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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

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Abstract

The vaginal microbiota, normally characterized by lactobacilli presence, is crucial for vaginal health. Members belonging to *L. crispatus* and *L. gasseri* species exert crucial protective functions against pathogens, although a total comprehension of factors that influence their dominance in healthy women is still lacking. Here we investigated the complete genome sequence and comprehensive phenotypic profile of *L. crispatus* strain BC5 and *L. gasseri* strain BC12, two vaginal strains featured by antibacterial and anti-viral activities. Phenotype microarray (PM) results revealed an improved capacity of BC5 to utilize different carbon sources as compared to BC12, although some specific carbon sources that can be associated to the human diet were only metabolized by BC12, i.e. uridine, amygdalin, tagatose. Additionally, the two strains were mostly distinct in the capacity to utilize the nitrogen sources under analysis. On the other hand, BC12 showed tolerance/resistance towards twice the number of stressors (i.e. antibiotics, toxic metals etc.) with respect to BC5. The divergent phenotypes observed in PM were supported by the identification in either BC5 or BC12 of specific genetic determinants that were found to be part of the core genome of each species. The PM results in combination with comparative genome data provide insights into the possible environmental factors and genetic traits supporting the predominance of either *L. crispatus* BC5 or *L. gasseri* BC12 in the vaginal niche, giving also indications for metabolic predictions at the species level.

DATA SUMMARY

The genome sequences of *Lactobacillus crispatus* BC5 and *L. gasseri* BC12 have been deposited under NCBI BioProject Accession PRJNA596958 and PRJNA596963, respectively. The authors confirm all supporting data have been provided within the article or through supplementary data files.

INTRODUCTION

The human vaginal microbial community is composed of several microorganisms, which establish complex interactions with the host. 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* [1]. Lactobacilli stimulate the preservation of vaginal homeostasis and protect the host mucosa from colonization and growth of different pathogens [2–4]. These protective functions have been associated with different action mechanisms, i.e. immunomodulation, production of antimicrobials, competitive exclusion, pH lowering and production of external vesicles [5–7]. Consequently, the decrease of lactobacilli abundance is usually coupled with the microbial community instability, the onset of different

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Keywords: *Lactobacillus crispatus*; *Lactobacillus gasseri*; *Lactobacillus* genomics; phenotype microarray; vaginal niche; comparative genomics.

Abbreviations: ANI, average nucleotide identity; CDS, coding sequences; KO, KEGG Orthologs; LC BC5, *Lactobacillus crispatus* BC5; LG BC12, *Lactobacillus gasseri* BC12; PM, phenotype microarray.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Eleven supplementary tables and two supplementary figures are available with the online version of this article.

pathologies and the shift from an eubiosis to a dysbiosis condition [8]. Among vaginal *Lactobacillus* spp. strains with health promoting activities, *L. crispatus* BC5 and *L. gasseri* BC12 have been extensively assessed for their antifungal, antibacterial and antiviral activities [2–4, 7, 9]. In addition, BC5 and BC12 strains were characterized in terms of metabolomic profiles during growth in MRS medium and for their interaction with epithelial cells [10, 11].

The microbial community composition in the vaginal niche is highly dynamic and influenced by several factors, such as hormones levels, pregnancy, urogenital infections and drug treatments [12]. A better understanding of factors that lead to the colonization and stability of *Lactobacillus* species in the cervicovaginal tract is urgent to develop novel strategies for woman health maintenance. In particular, despite the fact that several studies have analysed and compared lactobacilli genomics [1, 13, 14], a full comprehension of the efficiency in nutrients' utilization and resistance to stressors is lacking. In this context, phenotype microarrays (PM) provide insights into the metabolic profiling of microbial cells by assessing the utilization of nutrients and sensitivity to toxic compounds and chemical stressors [15]. Despite its limitations associated to *in vitro* approaches (i.e. exclusion of the effect of the host and other bacteria), PM is a high-throughput and standardized technology that can be combined with genome-wide analysis to unravel phenotypic and genotypic insights into ecological and functional aspects of clinical and environmental bacterial species [16–19].

In the present study, we performed PM and whole-genome sequencing of the two vaginal strains *L. crispatus* BC5 and *L. gasseri* BC12. The draft genomes of *L. crispatus* BC5 and *L. gasseri* BC12 were analysed to detect the genetic/genomic traits related to the PM features concerning these two strains' ability to utilize various carbon and nitrogen sources and to resist/tolerate hundreds of different chemical stressors. The analysis of these genetic features was also extended by performing a comparative analysis of all the genomes available in the database belonging to *L. crispatus* and *L. gasseri* species. The results of this study provide insights into the genotypic and phenotypic traits supporting *L. crispatus* BC5 and *L. gasseri* BC12 growth in the vaginal niche, also giving indications on the nutritional and stress factors that might influence their beneficial activity. Some metabolic considerations at the species level are formulated based on the conservation of phenotype-associated genetic traits in each core genome.

Impact Statement

Here we investigated the nutritional and stress-related genetic factors promoting the metabolic activity of *L. crispatus* BC5 or *L. gasseri* BC12 strains, which might influence their capacity to colonize and persist in the human vaginal niche successfully. Previous studies have reported the selective predominance of specific vaginal *Lactobacillus* species (including *L. crispatus* or *L. gasseri*). This seemed to be associated with diverse environmental and host factors; however, a total comprehension of factors that influence their dominance in human vaginal niche is still lacking. Our study applies comparative genome analyses and phenotype microarray assay to define the genetic/genomic traits related to the capabilities of *L. crispatus* BC5 and *L. gasseri* BC12 to utilize various carbon and nitrogen sources and to resist/tolerate hundreds of different chemical stressors. Starting from the results about these two model strains, pan- and core-genome analyses were performed to detect the phenotype-associated genetic traits that characterize each species, i.e. *L. crispatus* and *L. gasseri*. The findings presented here indicate that specific metabolic features and genetic traits distinguish *L. crispatus* from *L. gasseri*. Specific carbon and nitrogen sources as well as the presence of antibiotics, metals and other toxicants differently influence the metabolic activity of BC5 and BC12 strains, suggesting that distinct nutritional features and stress resistance capacities might play a role in the selective dominance of each *Lactobacillus* species in the human vaginal niche.

METHODS

Bacterial strains and culture conditions

L. crispatus BC5 and *L. gasseri* BC12, previously isolated from human vaginal swabs [2], were routinely cultured in de Man, Rogosa and Sharpe (MRS) broth under static conditions or on MRS agar plates supplemented with 0.05% (w/v) L-cysteine, at 37 °C in anaerobic jars containing Anaerocult A (Merck Millipore). Strains were stored at −80 °C in MRS medium containing 20% (v/v) glycerol.

Whole-genome sequencing and annotation

Genomic DNA isolation was performed from 2 ml overnight cultures of *L. crispatus* BC5 or *L. gasseri* BC12 using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the protocol 'Pretreatment for Gram-positive bacteria'. DNA quantification, integrity and purity were checked by Nanodrop spectrometer 1000-ND and on agarose gel. The genome sequencing was achieved using Illumina MiSeq technology, kit V3, 2×300 PE run, and sequencing reads were assembled using SPAdes [20]. *L. crispatus* BC5 genome was assembled into 50 contigs with an N50 of 104423 and genome coverage of 227×, whereas *L. gasseri* BC12 genome was assembled in 48 contigs with N50 of 106908 and overall coverage of 358×. Protein-coding genes and non-coding RNA (rRNA, tRNA and ncRNA) were identified using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP), Protein sequences were mapped to the KEGG orthology database. using KOfamKOALA combined with eggNOG [21, 22]. Antibiotic resistance genes and genetic variants associated to antibiotic resistance phenotypes were identified using the Comprehensive Antibiotic Resistance Database (CARD) in

strict mode [23]. Additionally, identification of antibiotic resistance gene families was further investigated using the stand-alone version of InterProScan v5.45–80.0 [24]. Manual curation was performed *ad hoc* based on literature information.

Phylogenomics and comparative genome analysis

Phylogenetic relatedness of BC5 and BC12 strains to *L. crispatus* and *L. gasseri* species was determined via average nucleotide identity (ANI) using the python module pyANI (https://github.com/widdowquinn/pyani) implemented in Anvi'o [25].

Comparative genome analysis was performed using all *L. gasseri* and *L. crispatus* strains available in the NCBI Assembly RefSeq database in May 2020. A pan-genome database was built using the Anvi'o pipeline [25] with an inflation parameter (*--mcl-inflation*) of 10 to identify protein clusters based on sequence similarity. In this respect, a soft-core genome was defined considering clusters present in at least 90% of genomes. Functional annotation of the proteins included in each cluster was finally performed using eggNOG [21]. Identification of gene clusters associated with vaginal *L. crispatus* and *L. gasseri* strains was performed using the Anvi'o script '*anvi-get-enriched-functions-per-pan-group*'. A gene cluster was considered significantly associated with the vaginal source when the calculated *adjusted P*-value was <0.001 and when present in at least 50% of the vaginal strains.

