The aim of this study was to provide new insight into the mechanism whereby the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) locates to cell walls of *Lactobacillus plantarum* 299v. After purification, cytosolic and cell wall GAPDH (cw-GAPDH) forms were characterized and shown to be identical homotetrameric active enzymes. GAPDH concentration on cell walls was growth-time dependent. Free GAPDH was not observed on the culture supernatant at any time during growth, and provoked cell lysis was not concomitant with any reassociation of GAPDH onto the cell surface. Hence, with the possibility of cw-GAPDH resulting from autolysis being unlikely, entrapment of intracellular GAPDH on the cell wall after a passive efflux through altered plasma membrane was investigated. Flow cytometry was used to assess *L. plantarum* 299v membrane permeabilization after labeling with propidium iodide (PI). By combining PI uptake and cw-GAPDH activity measurements, we demonstrate here that the increase in cw-GAPDH concentration from the early exponential phase to the late stationary phase is closely related to an increase in plasma membrane permeability during growth. Moreover, we observed that increases in both plasma membrane permeability and cw-GAPDH activity were delayed when glucose was added during *L. plantarum* 299v growth. Using a double labeling of *L. plantarum* 299v cells with anti-GAPDH antibodies and propidium iodide, we established unambiguously that cells with impaired membrane manifest five times more cw-GAPDH than unaltered cells. Our results show that plasma membrane permeability appears to be closely related to the efflux of GAPDH on the bacterial cell surface, offering new insight into the understanding of the cell wall location of this enzyme.

**Keywords:** *L. plantarum* 299v, surface-bound GAPDH, membrane permeability

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential intracellular glycolytic enzyme that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate. It also fulfills diverse functions unrelated to its glycolytic activity. In fact, GAPDH was found on the surface of Gram-positive [19, 23, 32, 27], but also Gram-negative, bacteria [11, 34, 17], fungi, and other eukaryotic organisms [2, 9, 14]. Until now, the mechanism that contributes to surface location of GAPDH has remained unknown. This cytoplasmic enzyme is considered to be a housekeeping molecule; it does not possess any signal sequences, cell wall-anchoring motifs, or hydrophobic membrane-spanning regions that could target it to a secretory pathway. Recent findings suggest that GAPDH and some other exported glycolytic enzymes, termed also as “anchorless proteins,” could reassociate with the cell walls after secretion by means of an undefined mechanism(s) [3, 7]. It has been suggested that once secreted on the surface, these enzymes can bind through charge and/or hydrophobic interactions to cell wall components [1]. In *Saccharomyces cerevisiae*, cw-GAPDH location into the cell wall seems to be dependent on environmental stress factors [10]. Indeed, starvation and/or a rise in temperature cause an increase of GAPDH activity at the cell wall surface that requires neither de novo protein synthesis nor the participation of ubiquitin yeast stress system. The same was observed for *C. albicans* [15]. In several pathogen organisms, this
enzyme is an immunodominant antigen [7, 29, 24], and/or serve as adhesins or invasins that may facilitate the colonization of host cells and establishment of infection by interaction with matrix extracellular components or by binding and activation of the plasminogen system [27, 28, 16, 3]. GAPDH was also reported on the surface of some nonpathogen lactobacilli [19]. This enzyme was identified in the extracellular proteome of Lactobacillus crispatus ST1 and seems to bind the plasminogen system and enhance its activation [19]. Recently, the occurrence of cell wall-associated GAPDH has been described on the surface of Lactobacillus plantarum LA318 and 423 strains, and found to be involved in the adherence of bacteria to gastric mucin and Caco-2 cells [21, 31]. L. plantarum is a widespread organism that is frequently isolated from plant and various fermented foods and is a natural inhabitant of the human intestinal tract. Some probiotic strains have been used to provide health benefits for the consumer [30], and several clinical studies have claimed the beneficial effects of L. plantarum 299v in functional bowel disorders [26].

