Comparative Genomic and Functional Analysis of *Lactobacillus casei* and *Lactobacillus rhamnosus* Strains Marketed as Probiotics

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Four *Lactobacillus* strains were isolated from marketed probiotic products, including *L. rhamnosus* strains from Vifit (Friesland Campina) and Idoform (Ferrosan) and *L. casei* strains from Actimel (Danone) and Yakult (Yakult Honsa Co.). Their genomes and phenotypes were characterized and compared in detail with *L. casei* strain BL23 and *L. rhamnosus* strain GG. Phenotypic analysis of the new isolates indicated differences in carbohydrate utilization between *L. casei* and *L. rhamnosus* strains, which could be linked to their genotypes. The two isolated *L. rhamnosus* strains had genomes that were virtually identical to that of *L. rhamnosus* GG, testifying to their genomic stability and integrity in food products. The *L. casei* strains showed much greater genomic heterogeneity. Remarkably, all strains contained an intact *spaCBA* pilus gene cluster. However, only the *L. rhamnosus* strains produced mucus-binding SpaCBA pili under the conditions tested. Transcription initiation mapping demonstrated that the insertion of an *iso*-IS*30* element upstream of the pilus gene cluster in *L. rhamnosus* strains but absent in *L. casei* strains had constituted a functional promoter driving pilus gene expression. All *L. rhamnosus* strains triggered an NF-κB response via Toll-like receptor 2 (TLR2) in a reporter cell line, whereas the *L. casei* strains did not or did so to a much lesser extent. This study demonstrates that the two *L. rhamnosus* strains isolated from probiotic products are virtually identical to *L. rhamnosus* GG and further highlights the differences between these and *L. casei* strains widely marketed as probiotics, in terms of genome content, mucus-binding and metabolic capacities, and host signaling capabilities.

L actic acid bacteria (LAB) are a phylogenetically related group of Gram-positive bacteria sharing as a common metabolic property the production of lactic acid as the main end product of carbohydrate utilization (1). Many LAB are traditionally used as culture starters in industrial dairy fermentations of raw materials, such as milk, vegetables, and meat. However, in recent years, specific LAB strains have been associated with health benefits and are marketed as probiotics in a highly successful way, reaching market volumes of over $100 billion (2, 3). Most of these marketed strains belong to the genus *Lactobacillus*, which represents the largest group of LAB, encompassing more than 100 cultivable bacterial species (4). They are found in a large variety of food-related habitats and are naturally associated with mucosal surfaces such as the oral cavity, vagina, and gastrointestinal (GI) tract.

Currently, strains belonging to the species *L. acidophilus*, *L. plantarum*, *L. johnsonii*, *L. reuteri*, *L. paracasei*, *L. casei*, and *L. rhamnosus* play a predominant role in the probiotics market, where they are known under proprietary brand names (5). Many of these LAB strains marketed as probiotics were selected according to their *in vitro* abilities to endure to the harsh physical-chemical environment of the human GI tract, i.e., low pH and high concentration of bile salts, and also for their remarkable adhesive properties on human mucus and antipathogenic activity (6). To demonstrate their health-promoting abilities, a number of *Lactobacillus* strains, including *L. rhamnosus* GG, have been successfully used in human interventions with subjects suffering from GI disorders and atopic dermatitis (7, 8). Comparative studies have shown that probiotic features and their associated health properties are strain specific and cannot be generalized, indicating that it

is essential to characterize the *Lactobacillus* strains at the genome level, as has been done for a limited number of paradigm probiotics (2). This has promoted rapid insights into the diversity, evolution, and molecular basis underlying the health benefits of these strains, resulting in a research area that has been termed probiogenomics (9). One of the most studied and widely marketed probiotic strain is the human isolate *L. rhamnosus* GG (commercialized under the name LGG). We have recently characterized this and another *L. rhamnosus* strain, LC705, at the genomic and phenotypic levels (10). This analysis identified candidate genes contributing to its adaptability in the intestinal tract, and the construction of dedicated knockout mutants contributed to establishing detailed gene-function relationships. Thus, specific surface macromolecules and their role in gastrointestinal fitness of *Lactobacillus* GG have been characterized. For instance, the long galactose-rich exopolysaccharide (EPS) molecules form a protective shield against antimicrobial peptides secreted by intestinal epithelial cells, promoting the survival of *L. rhamnosus* GG in the

TABLE 1 Strains used in this study

Strain	Functional product[a]	Product category	Source or reference
L. rhamnosus GG (ATCC 53103)	Gefilus product family	Buttermilks, yogurts, milk, fruit drinks, dairy drinks, and fermented whey-based drinks	Valio Ltd. culture collection
L. rhamnosus LrV	Vifit product family	Yogurts and drinkable yogurts	Friesland Campina
L. rhamnosus LrI	Idoform	Tablets	Ferrosan
L. casei BL23 (ATCC 393)	NA	Dairy product	35
L. casei LcY	Yakult	Fermented milk drink	Yakult Honsa Co.
L. casei LcA	Actimel	Fermented milk	Danone

[a] NA, not available.

intestinal tract (11). In addition, *L. rhamnosus* produces two secreted proteins, p75 and p40, reported to signal to the mitogen-activated protein kinase (MAPK) pathway in intestinal cells (12), which were found recently to be the glycosylated D-glutamyl-L-lysyl endopeptidase MspI and an essential cell wall hydrolase, Msp2, respectively (13, 14). Moreover, several surface proteins have been investigated because they mediate the interaction with human host, including the mucus-binding factor (MBF) (15) and the highly repeated protein MabA, which appears to contribute to biofilm formation (16). However, a major driver of adhesion to intestinal mucosa and biofilm formation is the mucus-binding pili of *L. rhamnosus* GG encoded by the *spaCBA-strC* gene cluster (10, 17, 18). These pili are protruding protein fibers consisting of multimers of SpaA, decorated by the mucus-binding protein SpaC and covalently linked to the peptidoglycan by the product of *spaB* (17). Comparative genome analysis has shown that *L. rhamnosus* and *L. casei* genomes are highly related (4). This is illustrated by the observation that not only *L. rhamnosus* but also *L. casei* strains produce highly identical MspI (p75) and Msp2 (p40) proteins that have similar functions in both species (19, 20). However, in spite of the fact that strains of *L. casei* are widely marketed as probiotics (5), the genomes of many commercial *L. casei* strains have yet to be reported. Currently, the complete genome sequences of *L. casei* ATCC 334 (21), *L. casei* BL23 (22), *L. casei* Zhang (23), *L. casei* LCW2 (24), *L. casei* W56 (25), and *L. casei* BD-II (26) are available, and some have been subjected to detailed comparative genome analysis (27, 28). However, only a very limited number of functional studies have been reported. The best-characterized strain is *L. casei* BL23, which was used in studies that indicated anti-inflammatory properties in an animal model of intestinal inflammation (29) and the capacity to bind extracellular matrix proteins (fibronectin and collagen), ascribed to the FbpA surface and other proteins that are also partly conserved in *L. rhamnosus* GG (30). Other documented properties of *L. casei* relate to its resistance to the stresses encountered during gastrointestinal passage mainly due to acid and bile tolerance (31–34).

