

Improved in vitro assay for determining the mucin adherence of bacteria sensitive to Triton X-100 treatment

Varvara Tsilia · Pieter Van den Abbeele ·
Tom Van de Wiele

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Abstract Mucin-associated microbiota are in relatively close contact with the intestinal epithelium and may thus have a more pronounced effect on host health. We have previously developed a simple mucin agar assay to simulate initial mucus colonization by intestinal microbial communities. Adherence of microbiota was estimated using flow cytometry after detachment with Triton X-100. In this study, the effect of this detergent on the cultivability of both virulent and commensal strains was investigated. Mucin attachment of selected strains was evaluated using the mucin adhesion assay. Bacteria were dislodged from the mucin surface by incubation with Triton or from the whole mucin agar layer using a stomacher. Mechanical extraction resulted in 1.24 ± 0.42 , 2.69 ± 0.44 , and 1.56 ± 0.85 log CFU/mL higher plate counts of *Lactobacillus rhamnosus*, *Bacillus cereus*, and *Escherichia coli* strains, respectively, than the chemical method. The sensitivity of bacteria to Triton varied among microbial species and strains. Among others, Triton inhibited the growth of *Salmonella enterica* LMG 10396 and *Pseudomonas aeruginosa* LMG 8029 on laboratory media, although these bacteria maintained their viability during this treatment. Only Gram-positive strains, *Enterococcus hirae* LMG 6399 and *L. rhamnosus* GG, were not affected by this detergent. Therefore, the mechanical method is recommended for the extraction of mucin-adhered bacteria that are sensitive to Triton, especially when followed by traditional cultivation

techniques. However, this approach can also be recommended for strains that are not affected by this detergent, because it resulted in higher recovery of adhered *L. rhamnosus* GG compared to the chemical extraction.

Abbreviations

AIEC	Adhesive invasive <i>Escherichia coli</i>
BHI	Brain heart infusion
CMC	Critical micelle concentration
INRA	National Institute for Agricultural Research
LGG	<i>Lactobacillus rhamnosus</i> GG
LPS	Lipopolysaccharide
A	Absorbance
PG	Peptidoglycan
PPBS	Potassium phosphate buffered saline
PPS	Physiological peptone solution
TX	Triton X-100
VBNC	Viable but nonculturable

Introduction

The mucus layer is a physical, chemical, and immune barrier that prevents the intestinal microbiota from coming in direct contact with the gut epithelium (Linden et al. 2008; McGuckin et al. 2011). The major structural components of the mucus layer are mucins that comprise a group of heavily O-glycosylated proteins (Bansil and Turner 2006; Lai et al. 2009). The abundance and diversity of mucin oligosaccharides represent potential binding sites for microbial adhesins (McGuckin et al. 2011; Juge 2012). Adhesion of bacteria on mucin receptors ensures prolonged transit time and offers a site of closer contact with the eukaryotic cells. This is thought

V. Tsilia · P. Van den Abbeele · T. Van de Wiele (✉)
Laboratory of Microbial Ecology and Technology (LabMET),
Department of Biochemical and Microbial Technology, Ghent
University, Coupure Links 653, 9000 Ghent, Belgium
e-mail: tom.vandewiele@ugent.be

V. Tsilia
e-mail: varvara.tsilia@ugent.be

P. Van den Abbeele
e-mail: pieter.vandenabbeele@ugent.be

to enhance the beneficial effects of probiotics, such as specific *Lactobacillus* and *Bifidobacterium* species (Elliott et al. 1998); however, it may also contribute to the virulence of pathogenic bacteria (Finlay and Falkow 1997; Sharon and Ofek 2000). Therefore, evaluation of the adhesion potential of autochthonous and allochthonous microorganisms is of extensive scientific interest.

Several approaches have been developed to investigate bacterial colonization on gut surfaces. In vivo studies require expertise and large investments. More importantly, they are limited by ethical constraints and the rigidity in sample accessibility, which usually results in end point measurements (Hartung and Daston 2009; Marzorati et al. 2011). On the other hand, in vitro adhesion setups can explain the underlying mechanistic basis of in vivo observations because they offer unlimited sampling potential and flexibility in the number or combination of factors tested (Macfarlane and Macfarlane 2007; Marzorati et al. 2009). HT-29-MTX sub-clones differentiate and secrete mucus upon induction with galactose and methotrexate, respectively (Lesuffleur et al. 1990; Hao and Lee 2004) but require more than 30 days for differentiation (Hao and Lee 2004). Additionally, co-incubation with intestinal bacteria is not recommended due to the high cytotoxicity of the microbial slurry (Parlesak et al. 2004). Therefore, application is limited to pure strains or short infection times hindering host adaptation or microbial metabolism (Marzorati et al. 2011). As a result, there is a need for alternative screening in vitro models to examine bacterial adhesion on mucosa surfaces.

