RESEARCH ARTICLE

Low cell surface hydrophobicity is one of the key factors for high butanol tolerance of Lactic acid bacteria

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Highly butanol-tolerant strains have always been attractive because of their potential as microbial hosts for butanol production. However, due to the amphiphilic nature of 1-butanol as a solvent, the relationship between the cell surface hydrophobicity and butanol resistance remained ambiguous to date. In this work, the quantitatively estimated cell surface hydrophobicity of 74 Lactic acid bacteria strains were juxtaposed to their tolerance to various butanol concentrations. The obtained results revealed that the strains' hydrophobicity was inversely proportional to their butanol tolerance. All highly butanol-resistant strains were hydrophilic (cell surface hydrophobicity < 1%), whereas the more hydrophobic the strains were, the more sensitive to butanol they were. Furthermore, cultivation at increasing butanol concentrations showed a clear tendency to decrease the level of hydrophobicity in all tested organisms, thus suggesting possible adaptation mechanisms. Purposeful reduction of cell surface hydrophobicity (by removal of S-layer proteins from the cell envelope) also led to an increase of butanol resistance. Since the results covered 23 different Lactic acid bacteria species of seven genera, it could be concluded that regardless of the species, the lower degree of cells' hydrophobicity clearly correlates with the higher level of butanol tolerance.

KEYWORDS

butanol tolerance, hydrophobicity, Lactic acid bacteria, surface layer proteins

1 | INTRODUCTION

Butanol represents one of the most promising biotechnological alternatives to petroleum derivatives that will be offered in the future market. As a 4-carbon alcohol, it has high combustion energy, low vapour pressure, excellent fuel characteristics, and could be produced by fermentation [1]. However, its biotechnological production will not be possible in industrial scale, until the problem of low microbial tolerance to butanol is solved. Over the last decade, a number of scientific studies are devoted to tolerance enhancement of both the "classical producers" of genus *Clostridium*, as well as the engineered non-natural butanol producing strains [2–5]. In a recent review, Peabody and Kao (2016) categorized the

tools utilized for butanol tolerance development into several groups: the use of genomic libraries and adaptive laboratory evolution; analyses of regulatory, proteomic, and metabolic cellular responses; an implementation of known mechanisms; and the use of advanced cellular modelling [6].

The harmful effect of butanol on the microbial cell is related to its nature of an organic solvent. So far, it was known that butanol is primarily accumulated in the cytoplasmic membrane leading to disruption of the phospholipid bilayer, thus increasing the cell membrane fluidity. It has recently become clear that butanol causes the destruction of the whole cell envelope by the release of lipopolysaccharides from the outer membrane, "leakiness" in both outer and inner membranes, and disruption in the peptidoglycan layer [7]. This is the reason why part of the mechanisms of microbial tolerance to butanol is common for both Gram-negative and Gram-positive bacteria. They include (i) adaptation of the cell

Abbreviations: CSH, cell surface hydrophobicity; LAB, Lactic acid bacteria; SLP, surface layer proteins

membrane composition and (ii) reverse transport of butanol by external medium energy-dependent active efflux pumps. Other mechanisms can also be reported such as *cis-to-trans* isomerization of unsaturated membrane fatty acids, increased content of saturated and long-chain fatty acids, modifications in the membrane phospholipid head groups, and accelerated biosynthesis of phospholipids [3,8]. Besides, Gram-positive bacteria possess additional and specific mechanisms of response to organic solvents stress: sporulation, and changes in cell morphology.

Poorly studied and unclear to date are the opportunities for whole cell envelope modifications. It is known that hydrophobicity of the outermost cellular structures has great influence on the cell's ability to adhere to surfaces or substrates, including the binding to toxic compounds. For instance, a mutant strain of *Pseudomonas putida* with increased hydrophilicity was shown to be more resistant to the hydrophobic toluene [9].

On the other hand, the amphiphilic nature of butanol as a solvent made obscure the level of hydrophobic interactions with the bacterial cell surface. 1-Butanol consists of a polar, hydrophilic hydroxyl residue and a nonpolar, hydrophobic hydrocarbon chain. Its hydrophobicity may be expressed by the logarithm of the octanol/water partition coefficient $\log P$ [10]. The value of $\log P = 0.88$ places 1-butanol in an intermediate position between the hydrophilic methanol (-0.77) and ethanol (-0.31), and the hydrophobic 1-octanol (3.0). This is the reason why the link between CSH and bacterial tolerance to butanol has remained ambiguous so far, thus making obscure the CSH levels that would provoke strains resistance.

Good model organisms to study these effects are the representatives of Lactic acid bacteria (LAB) by two main reasons. First, a number of highly butanol resistant strains of LAB were recently reported: *Lactobacillus brevis* [11], *Enterococcus faecium* [2,12], *Ent. faecalis* [13], *Pediococcus acidilactici* [2,14], *P. pentosaceus*, *Lb. plantarum*, *Ent. caseliflavus* [14]. Second, LAB group offers strains with CSH varying from 0 to 100%, well-studied with respect to the relationship between hydrophobicity and bacterial adhesion. The index of hydrophobicity depends on the molecular structure of the cell envelope. Main factors affecting total CSH include the presence of proteins, glycoproteins, teichoic and lipoteichoic acids, surface fibrils, and adhesins on the cell wall surface [15,16]. Several *Lactobacillus* species possess surface layer proteins (SLP) anchored to the cell wall that confer relatively high hydrophobicity on the *Lactobacillus* cell surface [17]. The surface of other LAB (for instance, *L. casei* ATCC 393) does not contain SLP, but is rich in polysaccharides instead, as this layer presents itself as a hydrophilic coating [18].