Phenotype microarray and data analysis

Phenotype microarray (PM) assay was performed on *L. crispatus* BC5 and *L. gasseri* BC12 using PM Technology (Biolog, Hayward, CA, USA) in anaerobic conditions. PM technology is based on reducing tetrazolium violet by cells with active metabolism and consequent formation of purple colour that was automatically recorded by a camera every 15 min [15]. Both strains were tested on Biolog microplates for their specific metabolic activity in the presence of 190 different carbon sources (PM1 and PM2A), 94 nitrogen sources (PM3B) and 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* strains were grown on MRS plates and then inoculated in PM plates described as follows: cells were removed from MRS agar plates using a cotton swab and inoculated in a solution composed of Inoculation Fluid (Biolog), DyeG (Biolog) and additive solution [the complete composition of the additive solutions and mixes are provided in Table S6 (available in the online version of this article)], at a cell density of *T* (transmittance)=65%. All plates were inoculated with 100 µl of cell suspension per well, and the anaerobic atmosphere was created with an Anaerocult P sachet (Sigma-Aldrich) placed together with each plate in a incubation bag sealed with tape [18]. Lastly, plates were incubated for 72 h at 37 °C in an Omnilog automated incubator/reader (Biolog).

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 [26]. For PM1-2A (carbon sources), PM3B (nitrogen sources), PM9-10 (Osmolytes and pH), PM data were referred to the value of the area under the curve (AUC) obtained per each compound during Omnilog data analysis (Table S5). In PM11C-20B (drug and chemical sensitivities), each compound was present in four 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 (Table S5).

Nucleotide sequence accession number

The whole genome shotgun projects of *L. crispatus* BC5 and *L. gasseri* BC12 have been deposited at DDBJ/ENA/GenBank under the accessions WUBT00000000.1 and WUBU00000000.1, respectively.

RESULTS

Phylogenomic analyses and genome annotation of *L. crispatus* BC5 and *L. gasseri* BC12

Draft genomes of 2064185 bp with a G+C content of 36.7 mol% for *L. crispatus* BC5 and 2020322 bp with a G+C content of 34.7 mol% for *L. gasseri* BC12 were obtained. These data were in line with the mean values calculated on 116 genomes of *L. crispatus* strains, and 40 genomes of *L. gasseri* strains retrieved from NCBI Assembly Database, i.e. size of 2.1 ± 0.18 Mbp for *L. crispatus* and 1.9 ± 0.26 Mbp for *L. gasseri;* % GC was 36.8±0.002% and 34.9±0.002%, respectively.

Phylogenetic relatedness of BC5 and BC12 to *L. crispatus* and *L. gasseri* species, respectively, was confirmed, being the ANI value >96.5% for both strains (97.6% for BC5 vs *L. crispatus* ATCC33820, 97.2% BC12 vs *L. gasseri* ATCC33323). On the other hand, in accordance with their belonging to a different species, the two strains shared an ANI of 75%.

Genome annotation analysis identified a total of 2015 coding sequences (CDS) and 81 RNA genes (16 rRNAs, 62 tRNAs and 3 ncRNAs) in BC5 and 1934 CDS and 76 RNA genes (10 rRNAs, 63 tRNAs and 3 ncRNA) in BC12. According to the NCBI Prokaryotic Annotation pipeline, 76 proteins in BC5 and BC12 were annotated with domain of unknown function (DUF) whereas 253 and 210 proteins were identified as hypothetical proteins (RefSeq assembly accession for BC5 and BC12: GCF_014654865.1 and GCF_014654855.1, respectively). According to KOfam and eggNOG, 1022 proteins for BC12 and 955 proteins for BC5 were assigned to the KEGG orthology database, i.e. KEGG Orthologs (KO) (Tables S1 and S2). Among those belonging to the major metabolic pathways, by directly comparing the two strains, BC5 and BC12 shared 174 functional orthologues (Table 1). On the other hand,

Table 1. 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		–

Continued

Table 1. Continued

KEGG metabolism	KEGG pathway name	Shared KO	LC BC5 unique KO	LG BC12 unique KO
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

* The full lists of KO associated to *L. crispatus* BC5 and *L. gasseri* BC12 protein coding sequences are reported in Table S1 and Table S2, respectively

unique KO in each strain were 129 and 99 in BC5 and BC12, respectively (Table 1).

Phenotype microarray of *L. crispatus* BC5 and *L. gasseri* BC12 and genetic traits associated with their phenotypic differences

Carbon sources

L. crispatus BC5 and *L. gasseri* BC12 showed metabolic activity in the presence of 61 and 54 carbon sources of different categories, respectively (Fig. 1a). Among these, both the strains displayed a preference for carbohydrates (over amino acids, carboxylic acids, amines, amides, esters, polymers, fatty acids). However, *L. crispatus* BC5 could utilize a higher number of sugars as compared to *L. gasseri* BC12 (35 by BC5 vs 32 by BC12 over the 71 carbohydrates under analysis). Furthermore, BC5 showed higher metabolic activities on 22 shared (i.e. utilized by both the strains) carbon sources (Fig. 1c) and the capability to utilize 18 compounds exclusively (indicated with * in Fig. 1a). 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, only the *L. crispatus* strain possesses genes coding for systems involved in their import (Table 2, Fig. 2). Concerning the higher metabolic activity on maltose, a maltose 6-phosphate glucosidase gene is present in BC5 genome but not in BC12 one (Table 2, Fig. 2). In α-glycosydes such as α-methyl-D-mannoside, BC5 possesses multiple copies of the genes encoding the multiple-sugar-metabolism (Msm) transport system, which are involved in the uptake of these sugars. The higher metabolic activity of BC5 on lactose and galactose can be associated with the different degradative pathways present in the two strains. BC5 possesses a beta-galactosidase gene (*lacZ*), which catalyses the conversion of lactose into D-galactose that can be further converted into α-D-glucose-6P (via Leloir pathway). Conversely, BC12 genome bears the genes of the tagatose-6P pathway, in which lactose and galactose are converted into glyceraldeyde-3P that can enter glycolysis. In consideration of sucrose, both BC5 and BC12 genomes possess the genes involved in converting sucrose into α-D-glucose-6P via D-fructose, but only the BC5 genome carries a levansucrase/invertase gene, needed for the conversion of sucrose into D-glucose and levan. The presence of an additional gene encoding the triose/dihydroxyacetone kinase (DhaK/DAK) solely in the BC5 genome might support the higher performance shown by this strain as compared to BC12. DhaK/DAK catalyses the direct conversion of fructose-1P into glyceraldehyde-3P via D-glyceraldehyde that might improve the sugar utilization kinetics.

L. gasseri BC12 showed higher performance as compared to *L. crispatus* BC5 only on a few carbon sources. In this regard, the *L. gasseri* strain showed higher metabolic activity on D-trehalose and the exclusive capability to utilize nine carbohydrates (indicated with $ in Fig. 1a). From a genotypic point of view, the presence in BC12 of a tagatose 1,6-diphosphate aldolase gene can support the capacity of only this strain to utilize D-tagatose (Table 2). Similarly, 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 BC12 to metabolize N-acetylgalactosamine efficiently. BC12's higher metabolic activity on trehalose is probably due to 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-6P as a substrate but with a different specificity than 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 [27].

The carboxylic acids' utilization profile was similar in the two strains (Fig. 1a), although BC5 showed higher metabolic activity on lactic acid and pyruvic acid. These findings, together with the higher respiration rate on glucose, indicate that BC5 strain has an improved homolactic fermentation efficiency as compared to BC12. Despite the low number of alcohols and C-source amino acids that were generally metabolized by the two strains, BC5 also showed a higher metabolic performance with these compounds (Fig. 1a, c). BC5 and BC12 strains were also 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 [28–30], the three fatty acids utilized by BC12 are derivatives of poly-oxyethylenesorbitan and can be used by some *Lactobacillus* strains as a source of unsaturated fatty acids [31]. Regarding 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 is able to utilize cyclodextrins and gelatin and only BC5 can metabolize inulin and mannan (Fig. 1a).