More robust mucin-based in vitro adhesion models do not usually incorporate a physiological host environment and use either commercial mucin (partially purified mucin from porcine stomach from Sigma-Aldrich) or mucin extract from the gastrointestinal tract of animals. Various fermentation systems have been developed to study the behavior of mixed intestinal microbial communities under condition simulating both the gut luminal and mucus environment. The mucus environment was incorporated in different forms, such as beads consisting of mucin solidified with sodium alginate packed in dialysis membranes (Probert and Gibson 2004), mucin soft agar in glass tubes (Macfarlane et al. 2005), or more recently mucin agar on microcosms (Van den Abbeele et al. 2012). Other simpler adhesion models employing passively immobilized intestinal mucus (Tuomola et al. 1999; Roos and Jonsson 2002; Rinkinen et al. 2003; Vesterlund et al. 2005) or mucin agar (Van den Abbeele et al. 2009) on multi-well plates also exist. These methods are particularly useful for high-throughput screening of pure cultures and are sufficient to investigate the effect of multiple (simultaneous or separate) parameters on the initial adhesion of bacteria on mucin.

Separation of bacteria from the mucin surface requires some sort of physical or chemical detachment or even lysis

of bacteria followed by an appropriate detection method. However, several detachment approaches, such as those employing detergents, may affect the bacterial integrity (Sheu and Freese 1973; Tsuchido et al. 1990), which is important when culture-based approaches are used for enumeration. The selection of the detachment approach also depends on the available detection methods. For example, some techniques, such as flow cytometry and radioactivity, are less readily accessible than others involving DNA extraction and plating.

We have previously developed a mucin adhesion assay (Van den Abbeele et al. 2009) for fast and accurate determination of the initial colonization of simulated mucus by intestinal microbial communities based on Triton X-100 (TX) extraction of attached bacteria followed by flow cytometry. In the present study, we examine a diversity of Gram-positive and negative microorganisms in respect to their susceptibility to this detergent using culture-based techniques to estimate the bacterial abundance. Some bacteria sensitive to TX were also tested with flow cytometry. Selected strains were evaluated for their ability to adhere to mucin agar layers using plating after detachment with TX or a mechanical approach with stomacher in order to compare the efficiency of the two extraction approaches. This method can be used routinely to elucidate the adhesion potential of bacteria, especially those sensitive to detergents, on mucin surfaces using culture methods.

Materials and methods

Bacterial strains

Bacillus cereus NVH 0500/00, *B. cereus* NVH 1230/88, and *B. cereus* NVH 0075/95 were kindly provided by Prof. M.H. Guinebrière (National Institute for Agricultural Research (INRA), France) and Prof. P.E. Granum (Norwegian School of Veterinary Science, Norway). *Escherichia coli* LF82 (adhesive invasive *E. coli*, AIEC) was given by Prof. A. Darfeuille-Michaud (INRA, France). *E. coli* LMG 2092, *Lactobacillus rhamnosus* LMG 18243 (LGG), *Enterococcus hirae* LMG 6399, *Salmonella enterica* LMG 10396, and *Pseudomonas aeruginosa* LMG 8029 were obtained from the Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology, Ghent University, Belgium (BCCM/LMG collection).

Growth conditions

A loopful of each stock bacterial culture from $-80\text{ }^{\circ}\text{C}$ was aseptically transferred in test tubes containing 10 mL of growth media. LGG and *E. hirae* LMG 6399 were cultured in MRS broth (Oxoid, England), while brain heart infusion broth (BHI, Oxoid, England) was used for the other strains.

All pre-cultures were incubated overnight at 37 °C (atmospheric headspace) without shaking.