The aims of this work are to elucidate the relationship between CSH and butanol tolerance, to clarify the level of CSH that supports butanol resistance, and to investigate the

PRACTICAL APPLICATION

Because of its excellent gasoline-like fuel properties (much better than ethanol), butanol is considered as one of the most promising future substitutes of the fossil fuels. However, low microbial tolerance to butanol is the main hindrance to its profitable biotechnological production. Lactic acid bacteria (LAB) are most resistant to butanol microorganisms. Here we reveal for the first time the clear correlation between hydrophobicity of LAB and their butanol tolerance. We showed that regardless of the species or genus of bacteria, the hydrophilic cell surface always ensures increased butanol resistance. This observation could be used for: 1) Fast and reliable distinction of resistant LAB strains based on CSH measurement; 2) Novel strategy to increase butanol resistance by manipulating the strains hydrophobicity; 3) Optimization of the fermentation broth and process parameters in order to support the hydrophilic state of the cells' surface (ionic strength, pH, redox potential).

opportunities for the natural and external CSH alterations in butanol environment.

2 | MATERIALS AND METHODS

2.1 | LAB strains and cultivation conditions

LAB laboratory collection of The Institute of Microbiology (Bulgarian Academy of Sciences), was screened for butanol-tolerant strains. The strains were isolated from traditional Bulgarian fermented foods (yogurt, cereals, sauerkraut, pickled vegetables, and Boza), collected previously [19–21], or obtained after enrichment by growth of the food samples in MRS supplemented with 3% v/v butanol. The reference strains were purchased from culture collections (ATCC, DSMZ, and NCDO) and are listed in Table 1. The strains were grown in MRS medium (Carl Roth GmbH & Co. KG, Germany) at 37°C, at micro-aerobic conditions, and preserved at -80°C , supplemented with 20% v/v glycerol.

2.2 | Butanol tolerance assay

Tolerance to butanol was assessed based on relative cellular growth rate in MRS medium (Carl Roth GmbH, Germany), supplemented with 1–4% v/v butanol (Sigma-Aldrich Chemie GmbH, Germany), and compared to the control MRS without solvent. To prevent evaporation, the fermentations were

TABLE 1 Relative growth rates of new LAB isolates and reference LAB strains in MRS medium containing 1% – 4% butanol (v/v). Growth at MRS without butanol is considered as 100%. The strains are ranked by their hydrophilicity, measured after cultivation in MRS

