5, *L. gasseri* BC12, and *L. gasseri* BC13) on cervico-vaginal and tonsillar *ex-vivo* tissues infected with HIV_{BAL} [87]. Inhibitory activities on HIV-1 replication were detected also in the presence of all the pathogens-derived EVs tested, with a dose-dependent effect. At the highest EVs amount tested (10^{10} EVs/mL), nontoxic effect was detected on MT4 cells, demonstrating that the inhibitory activity is not linked to MT4 cell death. Antiviral properties of *E. faecium* and *G. vaginalis* were comparable to inhibition activity exerted by *L. crispatus* BC3-derived EVs, in which the addition of $5x10^8$ EVs induced a reduction of the 60% in the $HIV_{LAI.04}$ replication on MT-4 cells [59]. However, the highest anti-HIV-1 activity was that reported for *L. gasseri* BC12, as demonstrated by viral inhibition values of 91.9 % [59]. Overall, HIV-1 inhibition or general antiviral properties had never been described for *S. aureus*, *E. faecalis* and *G. vaginalis*. On the contrary, some strains of *E. faecium* have been reported to exert an inhibiting effect against transmissible gastroenteritis coronavirus (TGEV) and Influenza A *in vitro*, as well as against astrovirus and rotavirus A *in vivo* [222–224]. Similarly to the results obtained with EVs of *L. gasseri* BC12, pathogen-derived vesicles lost their antiviral properties after proteinase treatment, suggesting that, also for those bacterial strains, proteins exposed on vesicles surface are involved in the anti-HIV-1 effect. In terms of mechanism of action, we found that HIV-1 virions pre-treated with EVs isolated from *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* were no more recognise by PG9 antibody, suggesting that the tested pathogen-derived EVs could obstruct the accessibility to the glycoprotein gp120 of HIV-1. Analogous interactions between EVs and HIV-1 Env were reported also for *L. gasseri* BC12 EVs (35.68% of HIV-1 virion captured with PG9 in confront to the control), while *L. crispatus* BC5 EVs (which are not active against HIV-1) were demonstrated to not interact with gp120 [59]. The inhibition of HIV-1 by *L. gasseri* BC12 through the secretion of EVs containing EFTU could be a feature shared with other bacterial strain/species and this hypothesis could explain the unexpected antiviral properties of pathogen-derived EVs and their similarities with EVs isolated from *L. gasseri*.

5. Conclusions and future perspectives

The microbial communities that populate the human body live in a perfect and fragile equilibrium based on the establishment of a complex interaction network with other microorganisms, with the host and with the environment. The variations of a single component perturbate the entire ecosystem and could lead to a deep rearrangement in the community composition with possible onset of different diseases. Among the different niches within the human body, the vaginal environment represents a good model due to quite lower microbial community complexity in comparison to other niches. Overall, vaginal microbiota of healthy pre-menopausal women is dominated by one *Lactobacillus* species among *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners*. These *Lactobacillus* species represent the first barrier against numerous urogenital pathogens and maintain niche homeostasis. On the other hand, shifts from *Lactobacillus*-dominated vaginal microbiota lead to the increase abundance in pathogenic species that is associated with the development of numerous diseases and leads also to an increased risk of other infections acquisition. In particular, HIV-1 infectivity and transmissibility increase in women affected by bacterial and other viral gynaecological infections. In this PhD thesis the following aspects were investigated: i) The environmental factors and genetic traits that lead to the dominance of either *L. crispatus* or *L. gasseri* in the vaginal niche; ii) The possible application of liposomes loaded with *L. gasseri* biosurfactants for the treatment and prevention of *S. aureus* biofilm infections; iii) EV-mediated competition strategies between lactobacilli-pathogen and pathogen-pathogen vaginal bacterial strains; iv) The mechanism of anti-HIV-1 effect exerted by *L. gasseri*-derived EVs and the antiviral properties of EVs isolated from opportunistic human pathogens. The conclusions related to each chapter are summarized on the basis of the results described and discussed previously.

The conclusions of the study on genotypic and phenotypic characterisation of *L. crispatus* and *L. gasseri* are:

- *L. crispatus* BC5 and *L. gasseri* BC12 have comparable genomic and genetic features as compared to those featuring each species. By comparing the genomes of the two species, our results indicate the bigger size of genomes of strains belonging to *L. crispatus* species as compared to the *L. gasseri* ones.
- *L. crispatus* BC5 metabolizes a higher number of carbon sources, while *L. gasseri* BC12 tolerates/resists to a greater amount of antibiotics, metals and other chemical stressors, suggesting different competition mechanisms between the 2 strains.
- The deciphering of metabolic activity of *L. gasseri* and *L. crispatus* strains in the presence of hundreds of different nutrients and environmental stressors provides important information for the comprehension of environmental factors that may influence the colonization of these

two species in the vaginal niche. Also, this knowledge could provide guidance to be taken into consideration for enhanced efficacy during probiotic formulation.