In this work, we report on the origin of the cell surface-associated GAPDH of the probiotic strain L. plantarum 299v. First, we isolated both cytoplasmic and cell wall-associated forms of the enzyme and established the structural identity of the two proteins in this strain. Then, we observed a change in the concentration of this housekeeping enzyme on the bacterial cell wall during growth and tried to investigate the physiological events underlying the exportation of active GAPDH to the bacterial cell surface. After ruling out the possibility of cw-GAPDH resulting from autolysis, we examined disturbances occurring at the single-cell level during L. plantarum 299v growth. Flow cytometry measurement (FCM) was used to assess membrane permeability and metabolic activity. Finally, a double labeling of bacterial cells with anti-GAPDH antibodies and propidium iodide was performed to establish a close relationship between plasma membrane integrity and cw-GAPDH concentration.

Materials and Methods

Strain and Culture Conditions
Strain L. plantarum 299v was obtained from commercial probiotic products (Probi AB, Sweden). The strain was grown in MRS broth (BD Difco, France) at 37°C.

Preparation of Bacterial Cell Wall Extract
Cells from a 200-ml overnight culture of L. plantarum 299v were harvested by centrifugation at 3,000 ×g and washed twice with cold PBS–citrate buffer, pH 4.5, suspended in 100 mM Tris buffer, pH 8.0, containing 1 mM PMFS, 5 mM EDTA, and incubated for 40 min at room temperature. After centrifugation at 3,000 ×g at 4°C, the supernatant was filtered through a 0.45-µm membrane, dialyzed, concentrated 10-fold, and stored at ~20°C.

Electrophoresis
Cell wall extract of 299v strain was separated by SDS–PAGE as described by Laemmli [22] in 10% polyacrylamide gel. Proteins were stained with Coomassie blue (G-250) or silver nitrate.

Peptide Mass Fingerprint of Cell Wall Extracted Proteins
Bands of separated cell wall proteins were excised from the SDS gel and submitted to in-gel proteinolysis [4 h at 37°C with 5 µg/ml trypsin (Sigma-Aldrich, France) in 100 mM NH4HCO3, pH 8]. The digestion was halted through the addition of 60% acetonitrile (v/v) – 0.1% (v/v) acetic acid in water. The peptide extract was concentrated to a final volume of 25 µl and stored at ~20°C. One µl of the peptide mixture was mixed with an equal volume of matrix solution [4 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile (v/v) and 0.1% (v/v) aqueous TFA] and deposited on the target plate of a MALDI-Q-TOF Premier instrument (Waters, Manchester, U.K.). Glu-fibrinopeptide (Sigma; m/z 1570.6774) was used for a single point Lock-mass (Waters, Manchester, U.K.) correction applied to all spectra. Calibrated spectra were submitted to database searches (Swissprot, NCBI) using the MASCOT mass mapping software.

Purification of Cell Wall and Cytosolic GAPDHs
Cells from an overnight culture of 299v strain were extracted with 100 mM Tris buffer, pH 8.0, as described above. The supernatant was concentrated 10-fold by ultrafiltration on a 10 kDa molecular mass cut-off membrane in a stirred UF cell (Millipore, U.S.A.) in the presence of 5 mM dithiothreitol (DTT). This extract was then treated with (NH4)2SO4 [85% (w/v) saturation] at 4°C for 18 h. After centrifugation at 16,000 ×g, the precipitate was suspended in 4 ml of 25 mM NH4HCO3 buffer, pH 7.5, containing 5 mM DTT, 5 mM EDTA, and 1 mM PMSF (buffer A) and dialyzed overnight at 4°C against the same buffer. The dialyzed sample was applied to a 5-ml Fast Flow blue Sepharose CL 6B column (GE Healthcare, Sweden) pre-equilibrated with buffer A at a flow rate of 2 ml/min. Bound proteins were eluted with a linear gradient of 0 to 10 mM nicotine amide adenine dinucleotide (NAD) in buffer A, pH 8.5 (Buffer B). Two ml fractions were collected and characterized for proteins concentration [5] and GAPDH activity. Homogeneity of the active fractions was checked by SDS–PAGE.