Species	Strain	Source	Relative growth in presence of butanol				CSH ^a
			1%	2%	3%	4%	
Isolates							
<i>P. acidilactici</i>	DF3	Milky beverage	~100.0	95.5 ± 3.4	55.7 ± 2.6	8.6 ± 1.8	0.0
<i>P. acidilactici</i>	Y1	Cereals	~100.0	91.9 ± 4.5	44.8 ± 5.8	6.2 ± 2.3	0.0
<i>P. acidilactici</i>	32	Probiotic drink	~100.0	92.5 ± 3.8	49.3 ± 5.4	6.2 ± 2.1	0.0
<i>P. acidilactici</i>	9	Grains	~100.0	91.6 ± 4.6	39.1 ± 5.5	6.1 ± 2.7	0.0
<i>P. acidilactici</i>	BB3	Boza	98.2 ± 3.4	91.4 ± 3.9	36.5 ± 4.2	5.9 ± 1.9	0.0
<i>Lb. delb. ssp. bulgaricus</i>	Y69	Yogurt	95.2 ± 4.3	89.6 ± 4.6	32.7 ± 3.8	2.0 ± 1.8	0.0
<i>Lb. delb. ssp. bulgaricus</i>	Ym2	Yogurt	93.7 ± 4.1	88.1 ± 4.2	30.4 ± 2.9	1.7 ± 1.1	0.0
<i>P. acidilactici</i>	ED	Boza	95.5 ± 2.8	90.8 ± 5.1	26.6 ± 3.6	5.1 ± 2.4	1.1 ± 0.6
<i>Lb. sakei</i>	3/30	Grains	95.1 ± 3.8	84.2 ± 3.2	16.5 ± 5.0	0.0	3.0 ± 1.0
<i>P. acidilactici</i>	57	Yogurt	88.2 ± 3.2	78.2 ± 4.6	16.9 ± 4.3	3.8 ± 2.1	8.2 ± 1.1
<i>Lb. helveticus</i>	Y22	Yogurt	90.9 ± 2.9	80.2 ± 2.8	17.9 ± 3.3	1.9 ± 1.2	8.0 ± 1.7
<i>Ent. faecium</i>	c1	Cereals	87.4 ± 4.2	74.9 ± 5.2	15.4 ± 4.6	2.0 ± 1.3	8.5 ± 1.6
<i>Lb. delb. ssp. bulgaricus</i>	Y32	Yogurt	89.6 ± 3.9	79.9 ± 3.5	17.3 ± 3.6	0.0	10.9 ± 0.5
<i>Ent. faecium</i>	c4	Cereals	88.6 ± 3.5	77.8 ± 3.8	17.1 ± 3.3	4.9 ± 1.8	12.2 ± 1.5
<i>P. acidilactici</i>	59	Yogurt	88.1 ± 3.1	76.4 ± 2.6	14.5 ± 3.7	2.9 ± 1.6	13.4 ± 0.8
<i>P. acidilactici</i>	33	Yogurt	86.5 ± 4.8	72.8 ± 4.8	13.5 ± 3.2	0.0	13.6 ± 1.5
<i>Ent. faecium</i>	H	Boza	85.3 ± 3.9	72.4 ± 3.5	12.1 ± 2.8	0.0	15.1 ± 1.5
<i>Lb. delb. ssp. bulgaricus</i>	Y17	Yogurt	89.4 ± 4.2	79.1 ± 4.2	17.1 ± 3.8	0.0	15.8 ± 1.1
<i>P. acidilactici</i>	Lin1	Boza	85.4 ± 4.4	71.1 ± 3.8	11.7 ± 3.0	0.0	15.8 ± 1.8
<i>P. acidilactici</i>	56	Yogurt	83.2 ± 4.8	70.6 ± 4.6	10.5 ± 3.4	0.0	17.1 ± 1.5
<i>Ent. faecium</i>	62	Yogurt	76.1 ± 5.3	64.8 ± 5.4	10.3 ± 5.1	0.0	18.3 ± 1.6
<i>Str. equinus</i>	D	Milky beverage	76.6 ± 5.1	64.9 ± 5.1	11.2 ± 2.9	0.0	18.9 ± 1.6
<i>Lb. plantarum</i>	Ym1	Yogurt	89.5 ± 5.4	71.1 ± 4.6	14.9 ± 3.4	0.0	21.1 ± 1.2
<i>Ent. faecium</i>	1.2	Grains	75.3 ± 4.9	64.2 ± 4.9	10.1 ± 2.1	0.0	22.5 ± 1.8
<i>Lb. plantarum</i>	S12	Sauerkraut	89.6 ± 4.8	68.8 ± 3.8	14.1 ± 3.3	0.0	24.2 ± 0.8
<i>Lb. delb. ssp. lactis</i>	Y12	Yogurt	74.4 ± 4.6	66.9 ± 2.9	11.1 ± 4.1	0.0	24.9 ± 1.0
<i>Lb. delb. ssp. bulgaricus</i>	Y10	Yogurt	73.2 ± 3.8	65.9 ± 3.1	10.2 ± 3.1	0.0	25.4 ± 1.2
<i>Lb. fermentum</i>	Y39	Yogurt	70.1 ± 3.3	63.5 ± 4.6	7.5 ± 1.8	0.0	25.8 ± 1.2
<i>Lb. plantarum</i>	Bom 816	Boza	71.3 ± 4.2	28.7 ± 4.8	0.0	0.0	46.2 ± 1.5
<i>Lb. pentosus</i>	N3	Boza	68.2 ± 3.1	26.9 ± 5.4	0.0	0.0	56.6 ± 1.7
<i>Lb. helveticus</i>	Y16	Yogurt	66.1 ± 3.4	18.5 ± 4.3	0.0	0.0	80.6 ± 3.2
<i>Lb. paracasei</i>	ml 25	Yogurt	65.3 ± 3.3	12.6 ± 3.2	0.0	0.0	83.5 ± 2.5
Reference strains							
<i>Lb. fermentum</i>	ATCC 14931		95.5 ± 4.3	84.4 ± 4.2	11.9 ± 1.5	0.0	0.5 ± 0.5
<i>Lb. casei</i>	ATCC 393		95.4 ± 3.5	84.6 ± 3.8	7.5 ± 1.8	0.0	2.8 ± 0.5
<i>Lb. paraplantarum</i>	ATCC 700211		95.3 ± 4.1	81.1 ± 5.1	9.9 ± 1.8	0.0	3.0 ± 0.9
<i>Lb. fermentum</i>	ATCC 14932		93.3 ± 5.1	75.5 ± 2.8	8.3 ± 3.1	0.0	3.1 ± 1.1
<i>Lb. delb. ssp. lactis</i>	ATCC 15808		91.2 ± 4.2	74.1 ± 3.2	8.2 ± 3.4	0.0	8.0 ± 1.0
<i>Lb. rhamnosus</i>	ATCC 7469		95.5 ± 3.2	79.5 ± 5.4	7.5 ± 2.2	0.0	12.7 ± 1.8
<i>Lb. plantarum</i>	NCDO 1193		82.6 ± 5.3	62.8 ± 4.6	4.7 ± 1.9	0.0	26.4 ± 1.5
<i>Lb. helveticus</i>	ATCC 27558		87.3 ± 4.9	42.2 ± 4.5	0.0	0.0	29.6 ± 1.7
<i>Lb. ruminis</i>	ATCC 25644		86.5 ± 4.6	38.8 ± 5.3	0.0	0.0	33.4 ± 2.8
<i>Lb. pentosus</i>	ATCC 363		79.4 ± 5.2	34.8 ± 3.7	0.0	0.0	38.2 ± 0.8

