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Article

Variability of Genetic Characters Associated with Probiotic Functions in *Lacticaseibacillus* Species

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Abstract: This study aimed to exploring the intra-species distribution of genetic characters that favor the persistence in the gastrointestinal tract (GIT) and host interaction of bacteria belonging to the species *Lacticaseibacillus* genus. These bacterial species comprise commercial probiotics with the widest use among consumers and strains naturally occurring in GIT and in fermented food. Since little is known on the distribution of genetic traits for adhesion capacity, polysaccharide production, biofilm formation, utilization of substrates critically important for survival in GIT, that influence probiotic characteristics, a list of genetic determinants involved in such functions was created by a search for specific genes involved in the above aspects in the genome the extensively characterized probiotic *L. rhamnosus* GG. The presence/absence and variability of each gene in other *Lacticaseibacillus* spp. genomes was assessed by alignment with the publicly available fully annotated genome sequences. Eighty-two gene loci were compared, and 49 of these were found to be absent in some genomes in a species or strain-specific mode. A set of genes was found to be conserved, indicating that all strains of the genus may exert some probiotic effects. Among the variable loci a taurine utilization operon and a α-L-fucosidase were examined for presence/absence in 26 strains isolated from infant feces by PCR based tests. Results were variable among the isolates, though their common origin indicated the capacity to survive in the intestinal niche. This study indicated that the capacity to exert probiotic actions of *Lacticaseibacillus* spp. depends on a conserved set of genes and is enhanced by variable genetic factors whose role is only in part elucidated. The selection of strains of the most promising probiotic candidates to be used in food is feasible by analyzing presence/absence of a set of variable traits.

Keywords: *Lacticaseibacillus* species; probiotic potential; genetic traits; presence in genomes

1. Introduction

Bacteria of the *Lacticaseibacillus* genus, in particular the species *Lacticaseibacillus casei*, *paracasei*, *rhamnosus* and *zeae*, comprise strains able to colonize the human gastrointestinal tract (GIT) and exert probiotic effects. These species are also adapted to fermented food environments, primarily dairy products, but also fermented foods of plant origin. Many studies have dealt with the demonstration of the beneficial effects of these bacteria and some of them exert probiotic effects being able to ameliorate or prevent different medical conditions [1–3]. Among these *L. rhamnosus* GG (ATCC 53103) is the most extensively studied [4]. These bacteria are commercially available as food supplements or in probiotic food products, but they also constitute the spontaneous microbiota of fermented foods of large consumption, including traditional products. In particular, *L. paracasei* strains constitute one of the main microbial components in traditional cheeses during and at the end of ripening. These foods can be a source of *Lacticaseibacillus* strains in numbers sufficient to influence host health [5]. However, strains that are part of the natural dairy microbiota are not characterized for their capacity to exert beneficial effects, so that this aspect can results highly variable and uncontrolled. The selection, among the

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autochthonous microorganisms, of *Lacticaseibacillus* bacteria with traits associated to probiotic functions and use of these as added cultures could confer health promoting properties to the dairy products also preventing the development of adventitious bacteria with undesirable characteristics.

Therefore, this study was focused on identifying genetic traits that, if present, can increase the potential of a bacterial strain belonging to the *Lacticaseibacillus* genus to behave as probiotics. To this aim, the genome of the most studied *Lacticaseibacillus* probiotic, *L. rhamnosus* GG, was used as a reference for identifying gene loci encoding for characteristics involved in survival in GIT and colonization capacity, adhesion to mucus or other host molecules, production of cell surface associated macromolecules, including exopolysaccharides (EPS) involved in adhesion and immune modulation [6]. All the gene loci to which any of the above functions could be assigned, based on the existing annotation or on protein databases and scientific literature consultation, were searched by Blastn in the other *Lacticaseibacillus* spp. completely annotated genomes. The variability in presence/absence of each gene, or gene cluster where appropriate, is presented. In addition, the presence/absence analysis of variable loci encoding taurine uptake and fucose utilization, as well as physiological features such as tolerance to bile salts and biofilm formation were determined for 26 fecal *Lacticaseibacillus* isolates. These properties were selected on the basis of previous evidences on their involvement in survival in the intestinal environment. Indeed, taurine utilization capacity conferred by the *tau*BAC gene cluster was suggested to increase bile tolerance and persistence in GIT [7], while utilization of L-fucose, one of the most common monosaccharides in glycans on mammalian cell surfaces and intestinal mucus, allowed by the presence of α-L-fucosidases, is advantageous for use of this sugar as carbon source in GIT [8].