Cytosolic GAPDH was extracted from pellets of lysed cells that were washed twice with buffer A, by means of mechanic crushing in a mini bead beater (Biospec Products, U.S.A.). Cell debris were sedimented by centrifugation at 16,000 ×g. The crude cell lysate was treated as described above for cell wall GAPDH.

Mass Spectrometry of Purified GAPDHs
The purity of cytosolic and cw-GAPDH proteins was assessed by means of LC-ESI-MS [150×4.6 mm C4 column (Vydac) coupled to an LCQ Advantage ion trap mass spectrometer (Thermo Fisher)]. For peptide mapping, after reduction (DTT), modification with iodoacetamide, and digestion with trypsin, peptides obtained from each purified GAPDH form were analyzed by LC-ESI-MS/MS [150×2.1 mm C8 column (Vydac) operated at 200 µl/min]. MS/MS data were acquired in an automated data-dependent mode with dynamic exclusion.

GAPDH Activity Assay
A GAPDH assay was performed to establish the presence of an active cw-GAPDH enzyme in L. plantarum 299v. Pellets from 1-ml culture, harvested at different stages of growth, were washed twice.
with PBS–citrate buffer, pH 5.0, and residual cw-GAPDH activity was measured by recording the absorbance at 340 nm according to Fersinid [12] after a 4-min incubation time with the substrate glyceraldehyde-3-phosphate. In addition, free GAPDH activity in the culture supernatant was monitored at each stage of growth. One unit of GAPDH activity corresponds to 1 µmole of NADH generated per minute.

**Molecular Mass Estimation for Native GAPDHs**
The molecular mass of native GAPDHs was estimated by size exclusion chromatography. Purified GAPDHs in 50 mM NH₄HCO₃ buffer, pH 7.8, containing 5 mM DTT, 5 mM EDTA, and 1 mM PMSF were applied to a Superdex 200 10/300 GL column (Tricorn, GE Healthcare) equilibrated with the same buffer at a flow rate of 0.5 ml/min. GAPDH-containing fractions were determined by enzymatic assay, and molecular mass was estimated according to a standard calibration curve established under the same conditions with a molecular mass marker kit (29–700 kDa, Sigma).

**Preparation of Polyclonal Antibodies Against Purified Cell Wall-Associated GAPDH**
Polyclonal anti-GAPDH antibodies were prepared by Proteogenix (Valparc, France). A New Zealand white rabbit was immunized with 2 mg of purified cw-GAPDH. Ninety days after immunization, serum samples were collected and GAPDH IgGs were purified on an affinity column.

**Cell Wall Location of L. plantarum 299v GAPDH**
Cell wall location of GAPDH was assayed by immunofluorescence. Cells from an overnight culture of 299v were washed with PBS buffer at pH 7.4 or 4.5 and then suspended at 10⁷ CFU/ml in PBS at the respective pH. After that, glass slides were coated with the cells, fixed with 3.5% (w/v) of paraformaldehyde, and incubated for 30 min at 37°C with the anti-GAPDH IgG as primary antibodies (d 1: 50), and with Alexa-488-conjugated goat anti-rabbit IgG (d 1:100) as a secondary antibody, incubated under the same conditions (Molecular Probes, The Netherlands). Alexa-488 fluorescence was tested pH independent, and stained bacteria were observed in a Zeiss confocal microscope (Carl Zeiss, Jena, Germany).