The pre-cultures were diluted ten times in fresh broth and incubated under the same conditions on a rotary shaker (110 rpm) until the absorbance (A) at 610 nm ranged between 1.5 and 1.8 (approximately 2.5 to 3.5 h for all strains, except for *P. aeruginosa* that required 18 h). Each culture was centrifuged at 5000g for 20 min at 4 °C and the pellet was washed two times with 0.1 mol/L potassium phosphate buffered saline (PPBS) at pH 7.0. The final $A_{610\text{ nm}}$ of the suspension was adjusted to 1.50 ± 0.05 using the same buffer.

Before use, the purity of all cultures was controlled by plating on BHI and LB (Sigma, USA) agar.

Survival of bacteria in Triton X-100 using plating

Each culture suspension was tenfold diluted in 0.1 mol/L PPBS pH 7.0 (control) or Triton X-100 (TX, Sigma, USA) to achieve a final concentration of 0.5 % w/v (8 mM) (Treatment). The TX solution was prepared in 0.1 mol/L PPBS pH 7.0. All samples were incubated at 37 °C for 30 min in an anaerobic jar at 110 rpm.

After incubation, bacterial concentration was determined by plating serial 10-fold dilutions of samples in physiological peptone solution (PPS). PPS consisted of 1 g/L neutralized peptone bacteriological (Oxoid, England) and 8.5 g/L NaCl (VWR, USA). *S. enterica* LMG 10396 and *P. aeruginosa* LMG 8029 were plated on LB agar, LGG and *E. hirae* LMG 6399 on MRS agar, and the other strains on BHI agar. All plates were incubated aerobically at 37 °C overnight, except for MRS plates that were grown under the same conditions for 1 d.

For each experiment, two biological replicates (different days) with at least two technical replicates each (same day) were used, except for *P. aeruginosa* and *E. hirae* for which three technical replicates were performed.

Survival of bacteria in Triton X-100 using flow cytometry

Control and treatment samples of *S. enterica* LMG 10396 and *P. aeruginosa* LMG 8029, collected after the 30-min incubation, were 100-fold diluted in 0.22- μm -filtered 0.1 mol/L PPBS pH 7.0 containing 1 % v/v of a fluorescent dye mixture. The dye mixture was prepared as described in De Roy and co-workers (2012) and consisted of SYBR® Green I nucleic acid stain (Invitrogen, USA) and propidium iodide (Sigma-Aldrich, USA) in order to differentiate between cells with intact and damaged cytoplasmic membranes. Prior analysis, the stained samples were incubated for 10 min in the dark at 37 °C.

An Accuri™C6 Flow Cytometer (BD, USA) equipped with a 20-mW 488-nm Solid State Blue Laser was used for the analysis. The generated green and red fluorescent light was

collected by the fluorescent channels FL-1 (533/30-nm band-pass filter) and FL-3 (670-nm long-pass filter), respectively. In order to eliminate irrelevant debris, the primary fluorescence (FL1-H) and secondary forward scatter (FSC-H) thresholds were set at 500 and 4000, respectively. The equipment was operated at medium flow rate (35 $\mu\text{L}/\text{min}$) and data were acquired for 30 s. Live cell counts were determined using BD CSampler software (version 1.0) by the number of particles represented on FL1-H versus FL3-H dot plots. The performance of the flow cytometer was checked prior to the analysis using Cyto-Count™, Count Control Beads (Dako, Denmark).

Mucin adhesion assay

Adhesion experiments were adapted by Van den Abbeele and co-workers (2009). Briefly, 1 mL of LGG, *B. cereus* NVH 0500/00, or AIEC LF82—prepared as described in the “Growth conditions” section—was placed in wells of 12-well plates (VWR, USA) containing 1.2 mL of autoclaved mucin agar pH 6.8. Mucin agar consisted of 5 % w/v porcine mucin type II (Sigma, USA) and 1 % w/v bacteriological agar (Oxoid, England). Multi-well plates were incubated at 37 °C in an anaerobic jar on a rotary shaker (30–50 rpm) for 90 min.

Bacteria extraction from mucin layer

After 90 min of incubation, the liquid phase was discarded and the mucin agar layer was rinsed two times with 0.1 mol/L PPBS pH 7.0 to remove loosely adhered cells. Bacterial cell extraction from the mucin agar was performed by two different methods—chemical and mechanical.