2. Materials and Methods

2.1 *Analysis of* Lacticaseibacillus spp. *genomes for genes involved in survival in GIT and adhesion*

The whole genome sequence of *L. rhamnosus* GG (GenBank acc. n. FM179322.1) was examined visually for the presence of genes involved in survival in GIT, polysaccharide production and adhesion on the basis of the predicted function indicated for each gene locus. Membrane and cell surface associated proteins without an assigned function were analysed by Blastp (https://blast.ncbi.nlm.nih.gov/), Interpro (https://ebi.ac.uk/interpro/) and UniProt (https://www.uniprot.org) search in order to derive a putative functional role. Only genes encoding proteins with a function assigned on the basis of the above analyses, or experimentally proven on the basis of scientific literature, were retained for analysis of the presence of homologs of the encoding genes in the available complete and fully annotated genomes of *L. casei*, *paracasei*, *rhamnosus* and *zeae* by Blastn. The latter species was considered for its close relatedness with *L. casei* [9]. The cut off values fixed for the definition of homology were at least 30% query coverage and sequence identity above 60%.

Polysaccharide production gene clusters were graphically represented by using the https://katlabs.cc/genegraphics/app.

2.2 *Bacteria strains and culture conditions*

Bacterial strains examined were isolated from children feces in a previous study [10] and assigned to the species *L. casei*, *L. paracasei*, *L. rhamnosus* and *L. zeae* according to the highest Blastn scores of their 16S rRNA gene sequences. *L. rhamnosus* GG ATCC 53103 was used as positive control in PCR amplification tests for genes *tau*B and the α-L-fucosidase gene LGG_02652. Lactobacilli were subcultured in MRS broth or agar (Biolife Italiana, Milan, Italy) at 37°C in aerobiosis for 48 h.

2.3 *Molecular techniques*

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DNA was extracted from 1 ml of fresh bacterial culture according to Amadoro et al. (2018). PCR tests for the screening of relevant genetic traits were carried out with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3')/1492r (5'- GGTTACCTTGTTACGACTT-3') targeted on the 16S rRNA gene, TauBF (5'-AGGSTCKGCATAGGC-3')/TauBR (5'-CATGTRGMYTAYTGTTAC-3'), targeted on the taurine uptake gene tauB, locus LGG_00172 and FucF (5'-KAACSACCCAGTCACT-3')/FucR (5'- GWCAGAACCAY-TACCG) targeted on the α-fucosidase gene, locus LGG_02652. The latter two degenerated primer pairs were designed on consensus nucleotide positions in the genes tauB and LGG_02652 after alignment of the homologous gene sequences by Clustal Ω (https://www.ebi.ac.uk/Tools/msa/clustalo/) for L. paracasei and L. rhamnosus and all the Lacticaseibacillus species, respectively. Primer specificity was checked by Blastn and their melting temperature and tendency to form dimers were optimized by the Eurofins Genomics (Ebersberg, Germany) Oligo Calculator (https://www.google.com/search?client=firefox-b-d&q=eurofins+oligo+calculator).

The expected length of the amplification product was 649 for L. paracasei and 665 bp for L. rhamnosus for TauBF/TauBR primers and 1220 bp for FucF/FucR primers. In the PCR reactions primers were used in 0.5 μM final concentration carried out with the Takara Bio EmeraldAmp GT PCR Master Mix (Diatech, Jesi, AN, Italy). PCR programs comprised an initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and elongation at 72 °C for 1 min. The annealing temperatures were 55°C for primer pair 27f/1492r and 50°C for the other primer pairs.

Sequencing of the amplification products was carried out after purification with the Wizard® SV Gel and PCR Clean-Up System (Promega Italia Srl, Milan, Italy) at Eurofins Genomics and the same primers used for amplification were used as sequencing primers.