The aim of the present study is to provide a comparative analysis of widely marketed probiotic *Lactobacillus* strains belonging to the species *L. casei* and *L. rhamnosus*. Hence, *L. rhamnosus* strains were isolated from the commercial products Vifit and Idoform, while *L. casei* strains were isolated from products branded as Yakult and Actimel. These strains were characterized at the genotypic and phenotypic levels for their carbohydrate metabolism and adhesive and immunomodulatory properties. The validity of this approach was confirmed by the high level of identity to the reported *L. rhamnosus* GG genome of the *L. rhamnosus* isolates from commercial products, testifying to the product stability of this widely used probiotic strain.

MATERIALS AND METHODS

Isolation of strains, growth conditions, and DNA extraction. The bacterial strains used in this study are listed in Table 1. *L. rhamnosus* GG (ATCC 53103) was obtained from the Valio culture collection (Valio Ltd., Helsinki, Finland), and *L. casei* BL23 (ATCC 393) that was cured of its lactose plasmid pLZ15 was kindly provided by the Institute of Agrochemistry and Food Technology (Valencia, Spain). *L. rhamnosus* strains were isolated as the dominant population from food and pharmaceutical products commercialized as carrying *L. rhamnosus* GG under the brand names Vifit (Friesland Campina, Netherlands) and Idoform (Ferrosan, Denmark), resulting in *L. rhamnosus* strains LrV and LrI, respectively. *L. casei* strains were derived from the food drinks branded as Yakult (Yakult Honsha Co., Japan) and Actimel (Danone, France) and were termed *L. casei* strains LcY and LcA, respectively. The isolation of strains LrV, LcY, and LcA was carried out by homogenizing 1 ml of product in 9 ml of sterile phosphate-buffered saline (PBS), while one Idoform tablet was dissolved in 10 ml of sterile PBS to isolate LrI. Isolation was carried out by generating single colonies via serial dilution and plating onto MRS broth (Difco BD, NJ) solidified with 1% (wt/vol) agar incubated anaerobically at 37°C for 48 h. Colonies of each product were selected, inoculated into MRS broth, and propagated overnight anaerobically at 37°C. An aliquot from each bacterial culture was used for chromosomal DNA extraction using a Wizard Genomic DNA purification kit (Promega, WI) according to the manufacturer's instructions.

Molecular typing. The identification of bacterial isolates to the species level was performed by amplification of the *tuf* gene, as described previously (36). Briefly, the *tuf* gene was amplified by PCR using a solution containing 10 mM Tris-HCl; 50 mM KCl; 1.5 mM MgCl$_2$; 200 μM each deoxynucleoside triphosphates (dNTPs) (Finnzymes, Finland); 10 pmol of primers PAR (5′-GACGGTTAAGATTGGTGAC-3′), CAS (5′-ACTG AAGGCGACAAGGA-3′), and RHA (5′-GCGTCAGGTTGGTGTTG-3′); 50 pmol of primer CPR (5′-CAANTGGATNGAACCTGGCTTT-3′); 25 ng of genomic DNA; and 2.5 U of Dynazyme DNA polymerase (Finnzymes, Finland) in a final volume of 50 μl. Multiplex PCR assays were run with a DNA Engine Peltier thermal cycler (Bio-Rad, CA). Amplification products were resolved by DNA gel electrophoresis (Sigma, MO), and gel was stained with ethidium bromide.

Fermentative profiling. The sugar degradation and other catabolic properties of the *Lactobacillus* strains were characterized by using an API CH 50 kit (Bio-Merieux, Marcy l'Etoile, France). All strains were grown until the logarithmic phase and then inoculated into API galleries according to the manufacturer's instructions. API galleries were incubated at 37°C for 48 h prior to colorimetric analysis.

Human mucus-binding assay. Adhesion assays of the *Lactobacillus* strains radiolabeled by [³H]thymidine were performed as described previously (37). In brief, Maxisorp microtiter plates (Nunc, Denmark) were coated with 100 μl of human intestinal mucus solution in PBS at final concentration of 0.5 mg/ml and incubated overnight at 4°C. The wells were washed with PBS to remove unbound mucus, and 100 μl of [³H]thymidine-radiolabeled bacterial suspensions at an optical density at 600 nm (OD$_{600}$) of 0.25 ± 0.01 was added. The microtiter plate was incubated at 37°C for 1 h. Next, wells were washed with PBS to remove unbound

bacteria and incubated at 60°C for 1 h with a 1% (wt/vol) SDS–0.1 M NaOH solution. The radioactivity of lysed bacterial suspensions was measured by liquid scintillation counting (Wallac 1480 Wizard 3 automatic gamma counter). The percent ratio between radioactivity values of bound bacterial suspension and the total bacterial suspension initially added to the well was used to measure adhesion to human intestinal mucus. For each strain, the binding assay was performed at least in triplicate. An antiserum-mediated mucus-binding assay was also performed for GG, LrV, and LrI in the presence of polyclonal SpaC antiserum exactly as described previously (10). A procedure similar to the one mentioned above was subsequently performed, and radiolabeled bacteria were added to intestinal immobilized mucus upon incubation with a 1:100 dilution of SpaC immune serum.

Bile salt sensitivity. All strains were propagated in MRS broth at 37°C anaerobically. Next, the bacterial suspensions were adjusted to an OD_{600} of 1.5 and further diluted in sterile PBS. Three microliters of samples was spotted onto MRS agar plates supplemented with 0.5% (wt/vol) Ox gall bile salts (Sigma, MO). Plates were then incubated for 48 h at 37°C under anaerobic conditions prior to visual examination.

Western blot analysis of cell wall proteins. Bacterial suspensions ($OD_{600} = 1$) were used to extract cell wall-associated proteins from the *Lactobacillus* strains. Cell pellets were washed once with PBS and disrupted mechanically by bead beating with sterile quartz beads (200- to 800-μm diameter; Merck, Germany). The cell wall fraction was resuspended in 500 μl of PBS, pelleted at $13,000 \times g$ for 30 min at 4°C, and subsequently digested for 3 h at 37°C in 50 μl of lysis buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mg/ml lysozyme, and 150 U/ml mutanolysin. Samples were mixed with 12.5 μl of 4× Laemmli buffer (Bio-Rad, CA) and denatured at 99°C for 10 min. Cell wall-associated proteins were separated by SDS-PAGE using a 10% (vol/vol) polyacrylamide gel and then electroblotted onto a 0.2-μm nitrocellulose membrane (Bio-Rad, CA). Polyclonal rabbit SpaA antiserum (1:10,000 dilution) (17) and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, United Kingdom) (1:10,000 dilution) were used as primary and secondary antibodies, respectively, in 5% nonfat milk–PBS. Membranes were blocked with 5% nonfat milk–PBS and washed with 0.05% Tween 20–PBS between incubations. Bands were visualized by using chemiluminescence according to the specifications of the supplier (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer, United Kingdom).