(Continues)

TABLE 1 (Continued)

Species	Strain	Source	Relative growth in presence of butanol				CSH ^a
			1%	2%	3%	4%	
<i>Lb. paracasei</i>	DSM 23505		75.6 ± 4.8	31.5 ± 4.3	0.0	0.0	42.6 ± 2.8
<i>Lb. delb. ssp. bulgaricus</i>	NCDO 2395		80.4 ± 2.6	25.5 ± 4.1	0.0	0.0	68.6 ± 2.1
<i>Lb. delb. ssp. bulgaricus</i>	DSM 20081		46.4 ± 3.8	9.2 ± 1.9	0.0	0.0	85.2 ± 3.5
<i>Lb. acidophilus</i>	ATCC 4357		16.1 ± 2.3	7.6 ± 0.8	0.0	0.0	90.0 ± 3.0
<i>Lb. acidophilus</i>	ATCC 4356		10.4 ± 2.1	6.5 ± 0.8	0.0	0.0	93.5 ± 3.5

^aThe CSH was estimated according to microbial adhesion to hydrocarbons method [22].

performed in 15-mL, tightly capped conical tubes, using 1% inoculum of cultures grown for 24 h. The cultivation was performed statically at 37°C, for 24 h.

The cell growth was monitored by measuring the OD of the culture at wavelength 590 nm using UV/VIS Spectrophotometer (Thermo ScientificTM), as the growth rates were calculated from the linear range of the exponential growth. The relative growth rates were calculated as a ratio between the specific growth rates in the presence of butanol, and those in the absence of butanol. All experiments were conducted three times.

2.3 | Hydrophobicity test

For estimation of the CSH of each particular LAB strain, the microbial adhesion to hydrocarbons method using hexadecane was performed. The strains were grown for 24 h and centrifuged at 10 000 rpm for 10 min. Bacterial pellets were washed twice and suspended in PUM buffer (0.1 M K₂HPO₄·3H₂O, 0.05 M KH₂PO₄, 0.025 M Urea, 0.001 MgSO₄·7H₂O, pH 7.1) at an OD₅₉₀ between 0.5 and 0.6. Hexadecane and the cell suspension in ratio 1:6 were vigorously mixed for 2 min. Then the mixtures were incubated at room temperature for 1 h until the full separation of the two phases. The absorbance of the lower aqueous phase was measured at λ₅₉₀, and the hydrophobicity level was calculated in percentage [22]. The test was carried out in triplicate for each strain.

2.4 | PCR amplification of 16S rRNA gene, sequencing, and phylogenetic analysis

Genetic identification of the strains was done by sequencing of 16S rRNA genes and comparison with NCBI database. Briefly, total genomic DNA was isolated from 24-hold cultures grown in MRS, using a GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, USA). The PCR amplification of 16S rRNA gene was done with primer pair 27F and 1492R [19] at final concentration of 10 pmol/mL, in QB-96 Satellite Gradient Thermal Cycler (LKB Vertriebs GmbH, Vienna, Austria), using

Phusion High-Fidelity Master Mix (Thermo Fisher Scientific Inc.), template DNA 2 ng/mL, under the following temperature profile: 95°C for 5 min, 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by final elongation at 72°C for 5 min. Sequencing of the obtained PCR fragments was done by Macrogen Inc. (Amsterdam, The Netherlands). Sequence alignment was done by Chromas LITE, version 2.1 and CAP3; the comparison with NCBI database was done by BLAST and ClustalW. The phylogenetic tree was built by the MEGA6 software [24]. The sequences are deposited in GenBank of NCBI with accession numbers from MH430030 to MH430036, MG437344 to MG437394, MG438466 to MG438490, KU513392, KU513399, KU513401, KU513405, and KU513409.

2.5 | S-layer protein extraction

The method of Lortal et al. (1992), with small modifications was used for SLP removal [25]. Cells of 5 mL 24-h old cultures were harvested by centrifugation at 10 000 rpm for 15 min at 4°C, washed twice with distilled water, and resuspended in 10 mL of 5 M LiCl solution. The mixtures were incubated for 30 min in ice bath to remove the S-layer of the cell surface of the strain, then centrifuged at 14 000 rpm for 15 min at 4°C. The supernatants containing the S-layer protein were collected. The proteins were precipitated with three volumes acetone, dialyzed for 4 h, resuspended in 100 µL PBS buffer, and subjected to SDS-PAGE analysis. The cell pellets were resuspended in 5 mL MRS broth, subjected to butanol-tolerance assay, and compared to control samples washed in sterile double distilled water.

2.6 | SDS-PAGE

SLP were visualized by SDS-PAGE using 10% Mini-Protean TGX Stain-Free Precast gel and MiniPROTEAN® Tetra cell (BioRad Laboratories, CA, USA), followed by gel staining with Coomassie Brilliant Blue R-250. Prestained Protein Marker (Crystalgen, USA) was used as a protein molecular weight standard.