Nitrogen sources

BC5 and BC12 strains could metabolize 13 and 14 nitrogen sources, respectively, out of the 95 compounds belonging to this PM category assay. They shared the capacity to utilize six of them, i.e. four amino acids, one amino sugar and alloxan (Fig. 1b). On the other hand, more than half of the nitrogen sources utilized were strain-specific (indicated with * or $ in Fig. 1b, c). From a genotypic point of view, the capacity of *L. crispatus* BC5 to utilize arginine could be related to the presence of the *arcA* gene in its genome encoding an arginine deiminase, which is able to convert arginine into citrulline and NH_3 (Fig. 2). However, BC5 did not possess the additional genes involved in the subsequent conversion of citrulline into ornithine [ornithine trans-carbamoylase (encoded by *arcB*), carbamate kinase (encoded by *arcC*),

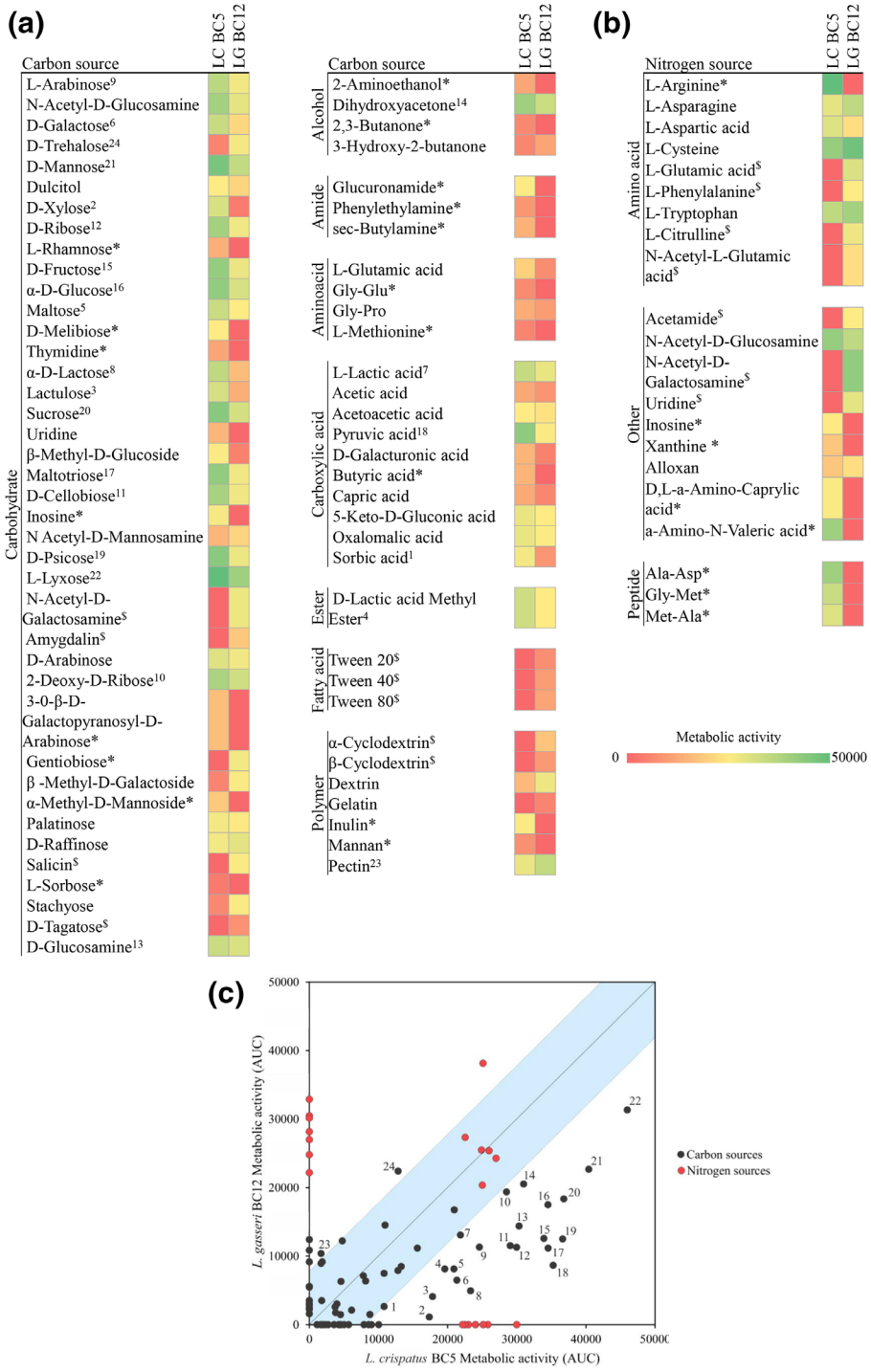

Fig. 1. Carbon and nitrogen sources metabolized by *L. crispatus* BC5 and *L. gasseri* BC12. Heatmaps represent metabolic activities of BC5 and BC12 in the presence of carbon sources (a) and nitrogen sources (b). (c) Correlation between the metabolic activities of *L. crispatus* BC5 and *L. gasseri* BC12 assessed in the presence of the carbon and nitrogen sources tested in PM assay. The metabolic activities of BC5 and BC12 are represented as dots on a 2D scatterplot. Plot bands represent mean (μA) and standard deviation (σA) from the mean of the activity difference (ΔA). The points located outside of the standard deviation band represent compounds inducing significantly different metabolic responses in BC5 and BC12. In particular, dots below and above the bands represent PM compounds inducing higher metabolic activity in BC5 compared to BC12 and higher metabolic activity in BC12 compared to BC5, respectively. Numbers in the plot represent the PM chemicals listed in (a). In (a) and (b), * indicates carbon and nitrogen sources metabolized only by BC5, $ indicates carbon and nitrogen source metabolized only by BC12.

Table 2. Genetic features of *L. crispatus* BC5 and *L. gasseri* BC12 involved in differentially metabolized carbon and nitrogen sources

Metabolism[§]	KO[†]	LC BC5 hits[‡] (no.)	LG BC12 hits[‡] (no.)	KO name
Galactose[6]/ Lactose[8]/Tagatose[§]	K00849	1	1	*galK;* galactokinase
	K00850	1	1	*pfkA;* 6-phosphofructokinase
	K00965	3	2	*galT;* UDPglucose-hexose-1-phosphate uridylyltransferase
	K01182	1	3	*malL;* oligo-1,6-glucosidase (non-direttamente coinvolta in metab galattosio)
	K01190	3	0	*lacZ;* beta-galactosidase
	K01220	0	3	*lacG;* 6-phospho-beta-galactosidase
	K01635	0	1	*lacD;* tagatose 1,6-diphosphate aldolase
	K01784	1	1	*galE;* UDP-glucose 4-epimerase (questa non-coinvolta in met galattosio)
	K01785	2	2	*galM;* aldose 1-epimerase
	K01819	0	2	*lacA;lacB*; galactose-6-phosphate isomerase
	K01835	1	1	*pgm;* phosphoglucomutase
	K02786	0	3	*lacF;* lactose PTS system EIIA
	K02788	0	3	*lacE;* lactose PTS system EIICB
	K20112	1	2	*gatA;* galactose PTS system EIIA
	K20113	0	1	PTS-Gal-EIIB (*gatB*); galactose-specific IIB
	K20114	0	1	PTS-Gal-EIIC (*gatC*); galactose-specific IIC
	K16209	1	0	*lacS*, *galP*, *rafP*; lactose/raffinose/galactose permease
Maltose[5]	K02777	2	1	*crr;* sugar PTS system EIIA component
	K02750	2	0	*glvC;* alpha-glucoside PTS system EIICB
	K01232	1	0	*glvA*; maltose-6'-phosphate glucosidase
Sucrose[20]	K02810	2	1	*scrA*; sucrose PTS system EIIBCA or EIIBC
	K01193	3	1	*sacA;* beta-fructofuranosidase
	K00847	1	1	*scrK;* fructokinase
	K01810	1	1	*pgi;* glucose-6-phosphate isomerase
	K01835	1	1	*pgm*; phosphoglucomutase
	K00692	1	0	*sacB*; levansucrase
	K00690	2	1	*sucP;* sucrose phosphorylase
N-acetyl-galactosamine[§]	K02744	0	2	*agaF*; N-acetylgalactosamine PTS system EIIA
	K02745	0	1	*agaV*; N-acetylgalactosamine PTS system EIIB
Fructose[15]	K02770	1	2	*fruA*; fructose PTS system EIIBC or EIIC
	K00882	1	3	*fruK*; 1-phosphofructokinase
	K01624	1	1	*fbaA*; fructose-bisphosphate aldolase
	K01803	1	2	*tpiA*; triosephosphate isomerase (TIM)

Continued

Table 2. Continued

Metabolism[$]	KO[†]	LC BC5 hits[‡] (no.)	LG BC12 hits[‡] (no.)	KO name
Melibiose*/ Maltotriose[17]	K10117	1	0	*msmE*; raffinose/stachyose/melibiose transport system substrate-binding protein
	K10118	1	0	*msmF*; raffinose/stachyose/melibiose transport system permease protein
	K10119	1	0	*msmG*; raffinose/stachyose/melibiose transport system permease protein
	K10112	4	2	*msmX*, *msmK*, *malK*, *sugC*, *ggtA*, *msiK*; multiple sugar transport system ATP-binding protein
	K07407	1	1	*galA*, *rafA*; alpha-galactosidase
Trehalose[24]	K02777	2	1	*crr*; sugar PTS system EIIA component
	K01232	1	0	*glvA*; maltose-6'-phosphate glucosidase
	K01226	0	1	*treC*; trehalose-6-phosphate hydrolase
Arginine*	K01478	1	0	*arcA*; arginine deiminase
Dipeptides/ Oligopeptides	K03305	1	0	di-/tripeptide transporter
	K15580	7	0	*oppA*, *mppA*; oligopeptide transport system substrate-binding protein
	K15581	1	0	*oppB*; oligopeptide transport system permease protein
	K15582	1	0	*oppC*; oligopeptide transport system permease protein
	K15583	1	0	*oppD*; oligopeptide transport system ATP-binding protein
	K10823	1	0	*oppF*; oligopeptide transport system ATP-binding protein