In the chemical approach, described by Van den Abbeele and co-workers (2009), mucin-adhered bacteria were detached during a 20-min incubation at 37 °C in an anaerobic jar on a rotary shaker (110 rpm) in the presence of 0.5 % TX. Subsequently, the mucin surface was rinsed three times with 0.1 mol/L PPBS pH 7.0, and the aliquots were pooled together with the TX solution.

In the mechanical method, the whole mucin layer was aseptically transferred with an ultraviolet sterilized spatula in a sterile bag containing 10 mL PPS, and the mixture was homogenized for 5 to 10 min in a stomacher. Adhered bacteria extracted with each method were quantified by plating on BHI agar (*E. coli* and *B. cereus*) or MRS agar (LGG) under the same conditions mentioned above. Each assay was performed in quadruplicate.

Statistical analysis

Statistical analysis was performed using RStudio 0.97.551 (<http://www.rstudio.com/ide/>) running R 3.0.0 (<http://www.R-project.org>). Normality of log-transformed data was

assessed with the Shapiro-Wilks normality test and further evaluated with normal Q-Q plots. For low number of observations ($n=3-5$), no distributional assumptions were made. Homoscedasticity for pairwise comparison was performed using bootstrap classical Levene's test (with correction factor) or bootstrap modified Levene's test (with modified structural zero removal method and correction factor) for normally and non-normally distributed data, respectively. Non-normally distributed data were compared with Wilcoxon rank sum test, while for normally distributed data, a two-sample t test was used or Welch modified t test if the homoscedasticity assumption was not met. In the case of multiple comparisons, the overall hypothesis was checked with ANOVA for normally distributed and homoscedastic data, Kruskal-Wallis rank sum testing for non-normally distributed and homoscedastic data, or weighted least squares analysis for the other cases. When the global null hypothesis of equality was rejected, post hoc pairwise t tests with Bonferroni correction were executed for the normally distributed data or pairwise Wilcoxon rank sum tests with Bonferroni correction for the non-normally distributed data. The significance level was set at 5%. The results are expressed as average values of log CFU/mL with their standard deviation.

Results

Survival of bacteria in TX

The behavior of different bacteria in the presence of 0.5 % TX was investigated. The diversity of microorganisms included Gram-positive (*B. cereus*, LGG, and *E. hirae* LMG 6399) and Gram-negative (*E. coli*, *S. enterica* LMG 10396, and *P. aeruginosa* LMG 8029) strains.

In most cases, bacterial concentrations after incubation in the presence of TX were lower than those in the control experiments (Table 1). The decrease was more pronounced for *B. cereus* strains compared to the other species studied ($p<0.001$), however, all three *B. cereus* strains were also differently influenced by TX ($p<0.001$). The largest difference between the control and the treatment was observed for *B. cereus* NVH 0075/95 and reached 4.42 ± 0.31 log CFU/mL ($p<0.001$). The effect of TX on the other strains was less pronounced and it was accompanied by a decrease of less than 2 log CFU/mL ($p<0.001$ for *E. coli* strains and *S. enterica* and $p=0.020$ for *P. aeruginosa*). In contrast, the Gram-positive LGG and *E. hirae* LMG 6399 were not susceptible to TX. For these strains, the difference between the control and the treatment was 0.04 ± 0.19 and -0.01 ± 0.06 log CFU/mL, respectively.

Survival of *S. enterica* LMG 10396 and *P. aeruginosa* LMG 8029 in 0.5 % TX was also evaluated using flow

cytometry, and live cells were determined empirically from FL1-H versus FL3-H dot plots (Table 1). A 30-min incubation of both strains in the presence of TX (treatment) did not result in extensive differences compared to the control experiment.

PPBS (control) maintained the viability of cells during incubation (no difference between 0 and 30 min) despite the method used to evaluate survival (results not shown).

Bacterial extraction after the mucin adhesion assay

A Gram-positive (LGG) and a Gram-negative (AIEC) strain with good attachment properties (Alander et al. 1997; Martinez-Medina et al. 2011) were chosen to compare the performance of the two extraction methods. In addition to LGG, which survives the chemical treatment, a TX-sensitive Gram-positive bacterium, i.e. *B. cereus*, was selected. None of the Gram-negative strains could survive well in the presence of TX; therefore, no additional strain was used.