2.4 *Biofilm forming capacity*

Two hundred μl of a 48 h culture in MRS broth were transferred in triplicate in a microtiter plate well and incubated at 37°C for 24 h. After the incubation the well content was aspirated and the well was washed trice with sterile saline. The well was filled with 200 μl of 99% methanol and kept for 5 min at room temperature. Methanol was aspirated and the well was let dry, before adding 200 ul of a 2% (w/v) aqueous solution of crystal violet (Merck Life Science S.r.l., Milan, Italy) and leaving in contact for 5 min. The colorant solution was removed and wells were let dry and washed several times with water. Finally, 160 μl of 33% (w/v) acetic acid were added and the optical density (OD) of the wells was read at 570 nm in a 1420 multilabel counter Victor 3 v plate reader (PerkinElmer Italia, Milan, Italy).

2.5 *Bile salt tolerance test*

Bile tolerance was assayed by determination of the transmembrane electrical potential ($\Delta\psi$) dissipation energizing cells as described by Taranto et al. (2006), then adding bile salts to 1.5% (w/v) final concentration. Safranin O (λ excitation 520 nm, λ emission 570 nm) 1.25 μM was used to determine changes in fluorescence with a LS50B spectrofluorimeter (PerkinElmer) and cells were completely depolarized by addition of 1 μM of the protonophore carbonyl cyanide-p-trifluoromethoxy-phenyl hydrazine (FCCP), as described by Pallotta et al., 2004 [11]. The experiments were carried out in triplicate.

3. Results

3.1. Distribution of genetic traits required for probiotic activity in Lacticaseibacillus *species*

The selection of genetic traits to be compared among strains in the genome of *L. rhamnosus* GG, after identification of some proteins with unassigned function by search in protein databases, resulted in the list reported in Table S1. Each gene locus in the list was aligned by Blastn to the completely annotated genomes of the species *Lacticaseibacillus casei*, *paracasei*, *rhamnosus* and *zeae* which are currently represented by 5, 53, 38 and 2 entries,

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respectively, in NCBI. The strain labelled as *L. casei* 12A was excluded from the analyses since it gave, for all analyzed loci, percentages of nucleotide identity and query coverage values dissimilar from the other *L. casei* strains and similar to the values obtained for *L. paracasei*, so that it is possibly misidentified.

Traits that are present in all genomes include the bile salt hydrolase (*bsh*) gene and indicates the common ability to detoxify bile salts conferred by this enzyme in *Lacticaseibacillus* species [12]. An exception is *L. paracasei* ATCC 334, in which a truncated copy of the gene is present. Surface antigens p40, p75, p60, MetQ and LGG_00503 are also conserved. Among these, p40 and p75 have a role in protection of inflammation and integrity of the intestinal epithelium [13] and in *L. paracasei* BL23 have cell-wall hydrolase activity and are secreted in microvesicles [14,15]. Antigen p60 has an immunomodulatory function [16], the myosin-cross-reactive antigen encoded by locus LGG_00503 may be also involved in the production of conjugated linoleic acid [17]. Other conserved genes are eleven glycosyltransferases for the biosynthesis of polysaccharides or lipopolysaccharides, a flippase-like protein LGG_00827, a lipotheicoic acid (LTA) synthase LGG_00830, a polysaccharide biosynthesis transport protein LGG_00851, the LiaX daptomycin-sensing surface protein LGG_00914, a PspC domain-containing protein that in *S. mutans* mediate biofilm formation *in vivo* [18], a toxin immunity protein LGG_01002, a lipopolysaccharide assembly protein LGG_01366, a FbpA, fibronectin binding protein, a AI-2E family transporter possibly involved in biofilm formation, a mucus binding protein MucBP LGG_01883, the first proteins of two gene clusters for polysaccharide production LGG_01990 and LGG_02036, a teichoic acid glycosylation protein LGG_02144, two PsaA putative adhesion lipoproteins and a polysaccharide transport protein LGG_02520.