FIGURE 1 Phylogenetic relationships between the new isolated butanol-resistant strains based on 16S rRNA gene sequences. The tree was constructed using the UPGMA method by the program MEGA6 [24]

FIGURE 2 Correlation between the relative growth rate of LAB strains in MRS medium containing 2% butanol and their CSH in MRS estimated by microbial adhesion to hydrocarbons method [22]. As 100% the growth of the respective strain in MRS was perceived. Seventy-four strains, tolerant to 2% butanol at a different extent were analyzed

NCDO 1193, and *Lb. delbrueckii* ssp. *lactis* ATCC 15808, which had a relative growth rate in the presence of 2% v/v butanol above 60% of the control. Thus, our results confirmed the reported so far high butanol stress resistance of LAB, exceeding the limits for several species. For instance, the first *Lb. delbrueckii* ssp. *bulgaricus* strains retaining 30% relative growth rate at 3%, and capable to grow at 4% butanol were isolated. The growth at 3% butanol obtained for *P. acidilactici* DF3 (55.7% relative growth rate) is the highest reported to date and place the species among the most butanol-resistant bacteria isolated so far [2,4,11]. Some of the novel strains reported here could be used as microbial cell factories for butanol production and as a valuable genetic resource for strains improvement.

3.2 | Impact of the CSH on LAB tolerance to butanol

LAB owned complicated cell envelope providing their adhesion to eukaryotic cells, biofilm formation, and co-aggregation with pathogens. It consists of a cytoplasmic membrane and a cell wall that is additionally surrounded by an external polysaccharide capsule or SLP [26]. The outermost layers on the cell wall determine to a great extent, the hydrophobic properties of the microbial cell. In general, CSH of LAB varies widely and is a strain-dependent feature. The highly hydrophobic *Lactobacillus* isolates

(*Lb. acidophilus*) have water contact angles above 100°, the extremely hydrophilic ones (*Lb. casei*) are with water contact angles of 19°. However, they may also be affected by environmental parameters as the ionic strength of the medium [18].

In order to define CSH of butanol-tolerant strains, microbial adhesion to hydrocarbons test by partition in hexadecane was used. The strains with a hydrophobicity index higher than 70% were classified as hydrophobic; with CSH between 36 and 70% are medium hydrophobic; those with an index below 35% are assigned as hydrophilic [23,27].

Our results revealed a clear correlation between the tolerance to butanol and CSH index. All highly butanol-resistant strains demonstrated a very low degree of hydrophobicity. The most resistant strains of *P. acidilactici* owned fully hydrophilic cell envelope, as with the quantitative increase of CSH index of the strain, decreased the percentage of relative growth rate at all the concentrations of butanol (Table 1). The same occurrence was observed for butanol-tolerant *Lb. delbrueckii* ssp. *bulgaricus* Y69 and Ym2, possessing CSH under 0.5%. Interestingly, both strains grew well at a concentration of 3% butanol, while the type strain *Lb. bulgaricus* NCDO 2395 hardly tolerated 2%, corresponding to the significant CSH index of 68.6%. Total low value of CSH of Y69 isolate is probably affected by the capability of the strain to form exopolysaccharide hydrophilic capsule (data not shown).

TABLE 2 LAB strains CSH measured after cultivation of the strains in MRS medium containing 0% – 4% butanol (v/v). The designation “NA” means “not available”, since the biomass formed by the strain at this butanol concentration was not sufficient to perform CSH measurement