TLR response assay. The HEK-blue hTLR2, HEK-blue hTLR4, and HEK-blue hTLR5 cell lines (Invivogen, CA), which constitutively express the Toll-like receptor (TLR) and an alkaline phosphatase gene fused to the NF-κB gene, were used in TLR response assays. All cell lines were grown and subcultured at 70 to 80% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g/liter D-glucose, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml μg/ml Normocin, 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated fetal bovine serum (Integro BV, Netherlands). For each cell line, an immune response experiment was carried out by splitting HEK-blue cells in flat-bottom 96-well plates and stimulating them by the addition of a bacterial suspension adjusted to an OD_{600} of 0.1 or TLR-specific ligands. The 96-well plates were incubated for 24 h at 37°C in a 5% CO_2 incubator. The receptor ligands PAM(3)CSK(4) (synthetic triacylated lipopeptide; 1 ng/ml for human TLR2 [hTLR2]), LPS-EB (lipopolysaccharide preparation isolated from *Escherichia coli* O111: Br; 1 ng/ml for hTLR4), and recFLA-ST (recombinant flagellin from *Salmonella enterica* serovar Typhimurium; 10 ng/ml for hTLR5) were used as positive controls, while maintenance medium without any selective antibiotics was used as the negative control. The activity of the secreted alkaline phosphatase, the product of the reporter gene fused to the NF-κB gene, was then determined by incubating 20-μl samples of the reporter cell line supernatant with 180 μl of QUANTI-Blue (Invivogen, CA) at 37°C, followed by measurement of the OD_{620}

after 1 h, 2 h, and 3 h. All assays were performed in triplicate for each sample.

Immunoelectron microscopy. *Lactobacillus* strains were grown to stationary phase and then used for transmission electron microscopy analyses. Sample preparation was done according to an immunogold-labeling protocol described previously (17). Briefly, drops of MRS-grown cultures were incubated on Formvar carbon-coated copper grids for 30 min at room temperature. Grids were washed three times with 0.02 M glycine in PBS and then incubated for 15 min with a blocking solution of 1% (wt/vol) bovine serum albumin (BSA). Next, polyclonal SpaA antibody was diluted 1:100 in 1% BSA, in which the grids were incubated for 1 h, and then washed with 0.1% BSA and incubated for 20 min with protein A gold conjugates (10-nm diameter). Grids were then washed several times in PBS, fixed for 5 min using 1% glutaraldehyde, washed again with MilliQ distilled water, and stained with a 1.8% methylcellulose–0.4% uranyl acetate solution. Grid visualization was carried out by using a Jeol 1200 EX II transmission electron microscope (Jeol Ltd., Japan).

Genome sequencing and bioinformatic analysis of *L. rhamnosus* and *L. casei* strains. Genomic DNAs of *L. rhamnosus* strains LrV and LrI and *L. casei* strains LcA and LcY were sequenced on a SOLiD sequencer platform (Life Technologies, CA). Sequence alignments were generated by mapping SOLiD color space reads to the *L. rhamnosus* GG genome (10) or the *L. casei* BL23 genome (22) as reference genomes, using SOLiD BioScope software (Life Technologies, CA) and SAM tools (38). In order to transfer annotation from a reference genome (GG or BL23) to an unannotated query genome, sequences were compared with "nucmer" to identify regions that share synteny; those regions were extracted out as base range in the query and base range in the reference genome (39). In-house custom-made scripts were used to transfer annotation. The nucleotide sequence identities between synteny blocks were more than or equal to 40%. In the case of the *L. rhamnosus* strains, initial detection of single-nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) was performed, and chromosomal regions with identified mutations were analyzed further. We considered only unequivocal SNPs with sufficient sequence coverage (>18) and verified them by PCR amplification using High-Fidelity Phusion DNA polymerase (Thermo Scientific, MA) according to the manufacturer's instructions. The PCR amplicons were then sequenced and compared to the reference *L. rhamnosus* strain GG genome. Orthologous genes between GG and BL23 genomes were calculated by using BLASTP (40) with the standard scoring matrix BLOSUM62 and an initial E value cutoff of $1 \times 10E04$. The score of every BLAST hit was set in proportion to the best score possible, the score of a hit of the query gene against itself. This resulted in a so-called score ratio value (SRV) between 0 and 100 that reflected the quality of the hit much better than the raw BLAST bit score (41). Two genes were considered orthologous if there existed a reciprocal best BLAST hit between the genes and both hits had an SRV of >35. Genomes were assigned to clusters of orthologous groups (COGs) by using rps-BLAST (reverse position-specific BLAST) and the NCBI Conserved Domain Database (CDD).

Primer extension. The transcriptional start site (TSS) and the promoter region of the SpaCBA pilus gene cluster were identified by primer extension according to a procedure previously described by Tu et al. (42). Briefly, 5'-6-carboxyfluorescein (FAM)-labeled cDNA was generated from 2 μg total *L. rhamnosus* GG RNA by using a FAM-labeled primer (5'-GTACCATTAGCATCGGTTTG-3') (Oligomer Oy, Finland) and a RevertAid Premium reverse transcriptase kit (Thermo Scientific, MA), according to the manufacturer's instructions. The cDNA mixture was then run on an ABI 3730 capillary sequencer in parallel with a Sanger sequencing reaction using the same primer.

RESULTS AND DISCUSSION

Isolation and metabolic characterization of the *Lactobacillus* strains. *L. rhamnosus* strains LrV and LrI were isolated from food and pharmaceutical products commercialized as carrying *L.*
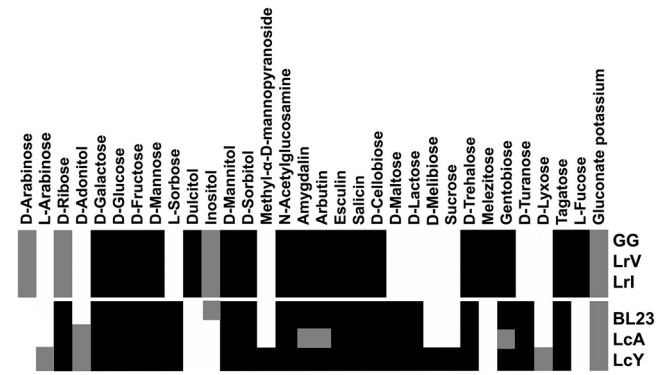
FIG 1 Metabolic profiles of the studied *Lactobacillus* strains. The fermentative capabilities of each strain are color-coded in black and gray for complete and partial carbohydrate utilization, respectively. Carbohydrates that were not fermented are shown in white. The results are those observed after 48 h of incubation. Not used by any of the strains were glycerol, erythritol, D-xylose, L-xylose, methyl-β-D-xylopyranoside, L-rhamnose, methyl-α-d-glucopyranoside, inulin, D-raffinose, amidon, glycogen, xylitol, D-fucose, D-arabitol, L-arabitol, 2-ketogluconate potassium, and 5-ketogluconate potassium.
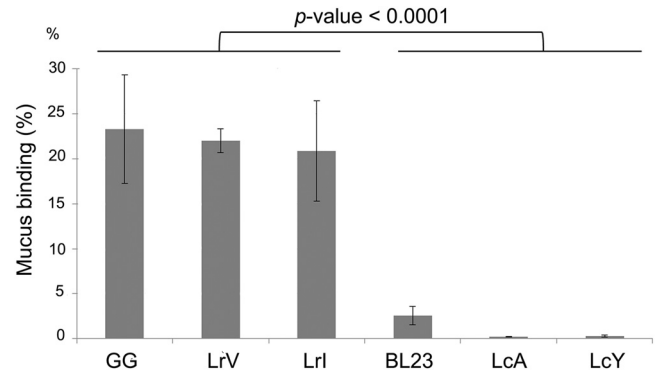