- The exhaustive genotypic and phenotypic characterization of both *L. crispatus* and *L. gasseri* strains done through the integration of whole genome sequencing, Phenotype Microarray and functional genomics could represent a model approach that can be applicable to other microorganisms populating the vaginal niche as well. In the future, analogue experiments on *L. jensenii* and *L. iners* should be done to help the full comprehension of environmental factors that positively influence all the vaginal *Lactobacillus* species that dominate the vaginal microbiota, and those elements that could advantage a specific species. Moreover, it will be possible to detect common factors that promote a vaginal niche enriched in lactobacilli and this will be important considering the protective role exerted by lactobacilli on vaginal niche. Furthermore, the extension of these approaches to the study of urogenital pathogens, such as *G. vaginalis*, could help the comprehension of infection disease onset as well as the development of specific medical therapies and prevention strategies.

The conclusions of the study on liposome loading *L. gasseri* biosurfactants as antibiofilm agent against *S. aureus* biofilm are:

- *Lactobacillus gasseri* BC9 biosurfactants exerts anti-biofilm activity against *S. aureus*, including different multi-drug resistant strains classified as MRSA. Both the two effects were not dose-dependent to BS concentration and in almost all cases the maximum inhibition was observed using a BS at the final concentration of 2.5 µg/mL. The formulation of liposomes loading BS (LP-BS) results stable among time and enhances antibiofilm activity exerted by BS.
- Both BS and BS-LP are considerable safe-compounds due to absence of toxic effects on human and murine fibroblasts.
- The anti-biofilm activity of *L. gasseri* cell-bound-BS encapsulated in liposomes represents a good example of novel applications for natural products. This study represents an innovative approach that could be considered as a starting point for future perspectives that spread from general to applied microbiology. Indeed, a characterization of the mechanisms by which BS exert their antibiofilm activities is currently under investigation. Regarding the medical application of BS, it could be interesting to test anti-biofilm activity of BS and BS-LP on other biofilm-forming human pathogens. Moreover, considering that BS used in this study did not kill bacterial cells, the formulations of liposomes loaded with BS and antibiotics (or other antimicrobial compounds) could represent a valid solution to contrast time pathogen that are growing at the same in biofilm and planktonic forms.

The conclusions of the study on vaginal lactobacilli and pathogen-derived extracellular vesicles effect on bacterial growth and HIV-1 infections are:

- Extracellular vesicles (EVs) released by lactobacilli do not influence the growth of the tested pathogens and lactobacilli growth is not regulated by pathogen-derived EVs. On the contrary, *E. faecium* and *E. faecalis* modulate the planktonic growth of *E. faecalis* through the secretion of EVs and in a dose-dependent way. The mechanisms of action by which EVs released by *E. faecalis* and *E. faecium* regulate *E. faecalis* growth are under investigation.
- Proteins exposed on the external surface of *L. gasseri* BC12-derived EVs are responsible of their anti-HIV-1 activity. Among the different proteins present in *L. gasseri* EVs, EFTU was demonstrated to inhibit HIV-1 replication *in vitro* dose-dependently but this protein seems to not efficiently bind HIV-1 virion. Currently, is under study whether EFTU alter the viral Env, gp120. The detection of EFTU in the EVs of numerous other bacterial species, together with its high aminoacidic conservation, may suggests a common anti-HIV-1 effect by these bacteria. The role of the other 13 protein present in *L. gasseri*-derived EVs on HIV-1 infection are under investigation.
- Conditioned medium and EVs isolated from opportunistic vaginal pathogens (*S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis*) cultures inhibited HIV-1 replication in a dose-dependent manner in *in vitro* studies. Similarly to EVs released by *L. gasseri* BC12, the protein component of the pathogen-derived EVs tested results to be essential for their antiviral activities. Moreover, all of the bacterial EVs tested impacted on the viral envelope protein, gp120. However, the exact mechanism still remains to be clarified.
- Pathogen-derived EVs discussed in this study are currently subjected to proteomic analysis to deeply describe their nature and to identify possible proteins involved in their anti-HIV-1 activity. Furthermore, other experiments with EVs isolated from *S. aureus*, *E. faecium*, *E. faecalis* and *G. vaginalis* are in progress, among these: a) HIV-1 inhibition on human cervico-vaginal *ex vivo* tissues; b) HIV-1 capture assay using other antibody specific for gp120 (PG16 and VRC01) or for gp41 (4B3).

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