§ Subscripts correspond to those reported in Fig. 1. Furthermore, * and $ indicate carbon and nitrogen sources metabolized in PM assay by either *L. crispatus* BC5 or *L. gasseri* BC12, respectively.
†GenBank locus tags associated to KO can be retrieved from Tables S1 and S2.
‡Number of genes assigned to the same functional orthologue group (from data in Tables S1 and S2).

membrane transport protein (encoded by *arcD*)] [32, 33], which explains the inability of BC5 also to utilize arginine as a carbon source. Furthermore, differently from BC12, BC5 could utilize di-peptides and alpha-amino fatty acids as nitrogen sources. The different nitrogen sources' utilization capability of BC5 and BC12 might be associated to the presence in each strain of a different dipeptides/oligopeptides' uptake system, i.e. the Opp system in BC5 and the DtpT in BC12 (Table 2), which might be involved in the intake of a specific range of oligopeptides due to affinity issues [34]. Regarding the nitrogen sources utilized only by BC12, no specific genetic determinants could be detected based on the genome functional annotation.

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 sulphate, 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 three 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 BC5 (Fig. 3). The presence of 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 (i.e. betaine and proline by BC12 and trigonelline by BC5) (Fig. 3).

Some genetic traits could be associated with the overall improved resistance and/or tolerance capacities of BC12 strain as compared to BC5 towards osmotic stressors. For instance, the presence of two copies of the potassium

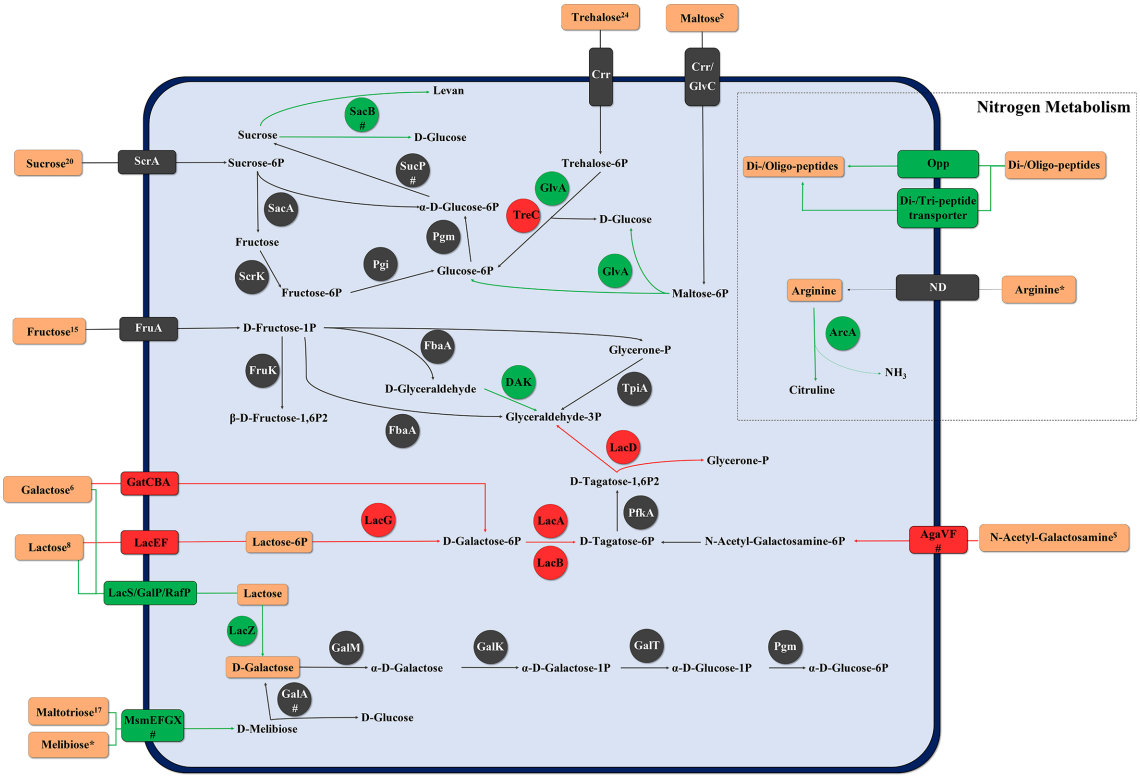

Fig. 2. Metabolic pathways of carbon and nitrogen sources differentiating BC5 and BC12 based on phenotype microarray (PM) and genome functional annotation. Metabolic reactions are indicated with coloured arrows: green for BC5-specific reactions, red for BC12-specific reactions, dark grey for reactions predicted to occur in both these strains. Most of the functions displayed are included in the core genome of each species with a few exceptions (indicated with #). The gene products are indicated in Table 2 except for DAK that stands for dihydroxyacetone kinase. Carbon and nitrogen sources are indicated within orange boxes with the same subscripts of Fig. 1 and Table 2. The box with ND indicates the absence of a specific gene predicted to encode that function by genome annotation.

uptake permease (KUP) gene in the BC12 genome might relate to the capacity of this strain to stand higher concentrations of KCl (Table 3). These genes encode a low affinity K^+ uptake system KUP possibly involved in the initial phase of osmo-adaptation [35]. 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 3). This genetic trait can be associated with *L. gasseri* strain's exclusive capacity to utilize the osmotic protectants L-proline and betaine. On the other hand, choline could not be used by BC12 to counteract osmotic stress, which might be due to a possible lower affinity of the Opu system toward this compound [36]. Genes encoding glycerol ABC transporters (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 as osmoprotectants to sustain the growth of the two strains in the presence of the highest NaCl concentration tested, i.e. 6% [37, 38].

Tolerance/resistance to chemical stressors including antibiotics and metals

Among the drugs and chemicals tested, BC12 was metabolically active in 86 chemicals/drugs, while *L. crispatus* BC5 showed resistance to 48 stressors (Fig. 4). In particular, both strains were resistant to 12 antibiotics out of the 91 antibiotics tested (Fig. 4) and both the strains were sensitive to aminocoumarin, lincomycin, nitrofurans, rifamycin and sulfonamides antibiotic classes. As compared to BC5, BC12 strain showed resistance to additional 20 antibiotics belonging to aminoglycosides, β-lactams, cephalosporines, fluoroquinolones, glycopeptides, macrolides, nitroimidazole and quinolones classes (Fig. 4). At the genetic level, the higher metabolic activity of BC12 in the presence of macrolides 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 3). BC12 was also found to be slightly more resistant than BC5 to some β-lactams (Fig. 4). 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