The variable genes follow a species-specific or a strain-specific distribution. Namely, some strains of *L. casei* do not have a FeoB for Fe(II) uptake encoding gene, the *L. paracasei* species lacks a β-N-acetylhexosaminidase, that is variable in *L. rhamnosus* strains, the TauE protein involved in taurine metabolism, the lectin-like protein LGG_00579, the cell surface protein LGG_00584, the MabA extracellular matrix binding protein, a modulator of adhesion and biofilm formation [19], the cell envelope-associated proteinase LGG_02734, lactocepin PrtR, able to selectively degrade pro-inflammatory chemokines and reduce inflammation in experimental IBD models [20] and the fibrinogen binding protein LGG_02282. Some *L. paracasei* strains lack the InlJ internalin LGG_02337. *L. casei* and *L. zeae* lack genes encoding the SpaCBA and SpaFED pili, a pilin subunit LGG_00422, the adhesion exoprotein LGG_02923 and five proteins containing the WxL domain [21]. These proteins are involved in single species biofilm formation and for some a lectin function was proven [22]. Other proteins not encoded in the genomes of *L. casei* and *L. zeae* are the extracellular complex proteins SpcA and B, the adhesin LGG_01590, one glycosyltransferase and the cell surface protein LGG_00578. The cell surface docked proteins encoded by the gene cluster LGG_01589 to LGG_01592 might be involved in adhesion, are highly conserved among S-layer-forming lactobacilli, are expressed constitutively and are specific to vertebrate-adapted species suggesting a role in adaptation to these hosts [23]. Worth of note is that the SpaCBA pilus has a variable presence in *L. rhamnosus*, while it was found to be present in all analysed *L. paracasei* strains with six of them having an additional plasmid encoded copy. In *L. paracasei* an additional pilin subunit D1, SpaA (LGG_00422), present in all genomes, is also found on plasmid in six strains. The remaining genes with an intra-species varying distribution are the SpaFED pili, the taurine metabolism system and proteins associated with EPS production. The role of the SpaFED pilus is not well defined and it was reported not to be expressed in *L. rhamnosus* GG [24,25]. The EPS production genes in *L. rhamnosus* GG are arranged in three gene clusters, namely loci from LGG_00278 to LGG_00283, loci from LGG_01990 to LGG_02005 and loci from LGG_02036 to LGG_02054. The first cluster comprises genes present only in some *L. paracasei* and *L. rhamnosus* strains. These are three ramnosyltransferases, a polysaccharide transporter Eps1 and an Eps2 protein involved in polysaccharide biosynthesis that in some strains are dislocated in different EPS gene clusters. Given the high variability in EPS production gene arrangement, the remaining two clusters are shown graphically in

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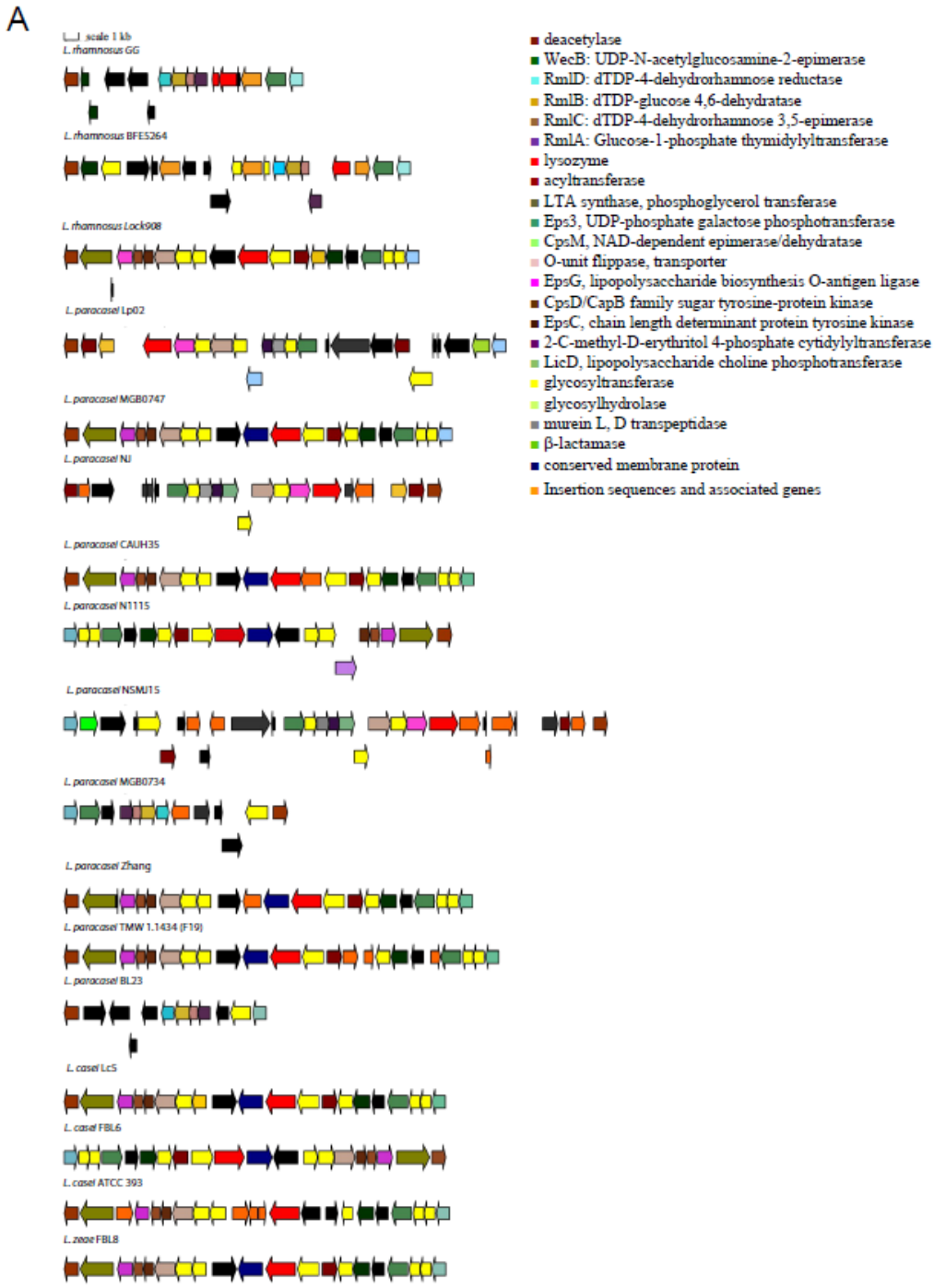
Figure 1 for strains representative of the different gene organization and identity observed.