**Cell Lysis and GAPDH Cell Wall Association**
A culture of L. plantarum 299v at the early stationary phase (12 h) was incubated for 15, 30, and 60 min at 37°C with an intracellular GAPDH. The latter, obtained from a crude mechanical cell lysate of the same culture, was added at a final concentration of 0.4 U/ml. Subsequently, cells from a 1-ml culture were harvested and washed twice with PBS–citrate buffer, pH 5.0, and residual GAPDH activity was measured on both washed cells and culture supernatants. In parallel, the stability of GAPDH added to a cell free supernatant was monitored by measuring its activity after the afore-mentioned periods. Prior to the deliberate addition of intracellular GAPDH at 12 h cell wall and released GAPDH activities were assayed during the growth time. Bacterial enumeration on MRS agar was performed both on washed cells and culture supernatants. In this way, the probes could be used to estimate simultaneously esterase activity (FDA) indicative of metabolic activity and propidium iodide for membrane damage. Data were analyzed with the Cell Quest software (Becton Dickinson).

**Statistics**
Statistical analyses of all data were performed using the Student’s t-test and reported as the mean±SD.

**RESULTS**

**GAPDH Identification and Characterization from L. plantarum 299v Cell Wall**
Surface proteins from L. plantarum 299v cells extracted with 100 mM Tris buffer, pH 8.0, were analyzed by SDS–PAGE. Six major detectable protein bands (numbered from 1 to 6 in Fig. 1A, line 1) were excised, submitted to in-gel proteolysis, and analyzed by MALDI–mass spectrometry. Identified proteins include well-characterized surface-exposed proteins like chaperone protein clpB (band 1), elongation factor G (band 2), chaperone protein DnaK (HSP70) (band 3), chaperomin GroEL (band 4), enolase (band 5), and GAPDH (band 6).

We purified 299v GAPDHs from (i) cell wall and (ii) cytosolic extracts by a single-step pseudo-affinity chromatography on Blue Sepharose CL 6B. SDS–PAGE analysis of the eluted fractions with GAPDH activity showed in each case a single band around 39 kDa molecular mass (Fig. 1A, lines 2 and 3). Cell wall and cytosolic GAPDH...
calculated specific activities were 22 and 10 U/mg, respectively. The two purified enzymes had identical retention times on a C4 reversed-phase column, and identical average molecular mass as indicated by LC-ESI-MS analysis. Both proteins exist in two forms: one with N-terminal methionine (theoretical average mass 36,438 Da, calculated from SwissProt entry Q88YH6), and, as expected owing to the presence of serine as the penultimate amino acid [18], a second form without N-terminal methionine (calculated mass 36,307 Da). In all cases, measured masses were in agreement with those calculated: respectively, 36,430 and 36,436±10 Da for cell wall and cytosolic forms with N-terminal methionine, and 36,306 and 36,312±10 Da for those without methionine. This result indicated that both cytosolic and cw-GAPDH primary structures were identical and did not bear post-translational modifications. A careful examination of tryptic peptide maps by LC-ESI-MS/MS confirmed this hypothesis. Indeed, identical chromatographic profiles and peptide sequences covering the whole primary structure were found in both cases (data not shown).

Next, we assessed the molecular mass of the native GAPDH forms. The multimeric state of both GAPDHs was probed by means of FPLC size exclusion chromatography. Both GAPDH forms eluted with an apparent molecular mass of 148 kDa protein, which corresponds to a tetrameric assembly (calculated: 145.6 kDa). These results were in agreement with those reported by Fothergill-Gilmore and Michels [13], where the authors indicated that cytosolic GAPDH has been remarkably conserved during evolution, and has a homotetrameric structure composed of a 35–37 kDa subunit.

**Cell Wall Location of L. plantarum 299v GAPDH**

Surface-exposed L. plantarum 299v GAPDH was revealed by indirect immunofluorescence after labeling with anti-GAPDH immunoglobulin. The surface location of this GAPDH was confirmed by indirect immunofluorescence of L. plantarum 299v cell wall. The cells were washed in PBS, pH 7.4, and phosphate-citrate buffer at pH 4.5, and revealed with anti-GAPDH immunoglobulin and Alexa-488 goat anti-rabbit IgG as secondary antibody (left). Phase-contrast images were given on right.
enzyme was pH dependent. Indeed, GAPDH was firmly bound to the 299v cell surface at pH 4.5, whereas it was released at pH 7.4 (Fig. 2). These results were in agreement with those reported for GAPDH of L. crispatus ST1 [1].

