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Role of the cell-wall structure in the retention of bacteria by microfiltration membranes

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A B S T R A C T

This experimental study investigates the retention of bacteria by porous membranes. The transfer of bacteria larger than the nominal pore size of microfiltration track-etched membranes has been studied for several kinds of bacterial strains. This unexpected transfer does not correlate to the hydrophobicity, neither to the surface charge of the microorganism, as suggested in previous reports. We conclude that, in our conditions, the kind of bacteria (Gram-positive or Gram-negative) is finally the most important parameter. As the distinction between those two types of bacteria is related to the cell-wall structure, we provide an experimental evidence, via the action of an antibiotic, that the cell-wall flexibility triggers the transfer of the bacteria through artificial membranes, when the pores are smaller in size than the cell.

1. Introduction

The retention of microorganisms is one of the key advantages of using membrane filtration for the production of drinking water. Ultrafiltration and microfiltration are known as efficient processes to remove bacteria and their selectivity is dominated by a sieving effect [1,2]. Nevertheless, several studies report leaks of bacteria through membranes with a nominal pore size smaller than the bacteria size [3–7]. Assuming the presence of a small number of abnormally large pores as compared to the average pore rating [3,4,8], is not sufficient to justify those results. In most cases the diameter of such “defects” remains smaller than the cell size. In this context, we may assume that the biological nature of the filtered particles is likely to induce specific behaviour and so to modify the expected transfer mechanisms. Therefore, the role of the cell properties, including their surface properties or their mechanical stiffness has been questioned in the present study, so as to understand to which extent this could explain the abnormal leaks observed through filtration membranes.

In cases of filtration of biological particles such as viruses or bacteriophages, several authors have pointed out that, due to their physico-chemical properties, electrostatic interactions may have

a strong effect on the retention predicted by considering only a size exclusion mechanism. For instance, Herath et al. [9] have shown that the rejection of MS2 and Q β (25 nm) by microfiltration membranes of 0.05 μ m mean diameter was larger for pH values close to their isoelectric point where bacteriophages are not charged anymore and thus are more likely to aggregate and to be rejected.

For bacterial cells, electrostatic interactions influence was also reported in case of deep filtration on granular bed. A low ionic strength or a high negative charge of the microorganism are sometimes recommended to facilitate the bacteria transport through a porous medium [10,11]. However, in membrane processes, electrostatic interactions appear early at the entrance of the particles into the pore, and thus the transposition of those last results to explain the unexpected leakage is not immediate.

In addition, the transfer of bacteria could be also originated from the mechanical properties of the bacterial cell-wall. However, only a few publications can be found on the effect of these properties on bacterial behaviour during filtration experiments. In this context papers already published on yeast and red blood cells have to be considered, since these are numerous, and meant to exhibit similar behaviours.

The deformability of biological particles during filtration experiments has already been invoked to describe the properties of filtration cakes resulting from the build up of retained cells at the membrane surface. Zydney et al. [12] study the extent of

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cellular blockage of membranes pores and the properties of a cake composed of red blood cells which are known for being highly deformable. Thereby, the cell bed is compressible which leads these authors to develop relationships between hydraulic permeability, porosity and compressive pressure based on experimental data obtained by filtration and centrifugation and including pore blockage effect. Then, they incorporate those relationships into a model for predicting the cross-flow permeation flux, assuming that the hydraulic resistance provided by cross-flow filtration of red blood cells was equivalent to that observed in an unstirred filtration system.

Numerous filtration studies of yeast suspensions have been published, in which the hydraulic resistance of cell deposits is evaluated as a function of the operating pressure. They conclude that the compressibility of the cells should be invoked to explain the observed discrepancy between experimental data and the data calculated using the classical Kozeny-Carman model. In this context, Meireles et al. [13] propose a numerical approach for modeling the hydraulic resistance of cakes obtained by dead-end filtration of baker's yeasts (*Saccharomyces cerevisiae*). These authors use a simplified version of the models developed by Smith et al. [14–16] which take the mechanical properties of the cells obtained by compression experiments and the osmotic equilibrium into account.

In a wider sense, several authors have made the assumption that, in terms of deformability, bacterial cells behave as yeasts. Then, by assuming bacteria deformation at constant volume, Hwang et al. [17] develop a model to evaluate the effects of operating conditions on the filtration performance based on Lu et al. [18] study of calcium-alginate gel particles. Their simulated results are in good agreement with experimental data obtained in cross-flow filtration of *Pseudomonas* species.