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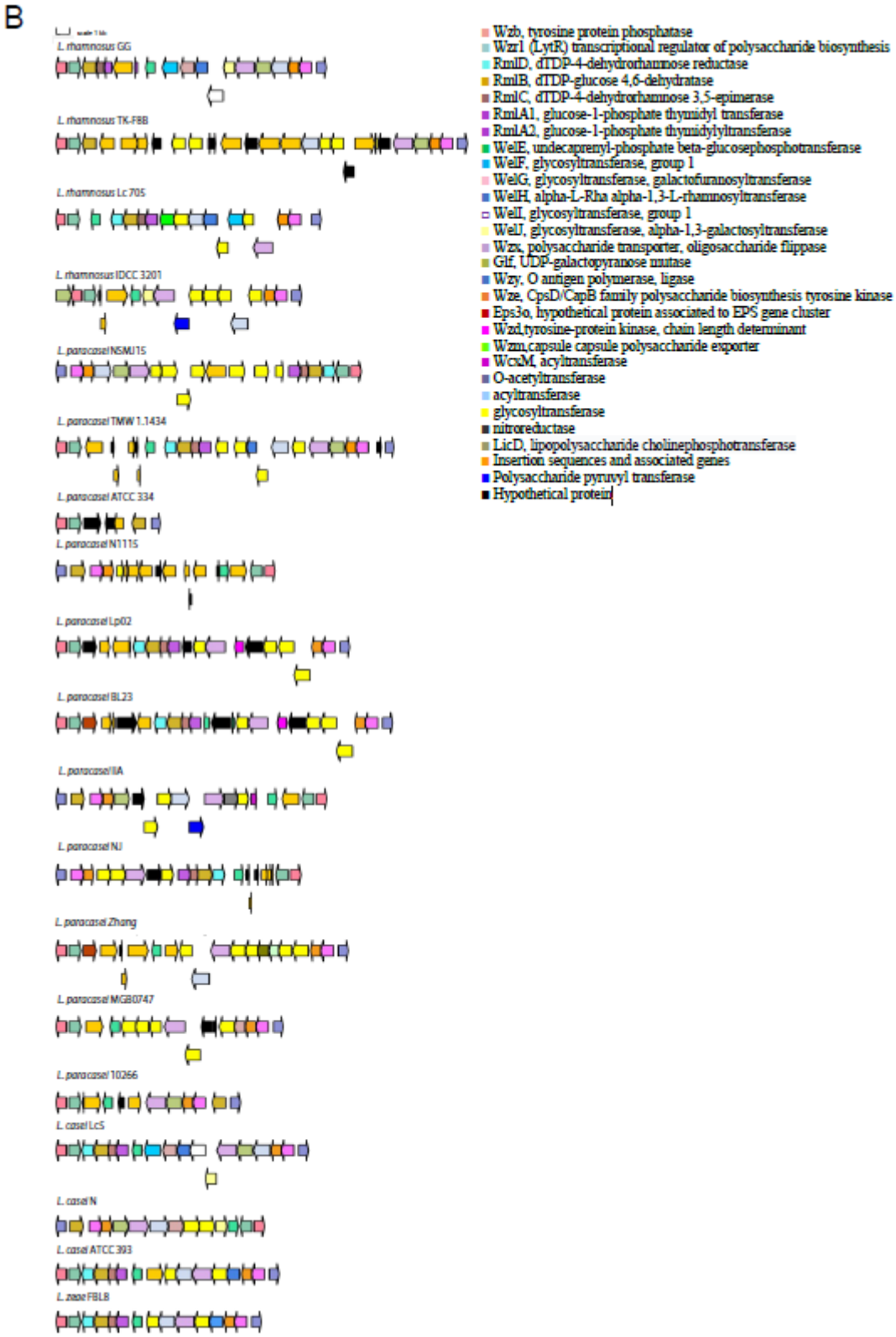