Cell Wall-Associated GAPDH Activity Change During Growth Phase
cw-GAPDH activity was assayed on intact L. plantarum 299v cells throughout bacterial growth. Simultaneously, culture medium pH and bacteria enumeration (CFU) were monitored and the concentration of residual glucose was quantified by high-performance liquid chromatography (HPLC) on a HPX87 column (BioRad) according to Morales et al. [25]. As indicated by the data in Table 1, cw-GAPDH activity was not constant during bacterial growth. First, we observed a 3-fold increase from the latency (3 h) to the end of the exponential phase (12 h), and then a strong 23-fold increase from 16 h to 24 h during the stationary phase. It is noteworthy that a high level of cw-GAPDH activity is concomitant with a low residual glucose concentration in the culture medium at the end of growth (Table 1). This increase in activity was not due to substrate (G-3-P) diffusion into bacteria cytoplasm during the enzymatic assay because this increase was not observed after cell washing with PBS, pH 7.4. SDS-PAGE analysis of cell wall extracts during L. plantarum 299v growth revealed a gradual increase of concentration of GAPDH and other surface-associated proteins (Fig. 1B). During the same time frame, GAPDH activity assay and SDS-PAGE analysis of L. plantarum 299v culture supernatants revealed the absence of released GAPDH (Table 1).

The ability of cytosolic GAPDH to reassociate with cell walls after bacterial autolysis was investigated. Cell lysate containing intracellular GAPDH was added to a L. plantarum 299v culture at an early stationary phase in order to simulate bacterial autolysis. The deliberately added GAPDH was rapidly inactivated in the culture supernatant. After 1-h incubation with the cell culture at 37°C, it had lost 90% of its initial activity without any concomitant increase in cw-GAPDH activity (Fig. 3). Moreover, the inactivation rate of this enzyme was identical when the latter was added to a 12-h cell free supernatant culture.

Next, we measured physiological characteristics of L. plantarum 299v cells during the growth phase. We used FCM and appropriate probes such as propidium iodide (PI) and fluorescein diacetate (FDA). PI is an exclusion dye

![Fig. 3. Cell lysis and GAPDH cell walls location.](image)

Culture of L. plantarum 299v was allowed to grow for 12 h and then incubated for 15, 30, and 60 min at 37°C with intracellular GAPDH at final concentration of 0.4 U/ml, obtained from a crude mechanical cell lysate. Residual GAPDH activity was measured on both washed cell and culture supernatant. Cell wall and released GAPDH activity was monitored during growth time before the deliberate addition of intracellular GAPDH. Assays were done in triplicate; error bars show standard deviation (SD).