FIG 2 Binding profiles of *L. rhamnosus* and *L. casei* strains expressed as percent adhesion to human intestinal mucus. The binding data are expressed as means ± standard deviations. The differences between data sets are considered significant ($P \leq 0.0001$).

rhamnosus GG under the brand names Vifit (Friesland Campina, Netherlands) and Idoform (Ferrosan, Denmark), respectively. *L. casei* strains LcY and LcA were isolated from the food drinks branded as Yakult (Yakult Honsha Co., Japan) and Actimel (Danone, France), respectively. Genomic DNA was isolated and used for 16S rRNA and *tuf* gene analysis (36) to confirm the correctness of their species identification (data not shown), which was corroborated by SOLiD genome mapping. Subsequently, the metabolic properties of the isolated *Lactobacillus* strains were compared with those of the well-characterized strains *L. rhamnosus* GG and *L. casei* BL23 (Fig. 1). Both isolated *L. rhamnosus* strains showed identical sugar utilization profiles characteristic of *L. rhamnosus* GG, including the capacity to convert L-fucose but the inability to use D-lactose or L-rhamnose (10). In contrast, the *L. casei* strains showed considerable variation, indicating that the isolates from probiotic products are not identical. Specifically, they all converted D-lactose, D-maltose, and L-sorbose but not L-fucose, while strain LcY, isolated from the Yakult product, could also utilize D-melibiose and sucrose (Fig. 1).

Bile salt resistance, mucus binding, and intestinal signaling of *Lactobacillus* strains. Several desirable traits for a potentially probiotic strain were analyzed in the *Lactobacillus* strains isolated from probiotic products in comparison with the well-studied strains *L. rhamnosus* GG and *L. casei* BL23. Bile salt resistance was tested in a plate agar system or medium containing taurocholic and glycocholic acids derived from the used Ox gall bile. All *L. casei* strains (BL23, LcY, and LcA) and all *L. rhamnosus* strains (GG, LrV, and LrI) were found to be resistant to 0.5% (wt/vol) Ox gall bile salts. This is in agreement with previous data on the bile sensitivity of lactobacilli (31, 43) and detailed information on the proteomic bile response of *L. casei* strains (32), including the two strains used to produce Yakult and Actimel (34) and *L. rhamnosus* GG (44). This indicates that all *L. casei* and *L. rhamnosus* strains possess functional bile resistance mechanisms, an essential trait for persisting in the intestinal tract.

The ability of a variety of *Lactobacillus* strains marketed as probiotics to adhere to human mucus was previously reported to be highly variable, with *L. rhamnosus* GG as the highest-binding

strain tested (45). Hence, we compared the properties of adhesion of all used *Lactobacillus* strains to human intestinal mucus (Fig. 2). Indeed, all *L. rhamnosus* isolates (GG, LrV, and LrY) showed very high-level mucus binding, while the *L. casei* strains showed only moderate binding (BL23) or virtually no binding (LcY and LcA) under the conditions tested.

Finally, we compared the capacity of the strains to signal via Toll-like receptors (TLRs) in a mammalian cell line. No significant signaling response was found via TLR4 and TLR5, which is line with the absence of their key ligands (the lipopolysaccharides and the flagellins, respectively) in these lactobacillus strains (data not shown). In contrast, specific and reproducible responses were obtained in a TLR2 reporter cell line, where the NF-κB response was determined via a reporter fusion (Fig. 3). Under the conditions tested, all *L. rhamnosus* strains (GG, LrV, and LrI) showed significant and similar signaling via TLR2, and *L. casei* BL23 showed a moderate response, but isolates LcY and LcA showed only background signaling in this *in vitro* system.

This different signaling response via TLR2 is remarkable since cell wall components such as peptidoglycan and lipoteichoic acids that signal to TLR2 are generally present in all lactobacilli. One possible explanation for this finding could be that the nonsignal-
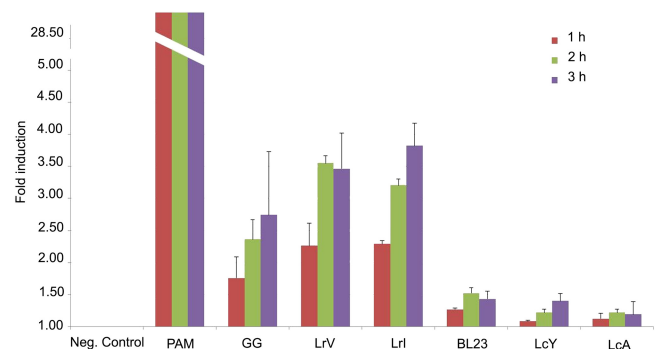

FIG 3 Response of HEK-blue hTLR2 cells to *L. rhamnosus* and *L. casei* strains. HEK-blue hTLR2 cells were stimulated with one of the six LAB strains for 24 h, after which the cell culture supernatant was incubated for 1 h, 2 h, and 3 h for detection of NF-κB activation. NF-κB-induced secreted embryonic alkaline phosphatase (SEAP) activity was measured by a spectrophotometer and converted to fold changes. The data are expressed as means ± standard deviations. PAM, PAM(3)CSK(4).
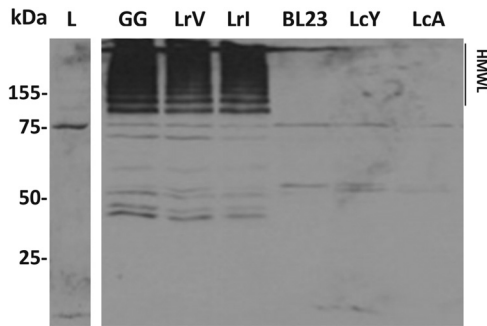
FIG 4 Immunoblotting analysis of cell wall-associated proteins of *L. rhamnosus* GG (lane 1), LrV (lane 2), and LrI (lane 3) and *L. casei* BL23 (lane 4), LcY (lane 5), and LcA (lane 6). The membrane was probed with polyclonal serum directed against the SpaA pilin subunit. HMWL, high-molecular-mass ladder; L, ladder.