Figure 1. Gene arrangement in the EPS production gene clusters starting from proteins orthologous to LGG_01990 (A) and LGG_02036 (B).his is a figure.

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3.2. Testing of genetic and physiological features in Lacticaseibacillus *isolates from feces*

As relevant features for adaptation to the intestinal environment, taurine and fucose utilization capacity were tested in 26 isolates identified as *Lacticaseibacillus* species by 16S rRNA gene sequences among lactobacilli from children feces obtained in a previous study [10]. Moreover, bile salt resistance was determined by a fluorimetric method. Examples of traces showing loss in fluorescence intensity of cells in presence of bile salts are shown in Figure 2.

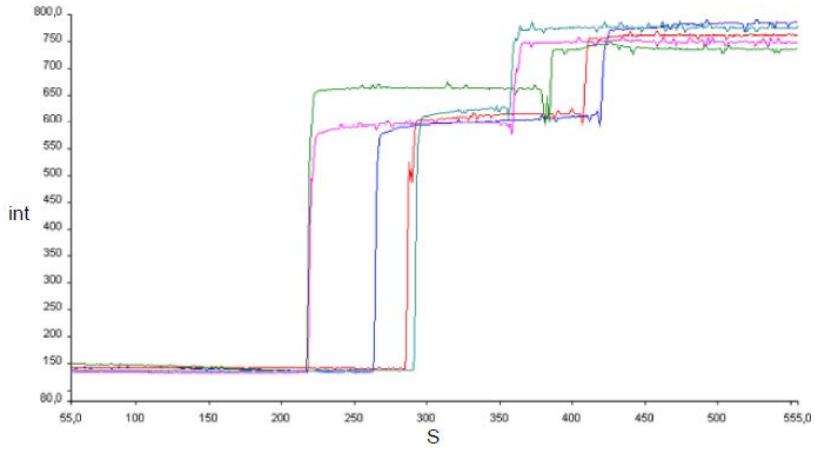

Figure 2. Fluorescence intensity variation for energized *Lacticaseibacillus* cells after addition of 1.5 % (w/v) bile salts.

Results for the tested properties are shown in Table 1. *L. rhamnosus* GG was used as a reference strain for the assays. The identity of the amplification products was confirmed by sequencing of those obtained from *L. rhamnosus* GG.

It is possible to observe that taurine utilization and fucosidase activity presence are neither shared by all the intestinal isolates nor present in most of them. Tolerance to bile salts was comparable among isolates, except for *L. zeae* J-7-2 that showed a more extensive membrane depolarization. Biofilm forming capacity was particularly strong for three strains, while the remaining isolates showed a biofilm forming capacity comparable to that of *L. rhamnosus* GG (n. 8) or lower (n. 15).

Table 1. Distribution of variable genes involved in survival in GIT in intestinal isolates of *L. casei*, *paracasei*, *rhamnosus* and *zeae*, percent dissipation of membrane potential in presence of 1.5% bile salts and biofilm formation extent.