Wells with mucin agar were inoculated with LGG, *B. cereus* NVH 0500/00, and AIEC at 8.87 ± 0.17 , 7.72 ± 0.11 , and 6.89 ± 0.67 log CFU/mL, respectively. Figure 1 shows the concentration of bacteria attached on mucin agar as determined by two different extraction methods. The extraction efficiency of the two techniques differs, resulting in consistently higher recovery of bacterial cells ($p<0.001$ for all strains) when the mechanical method was used compared to the TX-based approach (chemical method). The difference in adhered bacteria between the two techniques was 1.24 ± 0.42 , 2.69 ± 0.44 , and 1.56 ± 0.85 log CFU/mL for LGG, *B. cereus* NVH 0500/00, and AIEC, respectively ($p<0.001$ for all strains).

Discussion

In this study, three bacterial strains belonging to *L. rhamnosus*, *B. cereus*, and *E. coli* were tested for their ability to adhere on mucin agar. Bacteria attached on mucin were extracted from the surface using a detergent, i.e., TX, or from the whole mucin agar layer after mechanical detachment with a stomacher. The mechanical extraction method consistently resulted in higher bacterial plate counts than the chemical method for all strains tested. This suggests either that TX is not sufficient for the detachment of bacteria from the mucin agar or that it has a bacteriostatic or bactericidal effect.

We showed that the sensitivity of bacteria to TX varies among microbial species and strains (Table 1). However, the detection method employed to determine the effect of this detergent may lead to different assumptions for a given strain. For example, the plate counts of TX-treated *S. enterica* LMG 10396 and *P. aeruginosa* LMG 8029 were lower than those in

Table 1 Bacterial concentration after 30 min of incubation at 37 °C in 0.1 mol/L PPBS pH 7.0 (control) and in presence of Triton X-100 at a final concentration of 0.5 % w/v in the same buffer (treatment)

Strain	Gram reaction	Origin	Control log CFU or live cells per milliliter	Treatment	Detection method
<i>B. cereus</i> NVH 1230/88	+	Oriental stew (FP)	6.92±0.12	3.43±0.12*	Plating ^b
<i>B. cereus</i> NVH 0075/95	+	Vegetable stew (FP)	7.15±0.17	2.73±0.26*	Plating
<i>B. cereus</i> NVH 0500/00	+	Potatoes in cream sauce (FP)	7.14±0.20	4.72±0.44*	Plating
<i>L. rhamnosus</i> LMG 18243	+	Human feces	7.78±0.14	7.74±0.12	Plating
<i>E. hirae</i> LMG 6399	+	Unknown (possibly pig intestine)	7.86±0.04	7.87±0.05	Plating
<i>E. coli</i> LF82	-	Ileal lesion of CD patient	6.68±0.34	5.33±0.43*	Plating
<i>E. coli</i> LMG 2092	-	Urine ^a	6.71±0.34	5.55±0.70*	Plating
<i>S. enterica</i> LMG 10396	-	Human feces (FP) ^a	6.11±0.66	4.60±0.71*	Plating
<i>P. aeruginosa</i> LMG 8029	-	Infected outer ear ^a	7.974±0.010	7.846±0.009	Flow cytometry ^c
			6.40±0.29	5.00±0.59*	Plating
			8.234±0.014	8.136±0.011	Flow cytometry

FP food poisoning, CD Crohn's disease

*Treatment results significantly differ ($p < 0.001$ for all strains, except for *P. aeruginosa* that has $p = 0.020$) from the corresponding control of a given strain

^a Information obtained from BCCM/LMG collection

^b Analysis was performed by plating and the results are expressed as average log CFU/mL and accompanied by standard deviation

^c Analysis was performed by flow cytometry and the results are expressed as average log live cells per milliliter and accompanied by standard deviation

the control experiment, but there were no obvious differences in live cell counts during flow cytometry measurements. It is possible that TX treatment does not affect cell viability but prevents growth of bacteria on laboratory media, a physiological phenomenon referred to as “viable but nonculturable” (VBNC) state (Oliver 2005). Both *S. enterica* and *P. aeruginosa*, as well as *E. hirae* and *E. coli*, demonstrate VBNC responses in the presence of a natural stress (Oliver 2010). Interestingly, induction of VBNC state is accompanied by biochemical changes in the bacterial cell membrane and wall, including increased autolytic rate compared to normal cells (Signoretto et al. 2000; Oliver 2010). Therefore, the surfactant-based extraction assay (chemical method; Van den Abbeele et al. 2009) cannot be successfully applied to cultures susceptible to TX when it is combined with traditional culture-based techniques for the enumeration of attached bacteria. Even for strains that are resilient to TX, such as LGG, the recovery of adhered cells was better with the mechanical approach. TX was thus not sufficient enough to detach LGG from the mucin agar.