ing strains LcY and LcA lack the pili that are known to be present in *L. rhamnosus* GG (10) and that were recently found to be the primary factors involved in promoting intestinal signaling (46). *L. rhamnosus* GG pili are decorated by the mucus-binding protein SpaC (10, 17), and *L. rhamnosus* strains lacking the expression of pili do not display any mucus-binding ability, which may also explain the observed absence of high-level adhesion to mucus by the studied *L. casei* strains. Hence, we performed a comparative analysis of the genomes of the *L. rhamnosus* isolates (GG, LrV, and LrI) and the *L. casei* strains (BL23, LcA, and LcY). However, this showed clearly that the genomes of the *L. rhamnosus* strains (GG, LrV, and LrI) and all *L. casei* strains (BL23, LcA, and LcY) contained highly identical sequences for the *spaCBA-srtC* gene cluster, and therefore, we focused on a detailed analysis of the expression of these pilus genes.

Analysis of pilus gene-encoded cell wall-associated proteins. The pili of *L. rhamnosus* GG can be detected by using antibodies against the major pilus protein SpaA or the mucus-binding protein SpaC (17). Western blotting using polyclonal SpaA antibody (Fig. 4) showed that the cell envelope fractions of all *L. rhamnosus* strains (GG, LrV, and LrI) contained the protein multimers characteristic of pili with different sizes (17). In contrast, when the same experiment was applied to the *L. casei* strains (BL23, LcA, and LcY), no such SpaA multimers or even monomers were detected (Fig. 4). This was confirmed by overexposing the Western blots or by spotting whole cells, supernatants, or cell extracts of the *L. casei* strains followed by incubation with anti-SpaA or anti-SpaC antibodies (data not shown). Hence, we conclude that none of the *L. casei* strains produced the mucus-binding pili characteristic of *L. rhamnosus* strains GG, LrV, and LrI under the conditions tested.

Subsequently, we used immunogold-labeled anti-SpaA antibodies in an immunoelectron microscopy experiment with the aim to identify the ultrastructure of the pili (see Fig. S1 in the supplemental material). As expected, all *L. rhamnosus* strains produced similar pilus phenotypes characteristic of *L. rhamnosus* GG (17), while no such pilus structures could be identified in any of the *L. casei* strains. Altogether, these experiments indicate that while *L. rhamnosus* GG and its reisolates from probiotic products produce the mucus-binding pili, these are not present in *L. casei* BL23 or strains LcA and LcY isolated from the probiotic products Actimel and Yakult. We cannot exclude the possibility that pili in *L. casei* might be differentially regulated and therefore expressed

under *in vivo* conditions under the control of environmental factors, as seen for *Bifidobacterium breve* (47). However, exposure of both *L. casei* and *L. rhamnosus* strains to conditions mimicking the intestinal tract, i.e., incubation in the presence of bile salts and low pH, did not trigger the expression of pili in *L. casei* strains, whereas in *L. rhamnosus*, pili remained expressed (data not shown). Finally, the use of SpaC antiserum in mucus-binding assays abolished the mucus-binding ability of LrV and LrI to background levels, as previously reported for GG (10). This further supports the important role of the SpaCBA pili in the interaction with human intestinal mucosa.

Comparative genome analysis of *L. rhamnosus* and *L. casei* strains. To further characterize the *Lactobacillus* strains isolated from probiotic products, we determined their genome sequences and analyzed these sequences based on a comparison of the well-established genomes of *L. rhamnosus* GG and *L. casei* BL23 (10, 22). The 3-Mb genomes of the latter strains are similarly sized (10, 22), among the largest in the genus *Lactobacillus* (4), and include no plasmids, unlike *L. rhamnosus* LC705 (10) and *L. casei* ATCC 334 (21). Moreover, the genomes of *L. rhamnosus* GG and *L. casei* BL23 show a high degree of synteny, with only a few regions disrupted throughout the chromosome (as revealed by ACT [Artemis Comparison Tool] and Gepard dot plot alignments) (Fig. 5). These regions consist mostly of genomic islands encoding prophages, transposases, and sugar transport systems, illustrating the preponderant role of horizontal gene transfer in bacterial evolution, promoting metabolic diversity and life-style adaptability between different lactobacilli. Protein predictions indicated that a total of 2,180 proteins with a high amino acid identity score were shared (including the identical *spaCBA-srtC* gene cluster), while 836 and 835 proteins were specific for *L. rhamnosus* GG and *L. casei* BL23, respectively (Fig. 6). In contrast to *L. casei* strain BL23, the *spaCBA-srtC* pilus gene cluster in *L. rhamnosus* strain GG is located in a chromosomal region rich in transposase genes and poorly conserved among the publicly available sequenced genomes of *L. rhamnosus* strains, suggesting its acquisition by horizontal gene transfer. Also, the COG distribution revealed that a significant proportion of strain-specific genes were involved in carbohydrate transport and metabolism, supporting the observed metabolic differences (Fig. 5 to 7). We further examined some salient metabolic features of *L. rhamnosus* GG and *L. casei* BL23 genomes, i.e., utilization of D-ribose, L-sorbose, D-maltose, D-lactose, and L-fucose. The capacity to metabolize carbohydrates relies on functional transport systems and intact metabolic pathways. The maltose locus was found to be disrupted in *L. rhamnosus* GG by the insertion of a conserved coding region (LGG_00950) between the genes encoding the maltose-specific transport system (*malEFGK*) and the hydrolase (LGG_00949) (Fig. 7) (10). In contrast, *L. casei* BL23 has a continuous and intact maltose locus with no gene insertion (57), which is in agreement with its ability to convert maltose (Fig. 7). It is noteworthy that the insertional inactivation of the maltose locus found in *L. rhamnosus* strain GG is not present in all *L. rhamnosus* strains (data not shown), suggesting that only a sublineage of the *L. rhamnosus* species lost the ability to metabolize maltose. Metabolic profiling also indicated that *L. rhamnosus* GG cannot metabolize D-lactose, unlike *L. casei* BL23, which could be verified at the genome level. The *L. rhamnosus* GG genome possesses a lactose locus, where the genes encoding the antiterminator (*lacT*) and the phospho-β-galactosidase (*lacG*) are nonfunctional (10), whereas the homologous
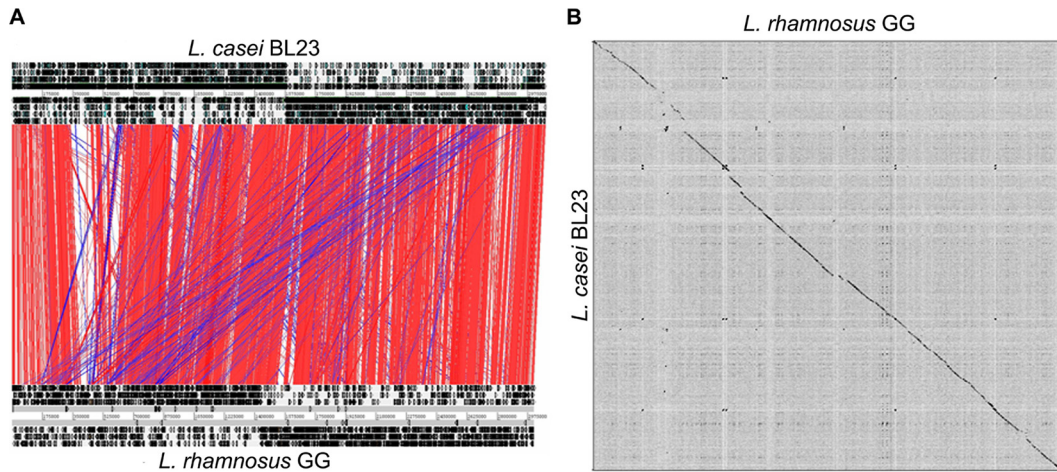
FIG 5 Genomic comparison of *L. casei* BL23 and *L. rhamnosus* GG. (A) ACT (Artemis Comparison Tool) comparison of *L. rhamnosus* GG (bottom chromosome) and *L. casei* BL23 (top chromosome) (55). Red and blue bars indicate similar regions between GG and BL23 (BLASTN hits), respectively, that have the same orientation or have been inverted. (B) Dot plot alignments of GG and BL23 using Gepard (56).