Isolate/ Strain	**Taurine utilization**	**α-L-fucosidase LGG_02652**	**% membrane potential dissipation**	**Biofilm formation (OD 620 nm)**
L. casei				
AB-15-6	+		77±0.5	0.028±0.03
C-15-1	+		75±0.5	0.034±0.01
C-15-1b	+	+	83.5±0.5	0.160±0.05
G-0-6	+		86±1	0.065±0.09
L. paracasei				
AN-15-1			87±0.5	0.436±0.05
AN-15-2	+		77±1	0.122±0.05
AN-15-3	+		80±0.5	0.051±0.02

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AN-15-4			87±0.5	0.068±0.01
J-7-1	+		83±1	0.115±0.03
J-7-3			79±0.5	0.534±0.09
J-15-4	+		75±1	0.034±0.07
P-7-13 ¤	+	+	77±0.5	0.133±0.02
6-15-1			71.5±0.5	0.043±0.04
L. rhamnosus				
AN-0-1			83±0.5	0.171±0.04
AN-7-1			76±1	0.111±0.05
AN-7-4	+		87±0.5	0.119±0.03
AN-21-1	+		82.5±0.5	0.032±0.02
AN-21-2		+	81±1	0.076±0.02
C-0-4			75±1	0.025±0.06
D-0-5	+	+	77±0.5	0.048±0.02
G-7-14	+		80±0.5	0.270±0.05
G-7-16	+		83±0.5	0.180±0.02
J-7-4	+		82±0.5	0.023±0.04
SA-7-6			83±0.5	0.613±0.07
Z-15-4	+	+	73±0.5	0.024±0.05
L. zeae				
J-7-2			95±1	0.084±0.02
L. rhamnosus GG	+	+	79±0.5	0.138±0.03

4. Discussion

This study highlighted that genetic characteristics that influence survival and persistence in GIT and probiotic effects exerted by bacteria of the *Lacticaseibacillus* genus are very complex. Genetic loci common to all the analyzed genomes encode for functions such as adhesion, bile resistance, polysaccharide production, fibronectin binding, cell-cell signalling and represent a constant endowment of genes that allow coping with the GIT environment to all bacteria of the species considered. An exception among the analysed genomes is represented by the dairy strain *L. paracasei* ATCC 334 (Acc. N. NC_008526.1), in which a non functional *bsh* gene is present and one of the main EPS production gene clusters is almost completely deleted (Figure 1B).

Among the common traits, the *bsh* gene product has particular relevance for probiotic function, since it has been shown to efficiently lower total and low-density lipoprotein cholesterol [26].

Another common trait that favors host colonization is the presence of the fibronectin-binding protein (FnBP) FbpA. This protein type binds fibronectin, a multidomain glycoprotein found in human body fluids, extracellular matrices and intestinal epithelial cells, that are a common target for bacterial adhesins in GIT. The FbpA from *L. paracasei* BL23 has been characterized and found to exhibit a strong affinity for immobilized fibronectin [27]. In *L. acidophilus*, a mutant with inactivated *fbp*A exhibited a significant decrease in adhesion to epithelial cells *in vitro*. While in pathogens, some FnBPs contribute to virulence, FnBPs in commensal and probiotic strains these proteins are essential for persistence in their ecological niches and might exert competition against pathogens for binding to fibronectin [28].

In most of the genomes, except for two *L. casei* strains, a FeoB protein, essential for the uptake of ferrous iron and gut colonization is encoded [29].

Proteins p75 and p40 present in all *Lacticaseibacillus* genomes, were found to mitigate intestinal inflammation through activation of the epidermal growth factor receptor [30],

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and upregulation of a proliferation-inducing ligand in the epithelium that stimulates the secretion of immunoglobulin A and relieves cytokine-induced apoptosis in the intestinal epithelial cells [31]. In addition, p75 and p40 stimulate epithelial cells to activate pathways that enhance their survival and barrier function to prevent bacterial translocation and the invasion by toxins [32].