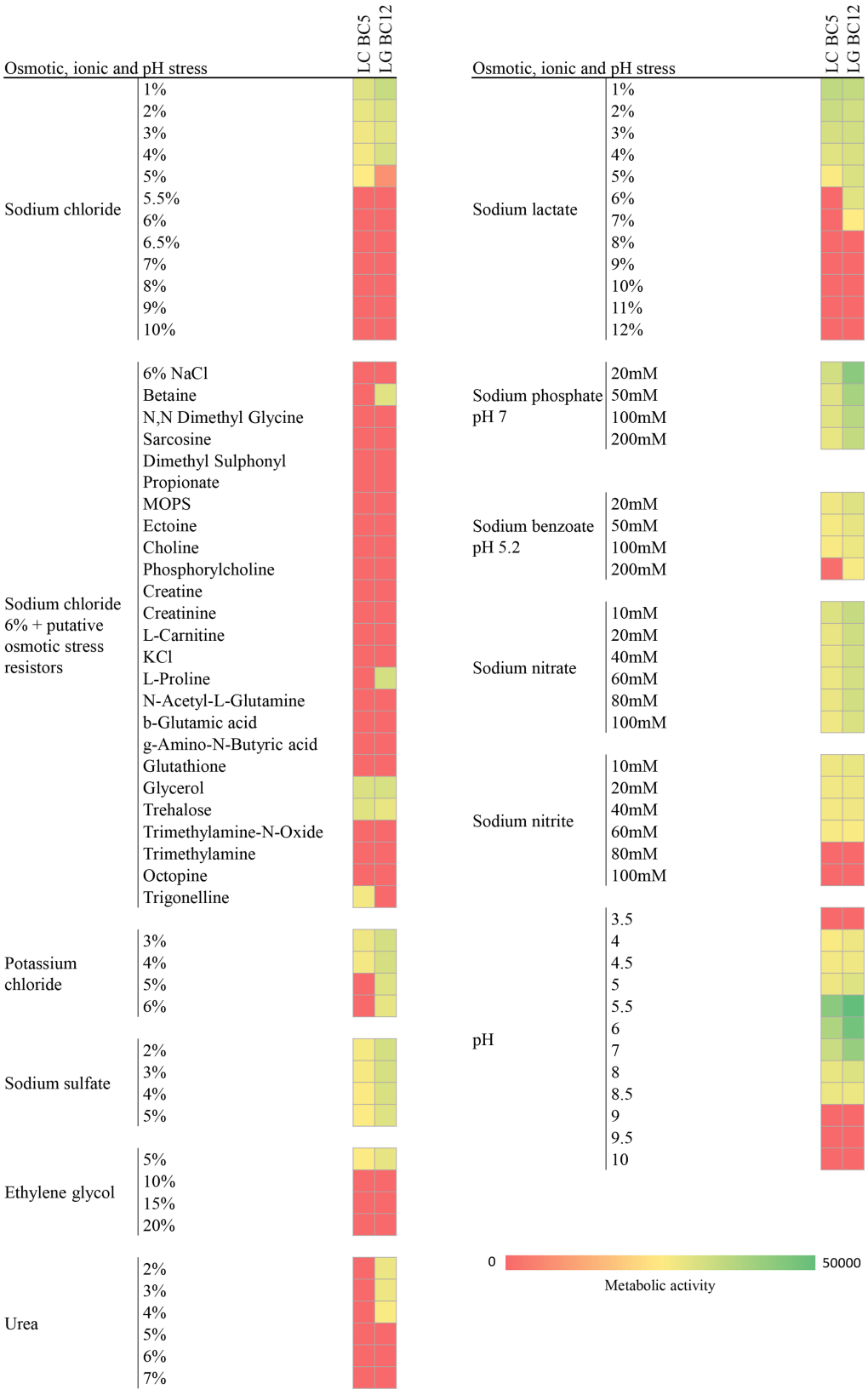

Fig. 3. Osmotic and ionic stress resistance/tolerance in *L. crispatus* BC5 and *L. gasseri* BC12. Heatmaps represent metabolic activities of BC5 and BC12 within a metabolic value range of 0 (red) – 50000 (green).

Table 3. Genetic features of *L. crispatus* BC5 and *L. gasseri* BC12 involved in stress response

Stress*	KO†	BC5 hits (no.)	BC12 hits (no.)	KO name
Osmotic	K05845	0	1	*opuC*; osmoprotectant transport system substrate-binding protein
	K05846	0	1	*opuBD*; osmoprotectant transport system permease protein
	K05847	0	1	*opuA*; osmoprotectant transport system ATP-binding protein
	K03549	1	2	KUP; system potassium uptake protein
	K03455/K03315	2	3	Monovalent cation / H(+) antiporter
Beta-lactam/ quinolones	K18104	0	1	*abcA*; multidrug resistance, efflux pump AbcA
Beta-lactams	–	4	7	beta-lactamase/penicillin binding proteins‡
Macrolide	K19350	2	1	lincosamide and streptogramin A transport system ATP-binding/permease protein
	K05685	0	1	*macB*, macrolide transport system ATP-binding/permease protein
Aminoglycosides	K00662	1	1	*aacC*; Aminoglycoside 3-N-acetyltransferase
	K19272	0	1	*aph*; aminoglycoside 3'-phosphotransferase
Fluoroquinolones	K18908	2	0	*mepA*; multidrug resistance, efflux pump MepA
Tetracyclin	K18220	1	1	Ribosome protection-type tetracycline resistance related proteins
Arsenic	K03892	1	0	*arsR*; ArsR family transcriptional regulator
	K00537	1	0	*arsC*; arsenate reductase
	K03325	1	0	*arsB*; arsenite transporter
Copper	–	1	1	*copY*; negative regulator of copper transport operon
	K17686	3	3	*copA*; P-type Cu^{+}transporter
	K07213	1	0	*copZ*; copper chaperon
	K01533	1	1	*copB*; P-type Cu2+transporter
Cobalt, zinc and cadmium	K16264	1	1	*czcD*; cobalt-zinc-cadmium efflux system
	–	0	1	*czrA*; ArsR family transcriptional regulator, zinc-responsive transcriptional repressor
Bile resistance	K01442	1	2	Choloylglycine hydrolase/bile salt hydrolase [EC:3.5.1.24]
	–	2	0	Putative Bile Salt Transporter§,‖

Continued

Table 3. Continued

Stress*	KO†	BC5 hits (no.)	BC12 hits (no.)	KO name
Others	K04078	1	1	chaperonin GroES
	K03687	1	1	molecular chaperone GrpE
	K04043	1	1	molecular chaperone DnaK
	K03686	1	1	molecular chaperone DnaJ
	K04077	1	1	chaperonin GroEL
	K04083	1	1	molecular chaperone Hsp33
	K01358	1	1	ATP-dependent Clp protease, protease subunit
	K03544	1	1	ATP-dependent Clp protease ATP-binding subunit ClpX
	K06149	2	1	universal stress protein A
	K11065	1	1	thioredoxin-dependent peroxiredoxin
	K08161	2	1	MFS transporter, DHA1 family, multidrug resistance protein
	K18926	2	4	MFS transporter, DHA2 family, lincomycin resistance protein
	K18936	1	1	MFS transporter, DHA2 family, multidrug resistance protein
	K00383	0	1	Glutaredoxin reductase

*Phenotypic data obtained for each stressors category and/or specific stressors are reported in Figs 3 and 4, except for 'bile resistance' and 'others'.
†GenBank locus tags associated to KO can be retrieved from Tables S1 and S2.
‡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.

are involved in the beta-lactams degradation and extrusion, respectively. Additionally, on the basis of previous findings, AbcA transporter can be associated with BC12 resistance towards polymyxin B [39] and the quinolone pipemidic acid. *L. gasseri* BC12 also showed higher resistance than BC5 to fluoroquinolones, except for ciprofloxacin, towards which both the strains showed the same tolerance. 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 for the quinolones efflux in *Lactococcus lactis* [40]. Regarding the genetic determinants associated with antibiotic resistance in *L. crispatus* BC5, this strain possessed two *mep*A genes encoding MATE family multi-drug efflux pumps possibly involved in the resistance to ciprofloxacin, gentamicin, streptomycin and kanamycin. With regard to the resistance to the three aminoglycosides shown in Fig. 4, both the strains carried the genes *aaaC3* encoding the aminoglycoside N3'-acetyltransferase (GRH99_07855) involved in gentamycin resistance mainly. At the same time, 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 with aminoglycoside resistance in other *Lactobacillus* spp. [41].

PM experiments testing the resistance/tolerance to 26 metals and metalloids showed that BC12 and BC5 strains were both metabolically active in the presence of four metals, although BC12 generally showed higher metabolic activity values. Further, the *L. gasseri* strain was resistant to other three metals indicated with $ in Fig. 4. From a genetic point of view, copper resistance could be associated with the presence of the operon *copYACB* in the genomes of both strains (Table 3). CopY acts as a negative regulator of the *cop* genes' expression while *copA* and *copC* encode soluble periplasmic Cu^{+}-binding proteins, which assist CopB in copper extrusion. *copB* encodes a copper-translocating P-type ATPase that shows similarity with lead, cadmium, zinc and mercury transporting ATPase, therefore being