Concerning the study of biological cell filtrability through membrane pores smaller than their size, most publications focus on red blood cells [19–21]. Scanning electron microscopy observations of the transfer of bovine red blood cell [22] through microfiltration membranes of average pore diameter of 0.3 and 0.4 μm reveal a considerable deformation of the cell enabling it to penetrate in pores much smaller than it. This ability results from the high flexibility of their bilipidic membrane. However, bacteria present a more complex cell-wall structure, composed not only of a lipid bilayer but also of a cross-linked polymer network responsible for mechanical strength and stiffness of their cell-wall which are larger than those of red blood cells. This is well reflected by the Young modulus: the one of the red blood cells is in the range of 0.3 to 3 $\times 10^5 \text{ N/m}^2$ whereas for bacteria it is around 10^7 to 10^8 N/m^2 [23]. In such conditions, further experimental investigations are necessary to evaluate if the phenomena documented for the red blood cells can be of any importance when standard membrane filtrations are applied to suspensions of bacteria, although the assumption has already been proposed to justify bacterial leakage of *Brevundimonas diminuta* through microfiltration membranes [22].

Bacteria deformability or more exactly volume reduction was observed in other cases. For instance, Mille et al. [24] investigated the behaviour and the viability of *Escherichia coli* suspended in glycerol solutions of osmotic pressure ranging between 26 and 133 MPa. When a bacterial cell is submitted to an increase in external osmotic pressure, a passive response mechanism is first observed: transfer of water leads to a reduction in cytoplasmic volume and consequently to a reduction in the cell volume. The cell viability is a function of the final volume which depends on the osmotic stress magnitude. This experimental study supports strong similarities between the possible response of bacteria to a mechanical pressure. Indeed, when submitted to compression, microorganisms are likely to loose part of their internal liquid. In cases of yeasts, Smith et

al. [14] have shown that this volume reduction comes with the cell deformation which magnitude is determined by cell mechanical properties such as cell-wall flexibility.

From this literature survey it appears that if the changes in shape and size of bacteria under stress have been well considered to explain deviation from ideality in filtration, less attention has been paid so far to the consequences of this phenomenon on the leakage of bacteria through micro or ultrafiltration membranes. The objectives of the present paper are to show the ability of various bacteria to pass through a membrane characterized by a nominal pore size smaller than the cell size at rest and to explore the possible causes for this transfer considering operating conditions (feed concentration and transmembrane pressure) and bacteria characteristics, such as size, shape, and surface charge or hydrophobicity, but also the cell-wall structure and flexibility. For this last purpose, we focused upon the fundamental distinction between Gram-positive and Gram-negative bacteria related to their cell-wall structure. As an experimental evidence, the cell-wall structure and flexibility of a Gram-positive bacteria was modified by using a biochemical treatment.

2. Materials and methods

2.1. Experimental setup and procedure

Filtration experiments were performed using the setup shown in Fig. 1, which consists of a 50 mL dead-end filtration stirred cell (Model 8050, Amicon) connected to a pressurised tank containing the bacterial suspension. The pressure on the permeate side was atmospheric under all conditions. The transmembrane pressure was set by a pressure reducing valve located on the feed side. The filtration cell contained a microfiltration membrane with an effective area of $1.34 \times 10^{-3} \text{ m}^2$. This setup has a small size which allows an easy disinfection and manipulations under laminar air flow.

Prior to the experiment, the membrane was disinfected by soaking in a solution of sodium hypochlorite at 200 ppm for 20 min and then thoroughly rinsed with sterile distilled water. The filtration cell was soaked in a more concentrated solution of sodium hypochlorite (1000 ppm) for 30 min. All other pieces of equipment were sterilized (20 min under 120 °C) and kept under laminar air flow to prevent contamination.

Each experiment was performed at room temperature in three steps. Sterile distilled water was first filtered through the membrane at a transmembrane pressure of $1.5 \times 10^5 \text{ Pa}$ so as to pack the membrane. This step was stopped once the flux had stabilized, after a filtration period of approximately 1 h. Then, the membrane permeability was determined. In the last step, the feed tank and the filtration cell were emptied and filled with the bacterial suspension to carry out the filtration run. The stirring rate was kept constant over all the experiments at 300 rpm. The transmembrane pressure was adjusted in the range: 0.2×10^5 to $1.0 \times 10^5 \text{ Pa}$. This range has been chosen to match the pressure used in drinking water production plants using membrane processes.