lactose locus in BL23 is intact (58) (Fig. 7). *L. casei* BL23 is also able to convert L-sorbose, which could be associated with the presence of an intact sorbose locus, *sorERFABCDG*, previously described by Yebra and colleagues (48). No such locus could be identified in *L. rhamnosus* GG, and only some genes homologous to *sorE*, *sorF*, and *sorG* were found scattered throughout the genome, indicating that *L. rhamnosus* GG does not possess the complete machinery necessary to utilize L-sorbose. Interestingly, *L. rhamnosus* GG does not use D-ribose as efficiently as BL23 (Fig. 1), which could be explained by the fact that the ribose-specific ABC transporter *rbsABC* is missing in GG compared to BL23 (Fig. 7). This also sug-

gests that *L. rhamnosus* GG uses alternative transporter systems, as it still converts D-ribose. Originally isolated from the human intestinal tract, *L. rhamnosus* GG is able to convert L-fucose, a sugar compound commonly present in the intestinal tract (49). A close inspection of both genomes revealed that *L. rhamnosus* has a fucose locus which is not present in *L. casei* BL23 (Fig. 7). Differences between *L. rhamnosus* GG and *L. casei* BL23 genomes are clearly reflected at the phenotypic level and show how different genetic events may contribute to the bacterial diversity within the same genus, i.e., loss, decay, or acquisition of a locus and gene mutation.

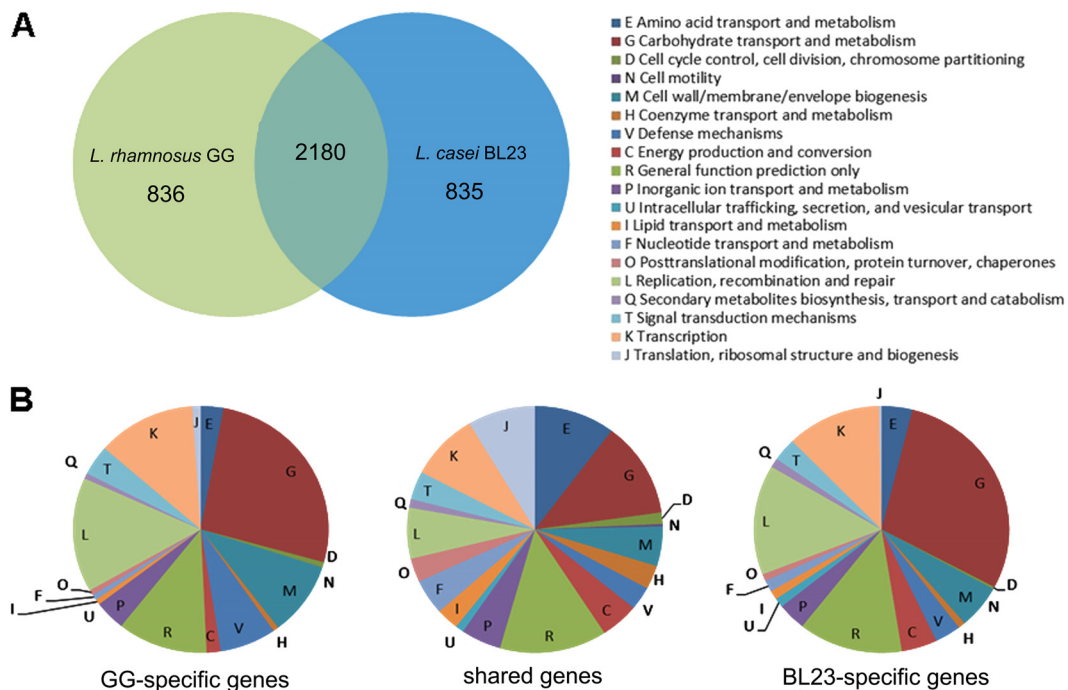Subsequently, the genomes of the *L. rhamnosus* strains (LrV