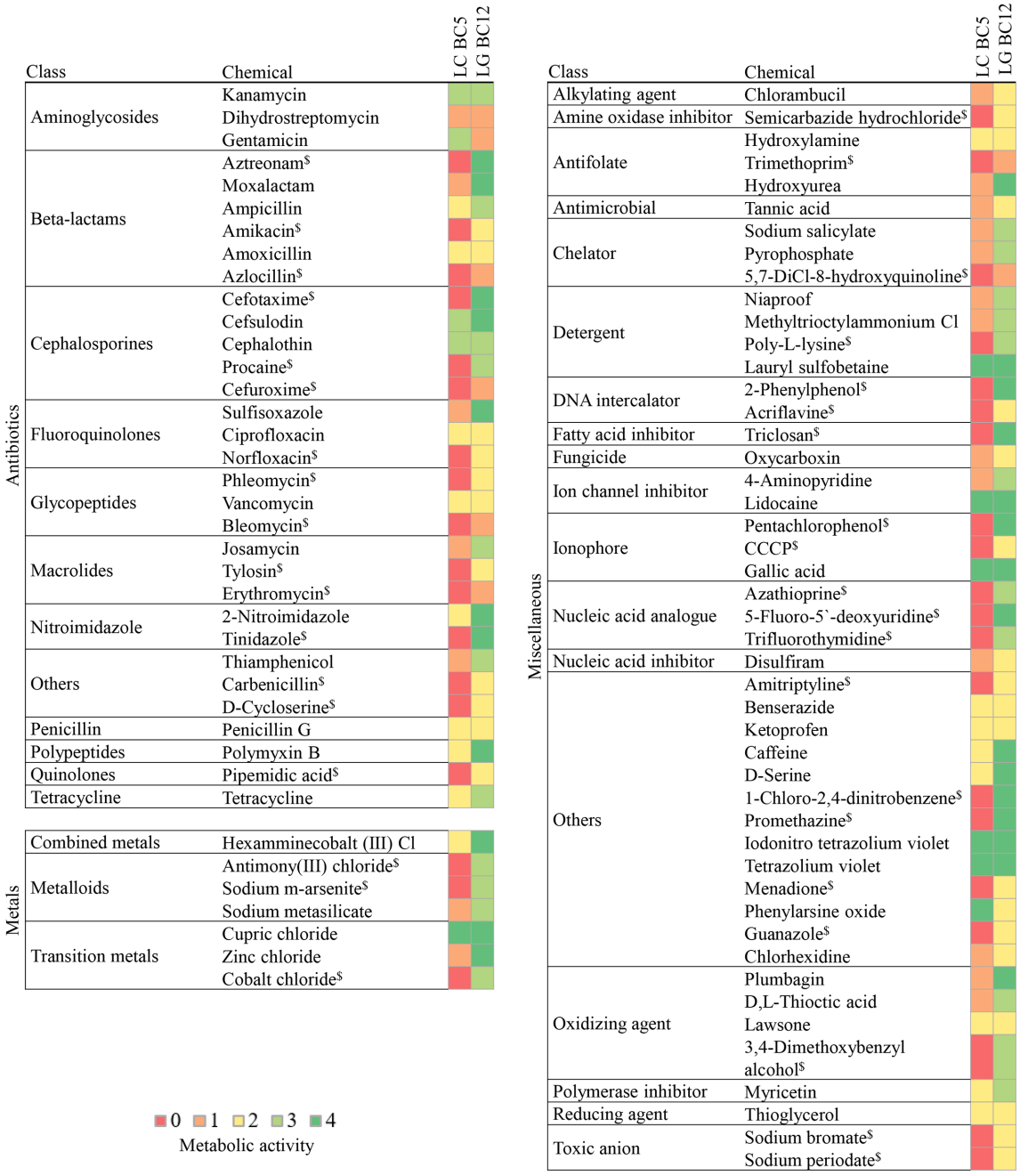

Fig. 4. Drug and chemical resistance of *L. crispatus* BC5 and *L. gasseri* BC12. Heatmaps represent metabolic activities of BC5 and BC12 in the presence of four different concentrations (from 1 to 4) of drugs and toxic chemicals. Only chemical compounds that allowed metabolic activity detection in at least one strain are reported. $ indicates the chemicals and drugs towards which only BC12 showed resistance/tolerance

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, including 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 [42]. This might explain the resistance to cobalt and the higher resistance to zinc shown by BC12 compared to BC5. On the other hand, the higher susceptibility to arsenite and antimonites shown by BC5 could not be explained at the genetic level as BC5 but not the BC12 genome carries *ars* genes (i.e. *ars*RBC operon), which are the genetic traits

typically associated to the bacterial resistance to arsenic and antimony [43]. Possible additional cationic antiporter 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 (Tables 3, S1 and S2) can support the ability of the two strains to resist 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 an additional 31 compounds (Fig. 4). 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. However, only the *L. gasseri* strain also possesses a glutathione reductase that might be involved in protection mechanisms from different physiological stressors, including toxic concentrations of metal ions, and osmotic and acid stresses [44]. Lastly, in both strains, a bile-salt hydrolase gene was found, which is related to still unclear tolerance mechanisms to these detergent-like biological molecules and possibly to other toxicants (e.g. detergent and antibiotic) that were tested in PM assay (Table 3). In BC12, two tandem genes encoding putative bile transporter genes (one partial and one complete) are organized in operon together with one of the two bile-salt hydrolase gene typical of lactobacilli strains isolated from the human environment [45].

Species-wide analysis of the genetic traits associated with the phenotypic differences between *L. crispatus* BC5 and *L. gasseri* BC12

A strain-level pan-genome analysis was performed to determine whether BC5 and BC12 key genes supporting the phenotype-genotype association were conserved in the core genome of the corresponding species. The pan-genome of the *L. crispatus* species was represented by 5609 clusters of homologue proteins, being 1334 shared across 90% of the strains (soft-core genome) and 38 only present in BC5 (Fig. S1). As compared to *L. crispatus*, the *L. gasseri* pan-genome included a lower number of clusters (i.e. 3268) with a soft-core genome of 1259 clusters and 34 BC12 singletons (Fig. S2).

Overall, most of the strain-specific features included in clusters of each soft-core genome, were linked to the metabolism of sucrose, N-acetyl-galactosamine, galactose, and lactose, maltose, fructose, melbiose, maltotriose, trehalose and D-tagatose (Figs 5 and 6, Tables S9 and S10). The exceptions are the sucrose phosphorylase genes that are present in both BC5 and BC12 but not in the soft-core of their respective species, and the levansucrase/invertase gene that was found in BC5 and only in a few members of *L. crispatus* species (Figs 5 and 6, Tables S9 and S10).

With regard to nitrogen metabolism, the BC5 arginine deiminase was identified in approximately 80% of the of *L. crispatus* strains, while most of the BC5 genes encoding di- and oligo-peptide transport systems were included in clusters within the *L. crispatus* soft-core genome (Fig. 5, Table S9).

Genes of *L. crispatus* BC5 and *L. gasseri* BC12 that were functionally linked to osmotic stress tolerance, heavy metals and resistance to antibiotics were generally conserved among members belonging to the corresponding species (Figs 5 and 6, Tables S9 and S10). Among the genetic features associated to stressors resistance, only the BC5 *ars* operon (*arsRBC*) occurred in a few *L. crispatus* strains, therefore not being included in the *L. crispatus* core genome (Fig. 5, Table S9).

Finally, we investigated whether key genes linked to the phenotypic differences between BC5 and BC12 were significantly correlated with the vaginal source. Within the *L. crispatus* pan-genome, we found 439 gene clusters to be more represented in strains isolated from the vaginal niche (Fig. S1, Table S3), among these, 66 gene clusters were also detected in BC5 (Fig. S1). Despite most of them were not assigned to KO groups, BC5 gene clusters associated with the vaginal origin were linked to amino acid metabolism and transport (e.g. arginine deiminase, Opp oligopeptide transport system), nutrient importer, pH stress tolerance, cofactor biosynthesis, defence system, environmental sensing (Table S11). In contrast, no gene clusters in the *L. gasseri* pan-genome were significantly correlated with the vaginal source (Table S4).

DISCUSSION

Persistence and colonization of *Lactobacillus* spp. in the human vaginal tract are dependent on a series of factors associated with the presence of other micro-organisms, the interactions with the host, the local nutrient availability and stress conditions. In this context, several studies have analysed possible mechanisms involved in vaginal niche adaptation, fitness and maintenance of *Lactobacillus* spp. mainly on the basis of comparative genomic studies correlating genomic features with the isolation niche [13, 46]. Conversely, only few data are available about the effect of different nutrients and stress conditions on the metabolic activity of *Lactobacillus* spp. strains. In this work, we investigated, using the high-throughput and standardized phenotype microarray assay, the metabolic response of the two strains *L. crispatus* BC5 and *L. gasseri* BC12 in the presence of a wide range of nutritional and stress factors; since some of these factors characterize also the vaginal niche, they may influence the relationship of symbiosis between the vaginal microbiota and the host. In particular, BC5 showed a higher capacity to utilize carbon sources (in terms of a number of metabolized compounds and metabolic activity values), while BC12 showed a general higher capacity to resist/tolerate stressors. This is in line with the results obtained from API test reported in a previous study that pointed out the capacity of *L. crispatus* strains to ferment a higher number of sugars as compared to *L. gasseri* strains [47]. Furthermore, the phenotypic diversity that we detected with PM assay was partially supported by differences at genetic/genomic level and related to the import and/or metabolism of nutrients or to the export and/or detoxification of stressors.