The permeation flux J (m s^{-1}) was measured by timed collection using an electronic balance (Ohaus) with an accuracy of $\pm 0.1 \times 10^{-5} \text{ m s}^{-1}$. For each run, bacterial feed suspension and retentate were sampled at the beginning and at the end of the experiment for subsequent analysis. Permeate samples were also collected periodically during the experiment in order to monitor the evolution of bacterial concentration. After each experiment, the membrane was replaced by a new one in order to avoid cross-contamination between runs.

Each experiment was performed at least twice or three times depending on the scattering and the reliability of the results.

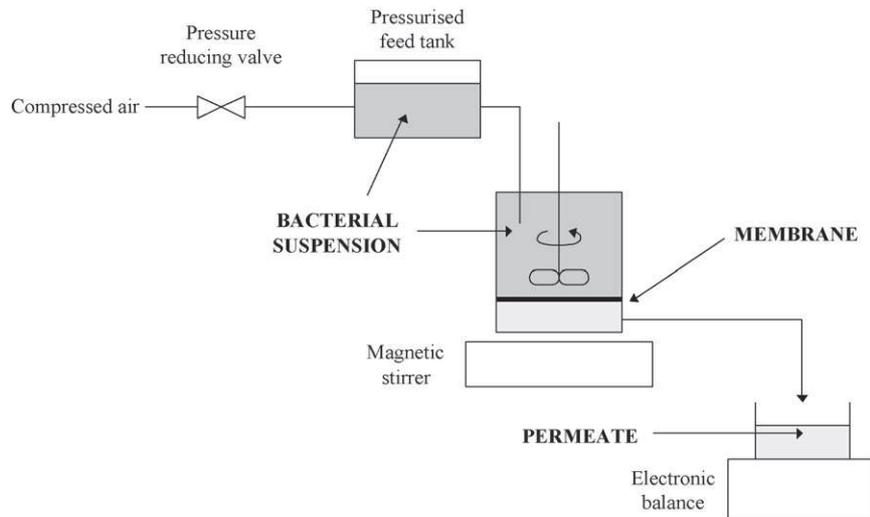


Fig. 1. Experimental setup.

2.2. Membranes

Polycarbonate track-etched microfiltration membranes supplied by Millipore were used for this study. This type of membranes was chosen for their well-defined pore geometry and size in order to minimize the effects of a pore size distribution. Challenge tests were carried out with membranes of nominal pore size of $0.4\ \mu\text{m}$. In each case, as shown in Fig. 2, the nominal pore size was smaller than the smallest dimension of the bacteria.

2.3. Bacterial suspensions and concentration evaluation during filtration

The six bacterial strains used in this study are listed in Table 1, where the bacteria size is as given in the literature. The choice of these bacteria is justified by the need of several strains of various morphological and structural characteristics. In addition, these strains fulfill two important experimental criteria: they are easy to cultivate (they need neither specific media nor specific atmosphere to be grown), their generation time is short which allows results after overnight incubation. Moreover, *E. coli* is a fecal indicator sys-

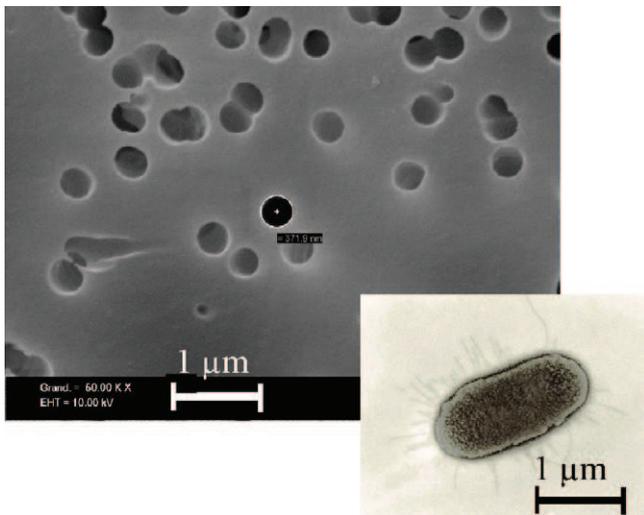


Fig. 2. Electron microscope images of track-etched membrane pore ($0.4\ \mu\text{m}$) and bacteria *Escherichia coli* ($2\ \mu\text{m} \times 1\ \mu\text{m}$) at the same scale.

tematically checked in potable water and *B. diminuta* is currently used to test microfiltration membrane efficiency.