FIG 6 Comparative genomic overview of *L. rhamnosus* GG and *L. casei* BL23. (A) Number of shared and strain-specific genes. (B) COG distribution of the different subset of genes shown in panel A.
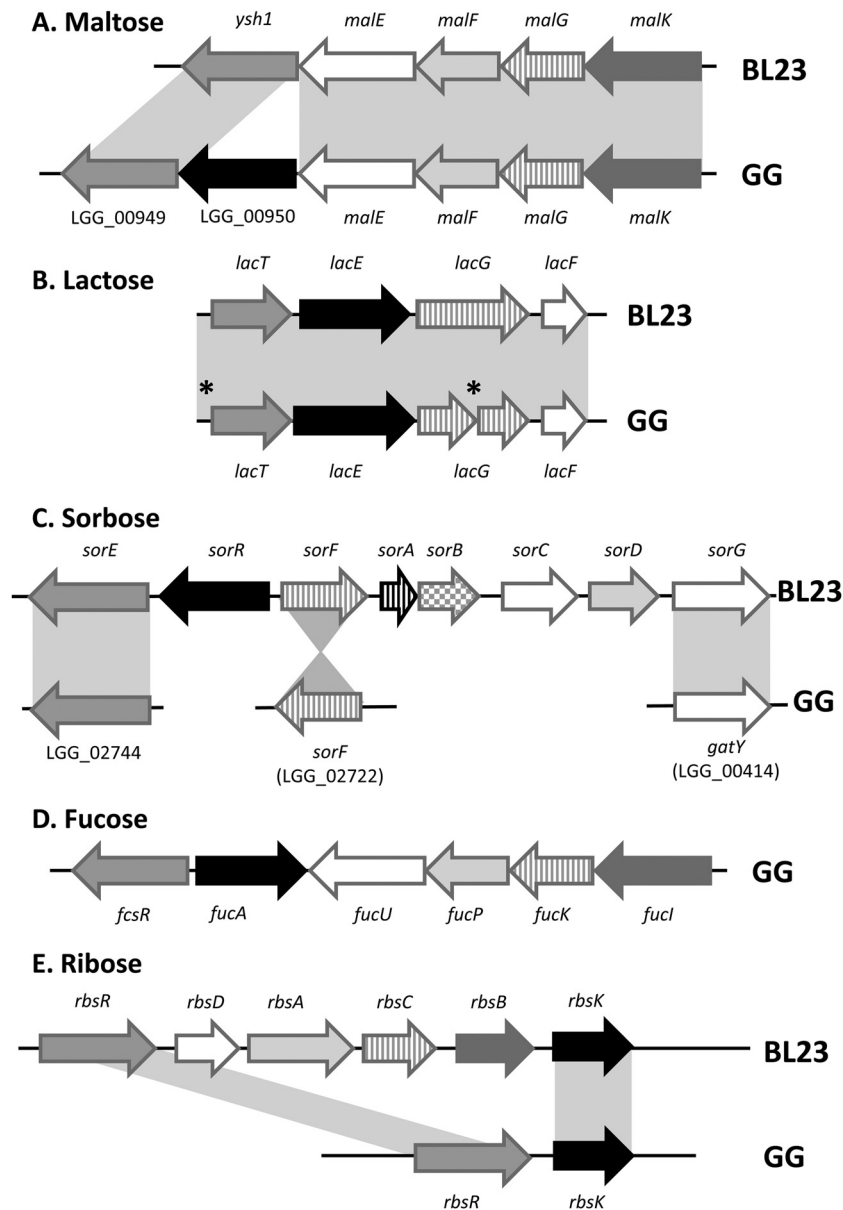
FIG 7 Metabolic cluster comparison between *L. rhamnosus* GG and *L. casei* BL23 genomes. Panels illustrate some of the genes associated with the indicated carbohydrates. Gray shading indicates homologous genes. The black asterisks indicate mutations previously reported (10).

and LrI) and the *L. casei* strains (LcY and LcA) were analyzed by SOLiD sequencing with paired-end and single reads (50-bp forward reads and 35-bp reverse reads), totaling 8.9 million to 12 million reads and amounting to over 100 Mbp for each genome. The SOLiD sequencing reads were mapped to the *L. rhamnosus* GG and *L. casei* BL23 genomes, providing sufficient information to gain insights into gene content, genetic order, and single-nucleotide polymorphisms. In the SOLiD mapping approach, tandem repeats, mononucleotide repeats, and low-complexity sequences present in the genomes may not be correctly mapped in some cases, as previously reported (50, 51). Using the annotation method (39), comparative analysis of the genomes of the *L. rhamnosus* isolates and that of *L. rhamnosus* GG (10) revealed strains LrV and LrI to be virtually syntenous at the genome level. We also

identified 4 and 2 SNPs to be present in LrV and LrI, respectively (Table 2). Remarkably, the 2 SNPs were identical and were located in intercistronic regions, suggesting that these might be hot spots for mutations. Strain LrV later acquired two additional SNPs, which are not expected to have an impact on the phenotype as observed in the present study. These SNPs were either located in a lipoprotein gene or affected the *glvA* gene, which is involved in dysfunctional maltose metabolism (Fig. 1). This illustrates the genomic stability of *L. rhamnosus* strain GG used in various food or other products, including Idoform and Vifit, but this also demonstrates the attention paid by the dairy industry to ensure the stability and integrity of stock culture propagation and use, i.e., inoculation with bacterial cells grown from a frozen and carefully preserved seed culture in large bioreactors and optimal handling

TABLE 2 Summary of SNPs identified in *L. rhamnosus* strains LrV and LrI[b]

SNP coordinate in GG	Mutation[a]		Nucleotide change	Gene	aa change	Description
	LrI	LrV				
1030390	×	×	T→G	LGG_01017	H294Q	Lipoprotein
1373568		×	G→A	NA	NA	Intercistronic region between converging LGG_1372 (conserved protein) and LGG_1371 (conserved protein)
2649651		×	G→T	NA	NA	Intercistronic region upstream of LGG_01853, ABC transporter, substrate-binding protein
2765383	×	×	G→A	LGG_02701	H98N	Maltose-6′-phosphate glucosidase GlvA

[a] × indicates the strain in which the mutation occurred.
[b] NA, not applicable; aa, amino acid.

in order to prevent any decline in cell viability. As shown by the very low numbers of SNPs found in both isolates, opportunities for evolutionary change in the culture over time are purposely kept to a minimum. It is noteworthy that in the course of this analysis, we identified two sequencing errors present in the original *L. rhamnosus* GG genome sequence (10), located at bp 615483 (T>C) and bp 1883242 (C>A). The deposited NCBI GenBank sequence (accession number FM179322) was corrected accordingly.

As no genomic information relating to the *L. casei* strains used in Actimel and Yakult was available, all SOLiD reads of strains LcA and LcY were mapped to the genome of *L. casei* strain BL23 (22). This showed that *L. casei* BL23 and strain LcA isolated from Actimel are highly similar. Using the annotation transfer method (39), all genes of strain BL23 were found to be potentially present in *L. casei* LcA, including the identical *spaCBA-srtC* gene cluster. A closer look at the consensus sequence shows that there were only 158 undetermined nucleotides, suggesting potential SNPs or InDels. These were not further addressed in this study, as they need more extensive high-resolution sequence analysis. Using a similar approach, a total of 34 genes from *L. casei* BL23 were not shared with strain LcY isolated from Yakult, indicating a further phylogenetic distance than strain LcA isolated from the Actimel product, which is reflected at a functional level when comparing the metabolic capacities of the *L. casei* strains (Fig. 1). The genes lacking in *L. casei* strain LcY include 34 genes encoding a prophage (see Table S1 in the supplemental material). However, the *spaCBA-srtC* gene cluster was intact in the genome of *L. casei* LcY. In addition, the large number of approximately 70 undefined nucleotides suggested more SNPs and InDels that differentiated *L. casei* strains BL23 and LcY. With the genomic resequencing approaches used here, we could not identify genes not present in BL23, but a more comprehensive high-resolution sequence analysis of all four strains, which is presently ongoing, indicated that we were able to cover 99 and 97% of the genomic information for *L. casei* strains LcA and LcY isolated from the Actimel and Yakult products, respectively (our unpublished data).