Membrane solubilization by surfactants, such as TX, occurs gradually. Initially, detergent monomers bind to the membrane, disrupt hydrogen bonds between the hydrophilic groups of phospholipids and the aqueous solution, and penetrate into the lipid bilayer. When a critical micelle concentration (CMC_{TX} 0.3 mM; Hafiz 2005) is reached, the structure is altered and the membrane is lysed resulting in mixed lipid-protein-detergent micelles. As the detergent concentration increases, binary complexes

(detergent-lipid, detergent-protein, lipid-protein) appear and eventually complete protein de-lipidation occurs. Once saturated, solubilization cannot proceed even if the surfactant concentration continues to increase. The concentration of TX used here (8 mM) largely exceeded the CMC; thus, membrane lysis can be expected. The bacterial cell wall may serve as a physical barrier for TX preventing loss of bacterial viability. Approximately 95 % of the cell wall of Gram-positive bacteria consists of multiple peptidoglycan (PG) layers, while Gram-negative bacteria have only a few PG layers (5–10 % of the cell wall) surrounded by a lipopolysaccharide (LPS) membrane. Structural variations between the cell wall of Gram-positive and Gram-negative bacteria may be responsible for differential responses in the presence of TX. However, we have shown that susceptibility to TX varied not only among bacteria with the same Gram reaction (LGG and *E. hirae* versus *B. cereus*), but also among the same species (e.g., *B. cereus*); therefore, there is no clear relationship between the cell wall type and the sensitivity to this detergent.

The observed sensitivity of *E. coli* strains (Table 1) is in contrast with previous results, which demonstrated that the growth of several *E. coli* was not affected by the presence of 0.4 % TX (Sheu and Freese 1973). The latter was solely attributed to the presence of an intact LPS layer (Birdsell and Cotarobl 1968; Sheu and Freese 1973), although TX could remove a significant amount of *E. coli* J-5 phospholipids and LPS without affecting structural stability (Schnaitman 1971a). Cell wall proteins of the same strain were not solubilized by TX (Schnaitman 1971a, b), due to hydrophobic