### Table 1. L. plantarum 299v growth: Evolution of some culture parameters and cw-GAPDH activity.

<table>
<thead>
<tr>
<th>Growth time (h)</th>
<th>OD$_{600}$ (nm)</th>
<th>CFU/ml</th>
<th>Glucose (g/l)</th>
<th>pH</th>
<th>cw-GAPDH activity (U/CFU)</th>
<th>Released GAPDH (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.38 (5.30±0.50)×10$^9$</td>
<td>15.73±1.32</td>
<td>5.86</td>
<td>(11.30±1.93)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.98 (2.80±0.10)×10$^9$</td>
<td>12.07±1.02</td>
<td>4.58</td>
<td>(12.90±0.97)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6.62 (6.40±1.15)×10$^9$</td>
<td>5.58±0.21</td>
<td>4.07</td>
<td>(18.90±3.63)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>8.09 (9.00±1.30)×10$^9$</td>
<td>2.95±0.12</td>
<td>3.86</td>
<td>(33.60±2.91)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>8.80 (7.10±0.73)×10$^9$</td>
<td>2.50±0.10</td>
<td>3.82</td>
<td>(40.40±4.59)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.20 (5.50±1.22)×10$^9$</td>
<td>2.20±0.11</td>
<td>3.79</td>
<td>(10.60±2.41)×10$^{12}$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.14 (3.46±0.55)×10$^9$</td>
<td>2.04±0.09</td>
<td>3.75</td>
<td>(9.13±1.46)×10$^{11}$</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*Released GAPDH in supernatant culture during L. plantarum 299v growth time.
ND: not detected.
that penetrates only damaged cells and subsequently increases their red fluorescence (FL3) on intercalating nucleic acids, and thus allows the testing of membrane permeability. FDA is an esterase substrate that penetrates cells and yields fluorescein (green fluorescence, FL1) on hydrolysis. Fluorescein is only retained by cells with intact membranes, thus allowing the estimation of their metabolic activity. The results are illustrated in Fig. 4A and 4B. The proportion of membrane-damaged cells increased gradually between the exponential phase (9 h) and the late stationary phase (24 h), as did cw-GAPDH activity over the same period (Fig. 4A). Furthermore, esterase activity evolved differently during growth; *L. plantarum* 299v cells presented a high level of green fluorescence from 6 h to 9 h and then metabolic activity diminished and remained almost constant during the stationary phase (Fig. 4A). Dot plots of FL1 versus FL3 (Fig. 4B) indicate that cells with damaged plasma membranes, PI⁺ subpopulations, from 12 to 24 h, seemed to maintain some metabolic activity compared with cells without any membrane integrity (48 h).

### Cw-GAPDH Location on Membrane-Damaged Cells

Polyclonal anti-GAPDH antibodies were used to probe the occurrence of GAPDH on the bacteria cell walls during the stationary phase (24 h). FCM analysis of *L. plantarum* 299v cells labeled with anti-GAPDH-Alexa 488 and PI revealed two cell subpopulations; namely, damaged cells (PI⁺) and non-damaged ones (PI⁻) (Fig. 5B). Cells labeled during the stationary phase with anti-GAPDH-Alexa 488 were found to be five times more common in the PI⁺ subpopulation than in the PI⁻ subpopulation (Fig. 5A). These observations prove that the cw-GAPDH concentration is significantly higher on the cell walls of damaged plasma membrane bacteria. These results are in agreement with the cw-GAPDH concentration and activity increases previously observed for *L. plantarum* 299v cultures taken during the stationary phase (Fig. 1B and 4A).

### Carbon Source Availability and cw-GAPDH Location

To discern whether the observed significant increase in cw-GAPDH activity during the stationary phase and carbon source depletion were interdependent, we supplemented the MRS medium with glucose. *L. plantarum* 299v cultures were fed either once at 9 h or twice at 9 and 12 h growth time (the final concentration of glucose after each feed was 20 g/l). CFU, cw-GAPDH activity, and membrane permeability were assessed after 24 h growth. When cultures were fed once or twice with glucose, the total number of bacteria increased 1.65-fold as compared with the control, but cw-GAPDH activity decreased significantly (up to 12-fold for a double glucose feed; Fig. 6). Similarly, the proportion of membrane-damaged cells was reduced by 30% and 70% under the same experimental conditions.

### Discussion

Six proteins were identified during this study of the cell surface of *L. plantarum* 299v, all of which have dual location both in the cytoplasm and on the bacterial surface. These proteins have low pIs and are weakly linked to *L. plantarum* 299v cell wall, since they are washed away at above pH 7.5. Among these proteins, enolase and GAPDH are referenced as housekeeping enzymes [29], and already known to be involved in a variety of functions independent
from their catalytic property (see introductory section). SDS–PAGE analysis of the surface proteins of *L. plantarum* 299v extracted at weak alkaline pH demonstrated that the presence of proteins from different functional categories onto the cell wall is modulated during growth time. This observation is in agreement with those reported by Kelly et al. [20] for *L. salivarius* cell wall-associated proteins, where the authors correlated the prominence of the 84-kDa band region, including DnaK, Ef-Ts and pyruvate kinase, with progression from the lag to stationary growth phase. A growing list of cytoplasmic proteins was identified on the surface of bacterial cell walls. However, the mechanisms involved in their location on the bacterial envelope remain unknown. These proteins contain neither signal sequences that could target them into secretory pathways nor cell wall-anchoring motifs. Authors have suggested that some of these proteins can reassociate with the cell wall after secretion [3, 7].