Species	Strain	CSH ^a			
		Butanol 0%	Butanol 1%	Butanol 2%	Butanol 3%
Lactic acid cocci isolates					
<i>P. acidilactici</i>	DF3	0.0	0.0	0.0	0.0
<i>P. acidilactici</i>	32	0.0	0.0	0.0	0.0
<i>P. acidilactici</i>	Y1	0.0	0.0	0.0	0.0
<i>P. acidilactici</i>	9	0.0	0.0	0.0	0.0
<i>P. acidilactici</i>	BB3	0.0	0.0	0.0	0.0
<i>P. acidilactici</i>	ED	1.1 ± 0.6	0.0	0.0	0.0
<i>P. acidilactici</i>	57	8.2 ± 1.1	1.8 ± 0.8	0.0	0.0
<i>Ent. faecium</i>	c1	8.5 ± 1.6	1.5 ± 0.8	0.0	0.0
<i>Ent. faecium</i>	c4	12.2 ± 1.5	8.2 ± 0.8	0.0	0.0
<i>P. acidilactici</i>	59	13.4 ± 0.8	4.6 ± 0.8	0.0	0.0
<i>P. acidilactici</i>	33	13.6 ± 1.5	10.6 ± 0.5	0.0	0.0
<i>Ent. faecium</i>	H	15.1 ± 1.5	11.1 ± 0.5	0.0	0.0
<i>P. acidilactici</i>	Lin1	15.8 ± 1.8	12.7 ± 0.5	0.0	0.0
<i>P. acidilactici</i>	56	17.1 ± 1.5	14.8 ± 0.5	0.0	0.0
<i>Ent. faecium</i>	62	18.3 ± 1.6	14.6 ± 0.8	0.0	0.0
<i>Str. equinus</i>	D	18.9 ± 1.6	14.2 ± 0.8	0.0	0.0
<i>Ent. faecium</i>	1.2	22.5 ± 1.8	16.4 ± 0.8	0.0	0.0
Lactobacillus isolates					
<i>Lb. delb. ssp. bulgaricus</i>	Y69	0.0	0.0	0.0	0.0
<i>Lb. helveticus</i>	Y22	8.0 ± 1.7	0.5 ± 0.5	0.0	0.0
<i>Lb. plantarum</i>	Ym1	21.1 ± 1.2	20.6 ± 0.8	9.8 ± 0.5	9.2 ± 0.5
<i>Lb. plantarum</i>	S12	24.2 ± 0.8	22.4 ± 0.8	10.4 ± 0.5	10.2 ± 0.5
<i>Lb. delb. ssp. lactis</i>	Y12	24.9 ± 1.0	21.9 ± 0.8	11.4 ± 0.5	10.8 ± 0.5
<i>Lb. delb. ssp. bulgaricus</i>	Y10	25.4 ± 0.8	22.6 ± 0.8	10.3 ± 0.5	9.4 ± 0.5
<i>Lb. plantarum</i>	Bom 816	46.2 ± 1.5	42.5 ± 1.0	29.1 ± 0.8	NA
<i>Lb. pentosus</i>	N3	56.6 ± 1.7	40.2 ± 1.0	29.5 ± 0.8	NA
<i>Lb. helveticus</i>	Y16	80.6 ± 3.2	61.5 ± 1.0	NA	NA
Referent <i>Lactobacillus</i> strains					
<i>Lb. fermentum</i>	ATCC 14931	0.5 ± 0.5	0.0	0.0	0.0
<i>Lb. casei</i>	ATCC 393	2.8 ± 0.5	0.0	0.0	0.0
<i>Lb. paraplantarum</i>	ATCC 700211	3.0 ± 0.9	0.2 ± 0.5	0.0	0.0
<i>Lb. fermentum</i>	ATCC 14932	3.1 ± 1.1	0.2 ± 0.5	0.0	0.0
<i>Lb. delb. ssp. lactis</i>	ATCC 15808	8.0 ± 1.0	3.8 ± 0.5	0.0	0.0
<i>Lb. plantarum</i>	ATCC 1193	26.4 ± 1.5	22.7 ± 0.8	11.6 ± 0.5	NA
<i>Lb. helveticus</i>	ATCC 27558	29.6 ± 1.7	26.3 ± 0.8	NA	NA
<i>Lb. pentosus</i>	ATCC 363	38.2 ± 0.8	36.6 ± 1.0	24.7 ± 1.0	NA
<i>Lb. delb. ssp. bulgaricus</i>	NCDO 2395	68.6 ± 2.1	51.4 ± 1.0	42.5 ± 1.0	NA
<i>Lb. delb. ssp. bulgaricus</i>	DSM 20081	85.2 ± 3.5	66.3 ± 1.0	NA	NA

^aThe CSH was estimated according to microbial adhesion to hydrocarbons method [22].

Regarding *Lb. helveticus* isolates, quite different strains' behavior was observed. Y22 had low CSH (~8%) and grew even at 4% butanol; Y16 was hydrophobic (~81%) and hardly tolerated 2% butanol. Considering the reference strains, the

more resistant to butanol strains also had the less CSH index. The strains possessing a high tolerance to 2% butanol *Lb. fermentum* ATCC 14931 and 14932, *Lb. delbrueckii* ssp. *lactis* ATCC 15808, *Lb. casei* ATCC 393, *Lb. rhamnosus* ATCC

7469, and *Lb. plantarum* NCDO 1193 own hydrophilic cell surface. *Lb. acidophilus* ATCC 4356, which had the highest CSH of 93.5%, tolerated hardly 2% butanol.

The correlation between hydrophobicity and butanol tolerance of the isolates is shown in Figure 2. It revealed a clear inverse proportional relation between CSH and the relative growth rate at 2% butanol. A large number of tested strains belonging to phylogenetically remote species allow stating that regardless of the species affiliation, the lower was the strain's CSH the higher was its butanol tolerance.

3.3 | Natural shift of CSH of LAB strains in butanol environment

The levels of hydrophobicity were estimated after cultivation of selected strains in media containing 1–4% v/v butanol. The results, presented in Table 2 showed obvious tendency of CSH decrease with increasing butanol amount. The most resistant strains remained hydrophilic in all trials. Others apparently attempted to reduce their level of hydrophobicity at increasing butanol concentrations, because the more hydrophilic cell envelope would prevent butanol adhesion to the microbial surface, i.e. the increased hydrophilicity would repulse butanol avoiding its penetration into the cell. This cells' response is consistent with a previous finding in *E. coli* K-12 mutants, which had a cell surface less hydrophobic compared to the parental cells, and displayed higher tolerance to organic solvents [28]. Similar adaptation mechanism to the toxic effects of organic solvents was described by Torres et al. (2009) for *B. licheniformis* S-86 [29].