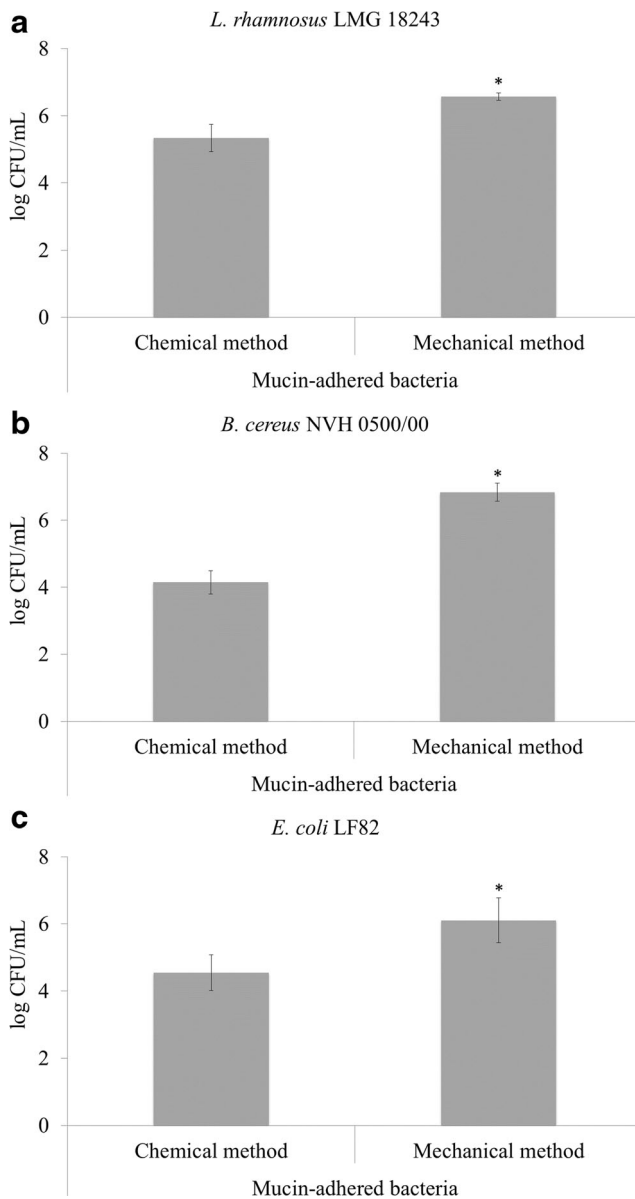


Fig. 1 Mucin-adhered bacterial counts of selected strains after 90 min of incubation at 37 °C estimated using two different extraction protocols. The results are expressed as average values of log CFU/mL (gray bars) accompanied by the standard deviation (error bars). **a** *Lactobacillus rhamnosus* LMG 18243, **b** *Bacillus cereus* NVH 0500/00, and **c** *Escherichia coli* LF82. The chemical method is described in Van den Abbeele et al. (2009), and the mechanical approach is the modified extraction method described in this article. Results obtained with the mechanical method were significantly higher (bars with asterisk) than those obtained with the chemical method for all strains tested ($p < 0.001$)

protein interactions between the cell wall and the LPS membrane (Schnaitman 1971a) rather than the PG layer itself (Sheu and Freese 1973). However, the protective role of LPS against TX is not universal (this study; Unemoto and Macleod 1975); and strain variations in the phospholipid type and the production of LPS may influence the responses to this detergent.

The thicker PG layer of *B. cereus* did not protect against the action of TX (Table 1), and this was supported by the documented susceptibility of the related *B. subtilis* to this surfactant (Sheu and Freese 1973; Tsuchido et al. 1990). In contrast, LGG (this study; Van den Abbeele et al. 2009) and *E. hirae* LMG 6399 were resistant to the same treatment. However, cell lysis of the latter strain has been previously observed at TX concentrations much lower than 0.5 % (Cornett and Shockman 1978; Tsuchido et al. 1990). Nevertheless, comparison among literature is restricted by the abundance of factors that affect the effectiveness of surfactants, such as the exposure time and the physical/chemical environment (pH, ion concentration (Tsuchido et al. 1990), and type), the culture growth phase (Schnaitman 1971b), the cell-to-protein ratio (Cornett and Shockman 1978), or the assay used to evaluate toxicity (this study; Dayeh et al. 2004). The ability of TX to solubilize membranes though is not solely responsible for surfactant-induced cell dissolution. An autolytic mechanism involving expression of bacterial PG hydrolases has been described (Cornett and Shockman 1978; Tsuchido et al. 1990) and is probably associated with neutralization or release of autolytic enzyme inhibitors/regulators (e.g., lipoteichoic acids and cardiolipin for *E. hirae*) in the presence of TX (Cornett and Shockman 1978; Cordwell et al. 2002). Autolysis caused by 0.05 % TX was limited in a methicillin-resistant *Staphylococcus aureus* compared to that of a double mutant lacking the autolytic regulators *mgrA/sarA* (Trotonda et al. 2009). A methicillin-susceptible *S. aureus* was rapidly lysed by TX, while the murein hydrolase mutant (Δatl) was not extensively affected (Bose et al. 2012). As expected, several cell wall hydrolases were found in the National Center of Biotechnological Information database associated to the bacterial species tested here, including the resilient LGG (GenBank: YP_005864803). More than 1500 hits for different *B. cereus* strains were obtained in the same search engine (results not shown); therefore, the release of autolytic enzymes from *B. cereus* in presence of TX cannot be excluded. Variations in the PG layers among different Gram-positive species could also be linked to TX sensitivity. For example, vancomycin-intermediate *S. aureus* was found with thickened cell wall which was associated with decreased hydrolysis (Boyle-Vavra et al. 2003).

In conclusion, the mechanical extraction method is recommended for the study of bacterial adhesion on mucin agar, especially when plating is used to estimate microbial counts. However, this approach is also applicable for strains that are not affected by TX, due to the observed higher recovery of LGG compared to the conventional extraction protocol. Because bacteria are extracted from the whole mucus instead of just the surface, microbiota that penetrate this layer can also be detected. The non-chemical approach reduces the experimental duration, and all the above contributes to the establishment of

a more reliable and universal method to evaluate bacterial adhesion to mucin.

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Conflict of interest The authors have declared no conflicts of interest.

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