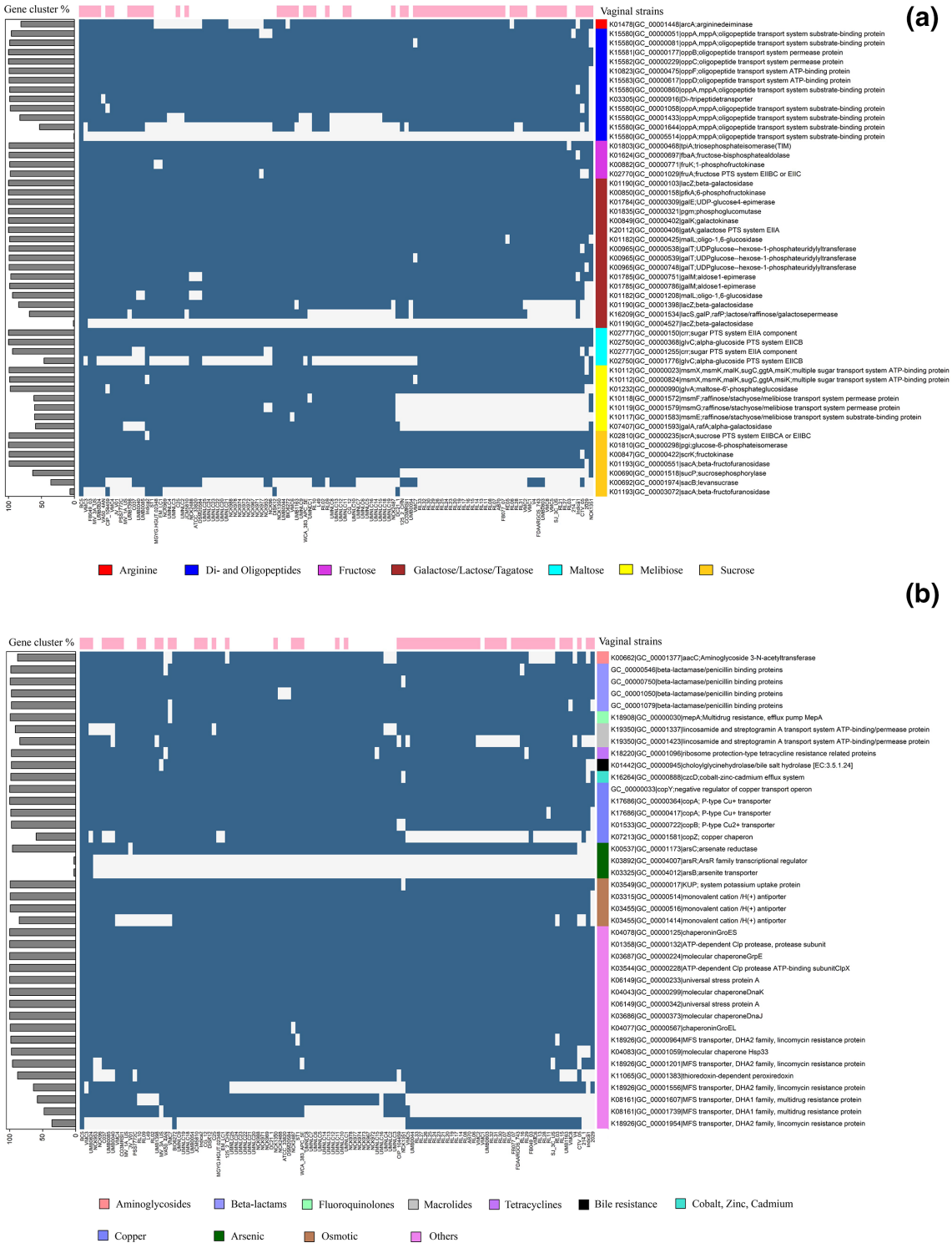

Fig. 5. Occurrence of *L. crispatus* BC5 genetic traits in *L. crispatus* pan-genome. Only genetic traits associated with the phenotypic differences between BC5 and BC12 in carbon metabolism (a) and stressors (b) are shown (these genetic traits correspond to those shown in Table 2). *Lactobacillus* strains isolated from the vaginal niche are shown in pink. Species-level conservation (in percentage) of each genetic trait is reported on the left of the heatmaps (as barplot). Columns are annotated according to the isolation source of the strains while rows are ordered according to the metabolic categories (indicated in the legends).

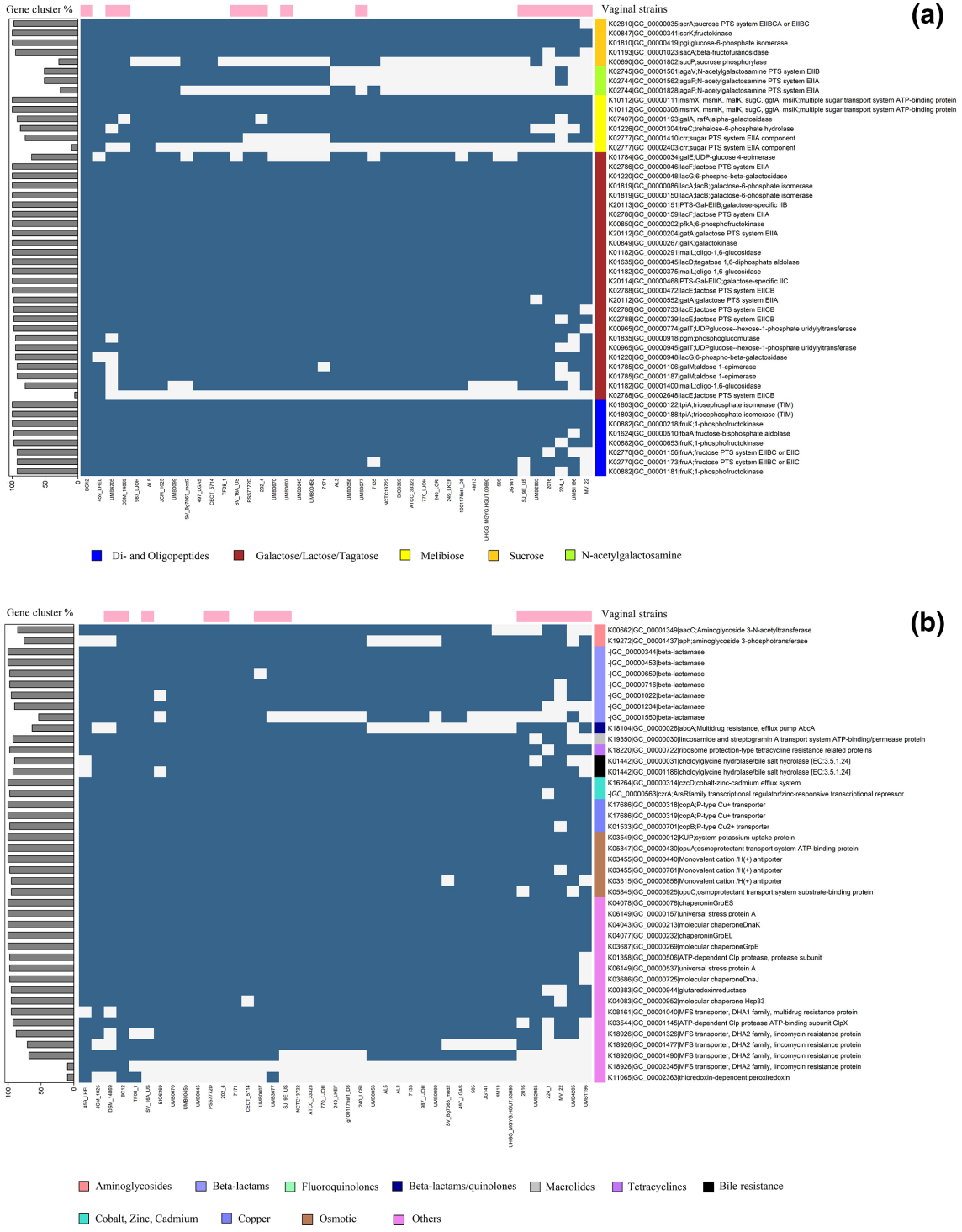

Fig. 6. Occurrence of *L. gasseri* BC12 genetic traits in *L. gasseri* pan-genome. Only genetic traits associated with the phenotypic differences between BC12 and BC5 in carbon metabolism (a) and stressors (b) are shown (these genetic traits correspond to those shown in Table 3). *Lactobacillus* strains isolated from the vaginal niche are shown in pink. Species-level conservation (in percentage) of each genetic trait is reported on the left of the heatmaps (as barplot). Columns are annotated according to the isolation source of the strains while rows are ordered according to the metabolic categories (indicated in the legends).

Most of these specific BC5 and/or BC12 genetic traits were also found in each species's core genome giving indications on the conservation of specific phenotype-associated genes among all the *L. crispatus* and *L. gasseri* strains. Among these, some of the genes of *L. crispatus* BC5 that are mainly associated with amino acid and carbohydrate metabolism/transport and pH stress tolerance were found to be significantly correlated with the vaginal source. Comparative genome analyses have been previously focused on the identification of habitat-specific traits in the vaginal lactobacilli genomes [48]. In line with our results, previous studies on some *L. crispatus* strains reported a correlation between genotypic/phenotypic features and isolation sources concerning both nutrient utilization and acid resistance [46, 47]. These authors also found some phenotypic features associated with possible niche-specific adaptation in *L. gasseri* strains (vaginal against intestinal) [47]. However, in our work, we have not detected specific genes in *L. gasseri* pan-genome that were significantly correlated with the vaginal isolation source, probably because of the limited number of genomes available for this species.