3.4 | Butanol tolerance increase by CSH decrease via SLP removal

CSH is complex phenomenon, usually determined by the presence of surface layer, monomolecular crystalline array, composed of non-covalently linked protein units. SLPs of lactobacilli have high content of hydrophobic amino acids, ranging between 31.9% (*Lb. gasseri* ATCC 19992) and 38.7% (*Lb. johnsonii* DSM 20553). Another typical feature of *Lactobacillus* SLP is the high number of positively charged amino acid residues, relatively low Mw (40–50 kDa) and the lack of glycosylation [30]. Species known as highly hydrophobic (and all containing Surface layer) are: *Lb. acidophilus*, *Lb. gasseri*, *Lb. brevis*, *Lb. paracasei*, *Lb. delbrueckii*, *Lb. crispatus*, *Lb. johnsonii* [26,30,31]. Contrariwise, LAB that are devoid of SLP, have usually hydrophilic surface, as *Lb. casei*, and *Lb. rhamnosus* [30].

The attempt to extract SLPs by LiCl method from the cells of the hydrophilic isolates of *P. acidilactici*, *Ent. faecium*, and *Lb. bulgaricus* failed, thus suggesting probable SLP absence. In contrast, SLP with molecular weight ~52 kDa were isolated from *L. helveticus* strains Y22, Y16, and ATCC 27558

FIGURE 3 SDS-PAGE profiles of the SLP (marked with arrows) of selected *Lactobacillus* strains extracted with 5M LiCl. Lanes and samples: 1) *Lb. helveticus* ATCC 27558, 2) *Lb. helveticus* Y22, 3) *Lb. helveticus* Y16, 4) *Lb. plantarum* NCDO 1193, 5) *Lb. plantarum* S12, 6) *Lb. plantarum* Ym1, 7) Pre-stained Protein Marker (Crystalgen, USA)

and demonstrated by SDS-PAGE (Figure 3). The extraction of SLP from *Lb. plantarum* strains S12, Ym1, and NCDO 1193 showed the presence of SLP-like proteins with Mw~40 kDa (Figure 3), similar to those of the non-specific adhesive proteins of *Lb. plantarum* strains [15].

The effect of SLP removal on butanol tolerance was investigated using selected SLP containing *L. helveticus* and *Lb. plantarum* strains (Figure 4), as samples with SLP removed (treated with LiCl) were compared with the water washed respective (untreated controls). CSH values after SLP removal evidently decreased in all cases (Figure 4A). The relative growth rate at 1–3% butanol (Figure 4B, C, and D) of the cultures with removed SLP demonstrated improved butanol tolerance, exactly correlating with CSH decrease. Regarding the strains Y22, S12, Ym1, and NCDO 1193, butanol resistance increase ranged from 3.3 to 10.4%. A dramatic double increase was observed in tolerance to 2% butanol of the hydrophobic *Lb. helveticus* strains Y16 and ATCC 27558. Moreover, both strains did not resist 3% butanol, but started tolerate it after SLP removal. Assuming, the role of SLP as an important factor in LAB hydrophobicity was confirmed. Novel approach to increase butanol tolerance of certain strains by SLP removal was suggested.

4 | CONCLUDING REMARKS

The findings presented here, are the first that connect the CSH with bacterial tolerance to butanol. A comprehensive study of a large and diverse microbial group (74 strains from 23 different LAB species), revealed the clear inversely proportional

FIGURE 4 The influence of S-layer proteins removal on the cell surface hydrophobicity (A) and the butanol tolerance (B, C, D).

Designations: LP – *Lb. plantarum*, LH – *Lb. helveticus*. The following strains were analysed: *Lb. plantarum* ATCC 1193, S12, Ym1; *Lb. helveticus* ATCC 27558, Y22, Y16. SLPs were removed by LiCl treatment [25]; these cultures were designated “treated”. Equal amounts of strains’ cells washed in sterile double distilled water were used as “untreated” controls. The relative growth rates of untreated and treated cells are compared in MRS medium, containing 1% (B), 2% (C), and 3% (D) butanol

relationship between CSH and butanol resistance. Moreover, an adaptive tendency of hydrophobicity decrease after exposure to rising butanol concentrations was observed. The role of SLP as an important factor in LAB hydrophobicity was confirmed. Proving that CSH may be both spontaneously, and artificially decreased, the present study opens up the possibility of its multiple alterations by biochemical, environmental, or bioprocess approaches, aiming to achieve higher butanol tolerance of the producing strains.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