Identification of the transcriptional start site of the *spaCBA* pilus operon. As all of the tested *L. rhamnosus* strains but none of the *L. casei* strains produced pili, in spite of the high level of conservation and sequence identity of the *spaCBA-srtC* pilus gene cluster (Fig. 8), we inspected the sequence upstream of this gene cluster (Fig. 8). A number of differences were evident, which may affect the expression of the pilus genes, notably those in the presumed promoter region. To define the transcription initiation of this cluster, we performed primer extension analysis, which re-

sulted in the identification of the promoter region of the *spaCBA* pilus gene cluster in *L. rhamnosus* GG. We observed that the transcriptional start site is located 47 nucleotides upstream of the *spaC* start codon (ATG). Putative −10 and −35 regions were proposed (Fig. 8). Interestingly, the putative promoter region identified in *L. rhamnosus* GG differs significantly from the sequences present in the *L. casei* strains: the differences result in a loss of the consensus −35 and −10 regions and the transcriptional start site (TSS). In spite of the fact that the *L. rhamnosus* promoter sequence does not resemble the canonical promoter, it shows high levels of expression of the pilus genes. The possibility that alternative sigma factors are used by this promoter is unlikely, as these have not been identified as a major control system in LAB (52). Moreover, the spacing between the predicted Shine-Dalgarno sequence and the initiation codon of the *spaC* gene is 2 nucleotides longer in the *L. casei* genomes, suggesting that apart from a transcriptional defect, translation would also be less efficient than that in *L. rhamnosus* strains. This would explain the absence of any detectable pili in the *L. casei* strains and correlate with the absence of mucus binding. In *L. rhamnosus* GG and the virtually identical strains LrV and LrI, an insertion sequence (IS) element is present upstream of the *spaC* gene, in contrast to the *L. casei* strains (27), suggesting that the integration of the IS element resulted in the activation of pilus gene expression in these strains but not in the *L. casei* strains. Such transcriptional activation is reminiscent of various other bacterial systems where gene expression is enhanced or altered by the introduction of IS elements (53, 54).

Conclusions. We characterized four probiotic-marketed strains at the genomic and phenotypic levels. The two *L. rhamnosus* strains, LrV and LrI, were virtually similar to GG in terms of genomes and phenotypes, showing the product stability and integrity of the widely used probiotic strain *L. rhamnosus* GG. Remarkably, the identification of SNPs in the genomes of the *L. rhamnosus* strains isolated from the Vifit and Idoform probiotic products may indicate the presence of hot spots for mutations. The two *L. casei* strains isolated showed more heterogeneity than *L. casei* BL23 regarding genome content and carbohydrate utilization. Interestingly, when looking at the presence of SpaCBA pilus structures in *L. rhamnosus* and *L. casei* strains by immunoblotting analysis, electron microscopy, and mucus-binding assays, only *L. rhamnosus* strains displayed functional pili that correlated with their mucus-binding abilities and possibly are responsible for the TLR2 response. Although characterized under *in vitro* conditions, our data indicate that *L. rhamnosus* strains are and remain piliated under different stress conditions, i.e., bile salts and low pH. In contrast, no expression of pili in *L. casei* could be observed under
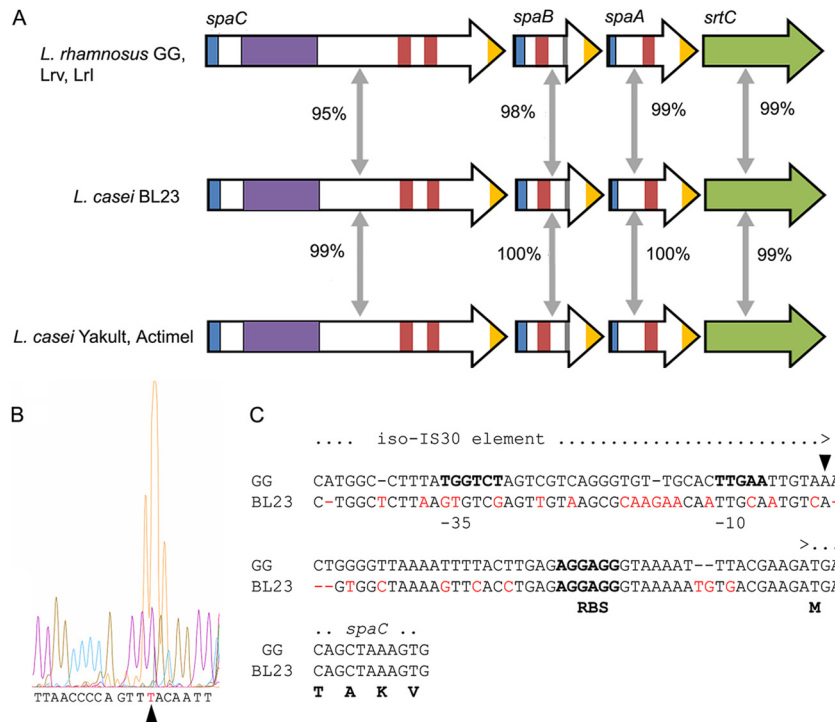
FIG 8 Comparison of SpaCBA pilus clusters from *L. rhamnosus* and *L. casei* strains. (A) BLAST results and corresponding amino acid conservation percentages for each gene. The presence of different motifs is color/pattern-coded as follows: green arrow for sortase, white arrow for pilus subunit, blue for secretion signal, yellow for the LPxTG motif, purple for the von Willebrand type A domain, and red for the Cna protein B-type domain. (B) Primer extension analysis of the SpaCBA pilus promoter. The peaks (yellow) detected during the analysis are indicated on the sequencing chromatogram. (C) Sequence alignment of the upstream region of the *spaC* gene in *L. casei* and *L. rhamnosus* strains with the position of the transcriptional start site, the putative −10 and −35 regions, and the ribosome-binding site (RBS). Nucleotides highlighted in red in the *L. casei* BL23 sequence differ from GG.

the conditions tested, even when mimicking intestinal tract conditions. This finding indicates that *L. casei* strains are not piliated when consumed by humans and may remain so when transiting in the intestinal tract. Although there is a possibility that environmental or stress factors might trigger pilus expression in *L. casei* under *in vivo* conditions, our present phenotypic and genomic data indicate that it is unlikely. The identification of the transcriptional start site of the *spaCBA* operon also suggested that the expression of pili was triggered by the insertion of an IS element in *L. rhamnosus* strains, in contrast to *L. casei* strains. The transposition of an IS element upstream of the *spaC* gene appeared to have a significant impact on the evolution of *L. rhamnosus* species by conferring a beneficial trait to colonize and persist in mucosa-associated niches, such as the human gastrointestinal tract.

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