Stock cultures of each bacterial strain were maintained on tryptone soy agar slants (Biomérieux, Crapone, France) at $4\ ^\circ\text{C}$. For preparation of inocula, bacteria were grown aerobically on tryptone soy agar plates at $37\ ^\circ\text{C}$ for two consecutive days. Colonies of the second 24 h culture were suspended in NaCl aqueous solution at $9\ \text{g/L}$ (corresponding to a ionic strength of $150\ \text{mmol/L}$) to obtain a concentration of about $10^8\ \text{cells/mL}$ as controlled by optical density at $640\ \text{nm}$. The use of an isotonic solution for bacterial suspensions avoids osmotic shock and maintains bacteria size equilibrium. Suspensions were then diluted down to $10^4\ \text{cells/mL}$ and this final suspension was used for microfiltration breakthrough assays. This concentration was chosen due to the bacterial concentration in raw water and in order to allow a direct detection measurement.

Tenfold dilution series of the retentate and feed samples were performed and $1\ \text{mL}$ of the retentate, the feed samples and dilutions was put into tryptone soy agar medium maintained in surfusion. Colony forming units (CFU) were enumerated after overnight incubation of the plates at $37\ ^\circ\text{C}$, considering dilutions with counts under $300\ \text{CFU}$. To control the permeate, the total volume was filtered through nitrocellulose filters (Millipore). The filter was then placed on a tryptone soy agar plate and incubated at $37\ ^\circ\text{C}$ for 24 h. The enumeration of CFU on the filter allows the determination of very small permeate concentrations.

The membrane retention efficiency is evaluated using the log reduction value (LRV) according to the following relationship:

$$\text{LRV} = \log \frac{C_r}{C_p} \quad (1)$$

where C_r and C_p are the bacterial retentate and permeate concentration (CFU/mL), respectively.

Table 1

Morphological and structural characteristics of tested bacteria and results obtained during filtration at $0.5\ \text{bar}$ of $10^4\ \text{CFU/mL}$ bacterial suspensions on $0.4\ \mu\text{m}$ track-etched membranes.

Bacterial strain	Reference	Gram	Shape	Size (μm)	LRV
<i>Brevundimonas diminuta</i> [34]	CIP 103020	–	Bacilli	0.8×0.5	5.5
<i>Pseudomonas aeruginosa</i> [35]	CIP 103467	–	Bacilli	1.6×0.8	3.2
<i>Escherichia coli</i> [36]	CIP 54127	–	Bacilli	2×1	3.2
<i>Staphylococcus aureus</i> [37]	CIP 53154	+	Cocci	0.8	>7
<i>Corynebacterium xerosis</i> [38]	CIP 5216	+	Bacilli	2×1	>7
<i>Micrococcus luteus</i> [38]	CIP 5345	+	Cocci	1.2	>7

Note that the bacterial viability and cultivability were controlled over the filtration test duration by evaluating the concentration of the feed suspension at the beginning and at the end of the run.

Moreover, we checked the mass balances including the bacteria in the permeate, those brought by the feed and those collected on the membrane surface. To evaluate the latter, the membrane was slightly shaken with sterile glass beads of 4 mm in diameter in a non-ionic surface-active agent (Tween 80 at 10%, Sigma–Aldrich). The bacterial concentration of the resulting suspension was determined by enumeration after 10-fold dilution series and inclusion in tryptone soy agar medium.

2.4. Determination of bacteria hydrophobicity and zeta potential

The bacteria hydrophobicity was evaluated by the Microorganisms Adherence To Solvents (MATS) method proposed by Bellon-Fontaine et al. [25]. Cell suspensions were prepared at 10^8 CFU/mL in Phosphate-Buffered Saline (PBS) at 0.15 mol/L. They were first washed three times in the PBS solution. Then, 1.2 mL of each cell suspension was placed into a glass test tube and 0.6 mL of the tested solvent was added. Four different solvents supplied by Sigma–Aldrich were used: hexadecane, decane, chloroform, ethyl acetate.

After the tubes were allowed to rest for 10 min at 30 °C, each one was vortexed for 90 s and left at rest for 15 min, during which time the two phases separated completely. The absorbance of the aqueous phase was then measured (640 nm). The blank consisted of PBS without cells. The results are expressed as the adherence degree (D) which is the proportion of the cells which were excluded from the aqueous phase, determined as follows:

$$D = \left(1 - \frac{A}{A_0}\right) \times 100 \quad (2)$$

where A_0 and A are the initial and final optical densities of the aqueous phase, respectively.

The bacteria zeta potential was determined by microelectrophoresis (Zetasizer 3000, Malvern). Bacterial suspensions were prepared in NaCl (9 g/L) aqueous solution as indicated in Section 2.3. Cell concentration used for those measurements was approximately 5×10^6 CFU/mL. The ionic strength and the pH of the support electrolyte were 150 mmol/L and 5.5, respectively.