1. Branduardi, P., de Ferra, F., Longo, V., Porro, D., Microbial *n*-butanol production from Clostridia to non-Clostridial hosts. *Eng. Life Sci.* 2014, 14, 16–26.
2. Pomaranski, E., Tiquia-Arashiro, S. M., Butanol tolerance of carboxydutrophic bacteria isolated from manure composts. *Environ. Technol.* 2016, 37, 1970–1982.
3. Green, E. M., Fermentative production of butanol - the industrial perspective. *Curr. Opin. Biotechnol.* 2011, 22, 337–343.
4. Liu, S., Qureshi, N., Hughes, S. R., Progress and perspectives on improving butanol tolerance. *World J. Microbiol. Biotechnol.* 2017, 33, 51.
5. Liu, X. B., Gu, Q. Y., Yu, X. B., Repetitive domestication to enhance butanol tolerance and production in *Clostridium acetobutylicum* through artificial simulation of bio-evolution. *Bioresour. Technol.* 2013, 130, 638–643.
6. Peabody, G. L., Kao, K. C., Recent progress in bio-butanol tolerance in microbial systems with an emphasis on *Clostridium*. *FEMS Microbiol. Lett.* 2016, 363, fnw017.
7. Fletcher, E. et al., Characterization of the effects of *n*-butanol on the cell envelope of *E. coli*. *Appl. Microbiol. Biotechnol.* 2016, 100, 9653–9659.
8. Torres S., Pandey A., Castro G. R., Organic solvent adaptation of Gram positive bacteria: Applications and biotechnological potentials. *Biotechnol. Adv.* 2011, 29, 442–452.
9. Kobayashi, H. et al., Outer membrane changes in a toluene-sensitive mutant of toluene-tolerant *Pseudomonas putida* IH-2000. *J. Bacteriol.* 1999, 181, 4493–4498.
10. McKarns, S. C. et al., Correlation between hydrophobicity of short-chain aliphatic alcohols and their ability to alter plasma membrane integrity. *Fundam. Appl. Toxicol.* 1997, 36, 62–70.
11. Berezina, O. V. et al., Reconstructing the clostridial *n*-butanol metabolic pathway in *Lactobacillus brevis*. *Appl. Microbiol. Biotechnol.* 2010, 87, 635–646.

12. Ting, C. et al., Screening butanol-tolerant *Enterococcus faecium* capable of butanol production. *Appl. Biochem. Biotechnol.* 2012, 168, 1672–1680.
13. Kanno, M. et al., Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. *Appl. Environ. Microbiol.* 2013, 79, 6998–7005.
14. Li, J. , et al., Screening and characterization of butanol-tolerant micro-organisms. *Lett. Appl. Microbiol.* 2010, 50, 373–379.
15. Ramiah, K., van Reenen, C. A., Dicks, L. M., Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. *Res. Microbiol.* 2008, 159, 470–475.
16. Krasowska, A., Sigler, K., How microorganisms use hydrophobicity and what does this mean for human needs? *Front. Cell. Infect. Microbiol.* 2014, 4, 112.
17. Van der Mei, H. C. et al., Cell surface hydrophobicity is conveyed by S-layer proteins – a study in recombinant lactobacilli. *Colloids Surf. B* 2003, 28, 127–134.
18. Vadillo-Rodríguez, V. et al., Dynamic cell surface hydrophobicity of *Lactobacillus* strains with and without surface layer proteins. *J. Bacteriol.* 2004, 186, 6647–6650.
19. Velikova, P. et al., Starch utilization routes in lactic acid bacteria: New insight by gene expression assay. *Starch-Stärke* 2016, 68, 953–960.
20. Petrova, P., Petrov, K., Direct starch conversion into L (+) lactic acid by a novel amylolytic strain of *Lactobacillus paracasei* B41. *Starch-Stärke* 2012, 64, 10–17.
21. Petrova, P., Emanuilova, M., Petrov, K., Amylolytic *Lactobacillus* strains from Bulgarian fermented beverage Boza. *Z. Naturf. C* 2010, 65, 218–224.
22. Rosenberg, M., Gutnik, D., Rosenberg, E., Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 1980, 9, 29–30.
23. Ocaña, V. S., Bru, E., de Ruiz Holgado, A., Nader-Macias M. E., Surface characteristics of lactobacilli isolated from human vagina. *J. Gen. Appl. Microbiol.* 1999, 45, 203–212.
24. Tamura, K. et al., MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 2013, 30, 2725–2729.
25. Lortal, S., van Heijenoort, J., Gruber, K., Sleytr, U. B., S-layer of *L. helveticus* ATCC 12046: isolation, chemical characterization and reformation after extraction with LiCl. *J. Gen. Microbiol.* 1992, 138, 611–618.
26. Papadimitriou, K. et al., Stress physiology of Lactic Acid Bacteria. *Microbiol. Mol. Biol. Rev.* 2016, 80, 837–890.
27. Ghatani, K., Tamang, B., Assessment of probiotic characteristics of lactic acid bacteria isolated from fermented yak milk products of Sikkim, India: Chhurpi, Shyow, and Khachu, *Food Biotechnol.* 2017, 31, 210–232.
28. Aono, R., Kobayashi, K., Cell surface properties of organic solvent tolerant mutants of *E. coli* K-12. *Appl. Environ. Microbiol.* 1997, 63, 3637–3642.
29. Torres, S. et al., Enzymatic synthesis of banana flavour (isoamyl acetate) by *Bacillus licheniformis* S-86 esterase. *Food Res. Int.* 2009, 42, 454–460.
30. Ävall-Jääskeläinen, S., Palva, A., *Lactobacillus* surface layers and their applications. *FEMS Microbiol. Rev.* 2005, 29, 511–529.
31. Bönisch, E. et al., Lipoteichoic acid mediates binding of a *Lactobacillus* S-layer protein. *Glycobiology* 2018, 28, 148–158.

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