To gain new insight into this dual location problem, we focused our work on the efflux of GAPDH from the cytoplasm to the external cell wall of *L. plantarum* 299v. First, we established the fact that cytoplasmic and cw-GAPDH forms are identical in *L. plantarum* 299v. No post-translational modifications were found for cw-GAPDH. Obviously, GAPDH is incorporated to the bacterial cell surface in its cytoplasmic native form. Furthermore, an increase in cw-GAPDH concentration during growth was confirmed by both activity measurements and SDS–PAGE analysis of surface-associated protein. cw-GAPDH increases gradually during growth, with remarkable upshift during the stationary phase. Moreover, neither GAPDH activity nor GAPDH protein were detected in the *L. plantarum* 299v culture medium. In addition, we report in this study that provoked cell lysis is not concomitant with increases in cw-GAPDH activity. Indeed, cytosolic GAPDH added to a culture media at the early stationary phase rapidly becomes less active without there being compensatory association with the cell surface. After demonstrating that the possibility of cw-GAPDH resulting from autolysis is unlikely, we examined disturbances occurring during *L. plantarum* 299v growth at single-cell level by means of FCM. By combining FCM analysis and GAPDH activity measurements, we found for the first time a relationship between increases in cw-GAPDH concentration and the amount of damage done to plasma membranes during bacterial growth. In fact, below its pl (the theoretical value is 5.3),
cytosolic *L. plantarum* 299v GAPDH flowing through altered membrane may well bind to bacterial negatively charged cell surface components, such as lipoteichoic acids [1], and so become trapped on cell walls.

Double labeling of *L. plantarum* 299v cells with anti-GAPDH and PI during the stationary phase has unambiguously confirmed that bacterial cells whose membrane was impaired have a significantly higher cw-GAPDH in content. It is worth noting that observed green fluorescence intensity following to cw-GAPDH labeling, is probably underestimated owing to steric hindrance preventing good cw-GAPDH accessibility and to loss of PAb anti-cw-GAPDH during the numerous washing steps.

Moreover, GAPDH incorporation into *L. plantarum* 299v cell wall and membrane plasma permeability were both delayed when glucose was supplemented during growth. These results suggest that cw-GAPDH concentration, cell membrane permeability, and carbon source availability are interdependent parameters. GAPDH incorporation into *S. cerevisiae* and *C. albicans* cell walls has been shown to be responsive to starvation and/or temperature upshift [10, 15]. Nevertheless, permeability changes in plasma membranes related to such environmental stresses were not reported in these cases. *Lactobacillus* cells may overcome the stress exerted by starvation or an acidic environment by glucose consumption [8, 6]. Indeed, the addition of glucose to MRS culture medium, done at exponential and early stationary phases, had a significant protective effect on the membrane integrity of *L. plantarum* 299v, and a concomitant decrease in cw-GAPDH activity was observed. However, further investigations will be needed to establish a direct relationship between the physiological effects of nutritional stress on membrane integrity on the one hand, and on the efflux and the entrapment of GAPDH on the other.

In conclusion, this study demonstrates that cytosolic and cell wall-associated GAPDH forms are identical and that plasma membrane permeability is closely related to the efflux and location on cell walls of GAPDH and probably of other cytoplasmic proteins. The functional significance of these proteins being present on the bacterial cell surface must be clarified, although they are likely involved in bacteria/host interaction. Furthermore, in light of recently published information about the predicted secretome of *L. plantarum* [4], we will continue our systematic process of identification and characterization of surface bacterial components. This genetic/biochemical approach should provide clues to the elucidation of the complex mechanisms of probiotic/host interaction.

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