L. crispatus BC5 and *L. gasseri* BC12 metabolic activities and genetic traits associated with the vaginal colonization/persistance

One of the most important aspects of microbial colonization of any environmental niche is utilizing the available nutrients for growth [49]. Within the context of vaginal 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^-) [50–52]. In particular, glycogen and glucose represent the major carbohydrates of the vaginal fluid, while mannose, maltose and glucosamine were found in much smaller quantities [53]. PM results indicated that BC5 strain could metabolize glucose, pyruvic acid and lactic acid at a higher rate indicating a higher lactic acid fermentation efficiency, compared to BC12. BC5 also showed higher metabolic activity in the presence of mannose [54], while the two strains equally utilized acetic acid and glucosamine. None of the two strains could directly utilize glycogen; however, BC5 showed higher metabolic activity in the presence of the glycogen debranching products (i.e. maltose, maltotriose and glucose), which can be released in the vaginal tract through depolymerization activity of amylases from the host or other bacterial strains [50, 55]. The previous study indicated that glycogen could be utilized as carbon and energy source by *L. crispatus* strains. This metabolic feature was associated with the expression of an active pullulanase type I with an N-terminal signal peptide probably involved in extracellular localization of the enzyme to degrade extracellular glycogen correctly [56]. Therefore, the inability of BC5 to utilize glycogen in PM might be due to amino acid deletion present in the N-terminal sequence of its pullulanase gene (data not shown). On the other hand, 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 the *L. gasseri* core genome, including BC12 (Table 2). In particular, in the case of maltose, a maltose 6-phosphate glucosidase activity can support the capacity of only BC5 to utilize maltose. This gene is included in an operon that was previously identified in all the 67 *L. crispatus* human strains under analysis and was predicted to be associated with the import and metabolism of trehalose [46].

Both of the strains could utilize the mucin-derived compound N-acetyl-glucosamine as both nitrogen and carbon source, while only BC12 could metabolize N-acetyl-galactosamine. These compounds might arise from mucin oligosaccharide degradation by both beneficial bacteria and pathogens, being mainly studied in the gastrointestinal tract [57, 58]. Unlike BC12, BC5 strain 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 microbiota, even under bacterial vaginosis condition in which a series of biogenic amines are produced by pathogenic bacteria [59–61].

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 the host, nitrogen is primarily available as free amino acids and proteins [62]. Among the amino acids that are normally present in the vaginal tract, aspartic acid and tryptophan could be utilized by both the strains [63], while only BC12 could metabolize glutamic acid as both carbon and nitrogen source and arginine was utilized as nitrogen source only by BC5. This latter capacity was associated with the presence in the 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 [32, 64]. 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. Interestingly, these genes were present in the *L. crispatus* core genome and significantly correlated with the vaginal origin of these species strains, possibly corresponding to a metabolic advantage for vaginal niche adaptation. The ability to efficiently assimilate complex nitrogen molecules was also described in a food-related *L. plantarum* strain. In particular, dipeptides efficiently sustained the growth of this strain under nutritional stress conditions [65].

Among those carbon sources that could not be utilized by none of the two strains but are important in the vaginal tract, it is noteworthy to mention glycerol. Glycerol did not seem to be utilized by any of the two strains in spite of the presence in their genomes of genes encoding a glycerol-3-phosphate dehydrogenase, a glycerol kinase and a glycerol facilitator protein. On the other hand, glycerol could be used as osmoprotectant by both BC5 and BC12. Glycerol is usually present at low concentration in the vaginal niche [50], however, it has been described as an underappreciated carbon source due to upregulation of glycerol pathways in *L. iners* under bacterial vaginosis conditions [66]. Furthermore, both the *Lactobacillus* strains could not use urea as carbon and/or 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 colonization and persistence of a bacterial population within a specific environmental niche. In particular, during their life as mutualistic organisms in the vaginal niche, lactobacilli are expected to encounter stresses related to antibiotics treatments and detergents. Antibiotics greatly influence the human microbiota and the microbial composition of the vaginal niche is known to undergo rapid modification during and after antibiotic treatments [67–69]. The evaluation of the survival capacity of autochthonous strains during antibiotic treatment may allow to predict their persistence in the vaginal niche and their metabolic advantage during specific antibiotic therapy [41]. A recent work about the antibiotic susceptibility of 182 *Lactobacillus* strains (including one type strain for each *L. gasseri* and *L. crispatus* species) reported that resistance to trimethoprim, vancomycin and kanamycin were common phenotypes within this genus. In this regard, in our work we found that only BC12 was resistant to trimethoprim and only when added at the lowest concentration, while both BC5 and BC12 strains were equally resistant to vancomycin and kanamycin. Generally, *Lactobacillus* spp. strains were found to be susceptible to low concentrations of β-lactams [69]. The apparent contrasting results on the resistance of BC5 and BC12 towards some β-lactams (including ampicillin and penicillin) might be due to the concentrations tested in the PM assay that are typically active towards Gram-negative bacteria and probably much lower than the MICs previously described [3]. From a genetic point of view, β-lactamase encoding genes were identified in both the strains and no mutations of the genes encoding penicillin-binding proteins (PBPs) were detected that could sustain a strong resistance phenotype [70]. Additional multidrug transport systems and oxidative stress response components might be involved in non-specific and multifactorial detoxification mechanisms of antibiotics and detergents [71]. In this regard, previous studies reported that deletion of one of the five transporters [generally annotated as a multidrug resistance (MDR) protein] rendered the mutant strains of *L. acidophilus* NCFM more sensitive to detergent-like molecules (i.e. bile) and some antibiotics [45, 72]. Furthermore, the induction of general stress response, the protection against oxidative damages, as well as the global glycolytic reorganizations were described to be involved in *Lactobacillus* resistance mechanisms to detergent-like molecules [45].

Besides antibiotics and detergents, the research interest in the resistance/tolerance of vaginal *Lactobacillus* spp. strains to osmolytes and other toxic compounds such as metal(loid)s, can be associated to their possible therapeutical application as probiotics [64, 65, 73, 74]. In the PM assay, both BC12 and BC5 strains showed the capacity to resist/tolerate some of these toxic compounds and chemical stressors, despite the general higher performance of the *L. gasseri* strain. In this regard, BC12 showed higher tolerance towards salts and molecules typically present in the vaginal fluid, such as KCl, sodium lactate (that is the dissociated form of lactic acid existing at neutral pH), and urea. Both the strains showed some genetic features associated with the possible accumulation of distinct compatible solutes that were conserved at the species-level, while only *L. gasseri* strains possessed the operon encoding the osmoprotectant transport system Opu that was previously characterized for its involvement in betaine and proline uptake in *Lactococcus lactis* [75]. In relation to metal resistance/tolerance, previous works have reported the capacity of *Lactobacillus* strains to tolerate heavy metals such as cadmium and lead [76] by reducing metal toxicity. In particular, bioaccumulation and biosorption were described to be possible detoxification strategies of *Lactobacillus* that can prevent the exposure of the human tissues to heavy metals [77]. The capacity of both BC5 and BC12 strains to resist copper, zinc and cadmium can be associated to the presence of *czc* and *cop* genes, whose molecular mechanisms have been extensively studied in model lactic acid bacteria [78].

In addition to the presence of specific genes associated with detoxification mechanisms that were the focus of the present study, specific SNP of the molecular targets or other cellular components can contribute to resistance/tolerance towards different stressors [79]. This aspect can influence the strain-level phenotype prediction driven by the detection of stress-related genetic traits in the species core genome or even in the strain genome. For instance, the presence of SNP in crucial cell-response components might justify the presence of specific resistance/tolerance in *Lactobacillus* spp. strains that did not show to possess the expected genetic determinants in their genomes [80]. Additionally, defective expression of the resistance genes can hamper the correlation between the resistance phenotype and genotype. This might explain the inability of BC5 to resist arsenic despite the presence of *ars* genes in its genome.

In conclusion, the results of our study provide useful indications on those factors contributing to the predominance of BC5 and BC12 strains in a common niche. Although we are aware of the restraints of our genetic dataset (which excludes uncharacterized genes), the strain-level differences that are known within *Lactobacillus* species and the limitation of functional assessment only based on genotype analysis, we believe that the results from this study can be used as the groundwork for further functional analyses at the species level also targeting other vaginal bacterial strains. Finally, in terms of health-promoting functionality that is well known for both BC5 and BC12 strains, our findings could provide guidance to be taken into consideration during the development of probiotic formulations for enhancing the therapeutic efficacy of these beneficial bacteria.

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Author contributions
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Conflicts of interest
The authors declare that there are no conflicts of interest.

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