The majority of the genetic determinants with a role in adaptation in GIT considered in this study resulted to be variable in the *Lacticaseibacillus* genomes. Among these, the sortase dependent pili SpaCBA and SpaFEG, with the first having a proven role in adhesion to mucus, collagen, biofilm formation and immune cell response stimulation, are included [33]. Similarly to what observed in this study, Douillard et al. [7] found that all the *L. paracasei* strains that they examined contained a SpaCBA pilus cluster. However, only the *L. rhamnosus* strains produced a functional SpaCBA, pilus since the insertion of an IS 30 element upstream of the pilus gene cluster had constituted a strong promoter that allowed pilus expression. On the other hand, they observed that some strains displayed mucus-binding capacity also in absence of SpaCBA pili, suggesting the existence of alternative mucus binding mechanisms.

The relevance of the SpaCBA pilus in *L. paracasei* physiology should be better elucidated. This is present in two copies in some *L. paracasei* strains, among which strain LP10266 (Acc. n. NZ_CP031785.1 and n. NZ_CP031786.1) was recently shown to exhibit increased adhesion capacity. This strain was isolated from a patient with endocarditis and it was hypothesized that increased adherence can represent a virulence trait [34]. High adherence determined by a plasmid encoded SpaCBA pilus was reported also for a *L. paracasei* strain isolated from raw cow's milk [35].

The SpaCBA pilus resulted absent in *L. casei* and *L. zeae* genomes analyzed, but this can be attributed to their low number. This might explain why the *tau*B gene, absent in the five *L. casei* genomes analysed, was instead detected in the fecal isolates.

However, in general, the strains of these two species analysed in this study are defective of many important adaptive traits. This might explain their infrequent occurrence in different ecological niches compared to *L. paracasei* and *L. rhamnosus*.

The taurine uptake system is another variable trait, that, based on the genome analysis of 19 *L. casei*/*paracasei* strains, was proposed to be lost by strains adapted to dairy niches [36]. However, in this study, it appeared to be absent also in strains of intestinal origin, indicating that it may be not essential, at least in the short term, for survival in GIT.

The α-L-fucosidase found to be absent in some genomes in this study and corresponding to the experimentally characterized LCABL_28270 in *L. paracasei* BL23 (GenBank acc. n. FM177140) (Rodríguez-Díaz et al., 2011) was also proposed to be lost by dairy strains [7]. However, in this study, it appeared to be present only in a minority of fecal isolates.

A great variability was displayed by the EPS production gene clusters. This observation explains the diverse EPS types produced by *Lacticaseibacillus* strains and experimentally characterized [37,38]. Given the multiple actions exerted by these macromolecules, such as immune modulation, antioxidant properties [37], enhancement of the hydrophobicity of bacterial cell surface which increases binding to the intestinal mucosa, biofilm formation, variability in composition of these macromolecules can create a variability of the effects that these bacteria can exert and deserve to be defined to the strain level.

MucBP proteins were found to be present in most genomes. One of these, namely the LGG_02337 is an adhesin distributed on the whole cell surface, participates to adhesion of *L. rhamnosus* GG to mucus, and was recognized to be involved in pilus-mediated mucosal adhesion [39].

Finally, proteins with a WxL C-terminal domain, shown to possibly form a cell-surface protein complex involved in the degradation of plant polysaccharides in other lactobacilli, have a lectin-like function [40]. One protein of this group was found to be responsible of adherence of *L. rhamnosus* GR-1 to the vaginal epitelium [41].

5. Conclusions

This study highlighted that all the *Lacticaseibacillus* spp. genomes analyzed comprise a common set of genes that could favour probiotic functions to be exerted by most of the microorganissms belonging to this genus. However, another set of gene loci is variable and can confer increased colonization capacity and beneficial host interaction activities to some strains. The presence of genetic traits relevant for survival in GIT, namely taurine and fucose utilization, as well as bile salt tolerance and biofilm formation, were found to be variable in faecal isolates, showing that a complex of features, not single traits, play a role in adaptation to the intestinal niche. Numerous traits emerged whose functional role is still little explored so far. This study resulted in the identification of variable genetic elements to be collectively analyzed in the preliminary the selection of *Lacticaseibacillus* natural strain to be used to increase the beneficial properties of fermented products. This preliminary analysis based on variable genetic traits must be followed by whole genome sequencing for the selected probiotic candidates, in accordance with the current European food safety guidelines [42]. his section is not mandatory but can be added to the manuscript if the discussion is unusually long or complex.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1.

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Conflicts of Interest: The authors declare no conflict of interest.

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