2.5. Bacterial cell-wall modification

In an attempt to determine the role of the peptidoglycan layer from the cell-wall structure in bacteria retention by porous membranes, we have treated *Staphylococcus aureus* CIP 53154 with amoxicillin (Sigma–Aldrich). Amoxicillin is an antibiotic belonging to β -lactams which avoids cross-linkages between the peptidoglycan polymer chains (the network responsible for the mechanical strength and stiffness of the cell-wall). As a consequence, there is a less significant structuring of the peptidoglycan, leading, at subinhibitory and sub-lethal concentrations, to the improvement of the bacterial cell-wall elasticity without altering the viability and cultivability of the microorganism [26].

In a first instance, the Minimal Inhibitory and Bactericidal Concentrations of amoxicillin (MIC, MBC) were determined against the tested strain using a trypticase soy broth micromethod followed by a subculture on trypticase soy agar. Then the viability and the cultivability of a bacterial suspension at 10^4 CFU/mL were checked by numeration on trypticase soy agar after 1 h 30 min to 3 h of contact with amoxicillin at a MIC/2 concentration (5 ng/mL). In these conditions of contact time and antibiotic dose, no cell lysis or cultivability loss was observed.

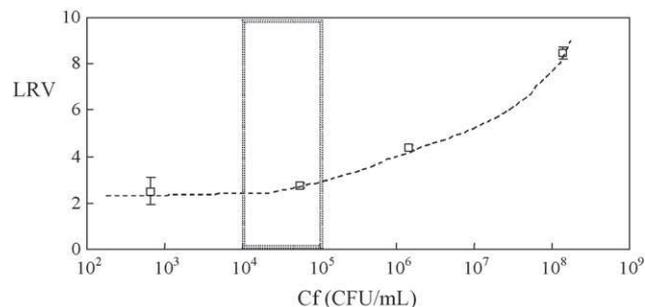


Fig. 3. Evolution of the log reduction value (LRV) during filtration at 0.5 bar of *E. coli* on 0.4 μ m membranes versus the suspension feed concentration (Cf).

Finally, for the filtration assay, bacterial feed suspension at 10^4 CFU/mL was kept in contact with amoxicillin at 5 ng/mL (MIC/2) during 1 h 30 min before the filtration run which duration was 1 h 30 min. The numeration for both permeate and retentate samples were performed as described in Section 2.3. As 1 mL of sample was put into 19 mL of tryptone soy agar medium maintained in surfusion, this dilution stopped the action of amoxicillin, all the more that we were already working at subinhibitory and sub-lethal concentrations. Viability controls of the treated cells were performed in these conditions at the beginning and at the end of each test and compared to the initial feed sample of non-treated bacteria dispersions, kept as a reference.

3. Results and discussion

3.1. Unexpected bacterial leakage

The first step of our study was to evidence unexpected bacterial leakage with one system “bacteria/membrane pore size”. For this purpose, we selected *E. coli* CIP 54127 because (i) this strain is one of the easiest to grow and (ii) it presents well-known characteristics, and we used a 0.4 μ m pore diameter membrane. As shown in Fig. 2, the membrane average pore size remains smaller than bacteria which are approximately 2μ m \times 1μ m in size.

The results of those preliminary experiments show that independently of the operating conditions we have explored, the bacteria was detected in all the permeate samples. Thus, in spite of an unfavourable size ratio, *E. coli* is likely to pass through a 0.4 μ m polycarbonate track-etched membrane. We also notice that the magnitude of this unexpected transfer depends on the applied operating conditions. Therefore, next section focuses on the effect of various operating parameters on bacteria transfer.

3.2. Operating conditions

Since in a dead-end filtration regime, the microorganism concentration in the retentate is not constant all over the duration of the trial, we studied the effect of the concentration upon the bacteria rejection. A set of four independent experiments were performed at constant transmembrane pressure (0.5 bar) using 0.4 μ m polycarbonate track-etched membranes with various *E. coli* feed concentrations: 6.6×10^2 , 5.5×10^4 , 1.4×10^6 and 1.4×10^8 CFU/mL. The log reduction values displayed in Fig. 3 are those obtained at the very beginning of the trial in order to avoid the effect of the concentration factor in the filtration cell. These results show that, beyond 10^5 CFU/mL, the log reduction value strongly increases with the bacterial feed concentration. A low concentration makes the bacteria transfer through the membrane easier. We may assume that aggregation phenomena are responsible for the observed improvement of the bacteria rejection at high concentration level,

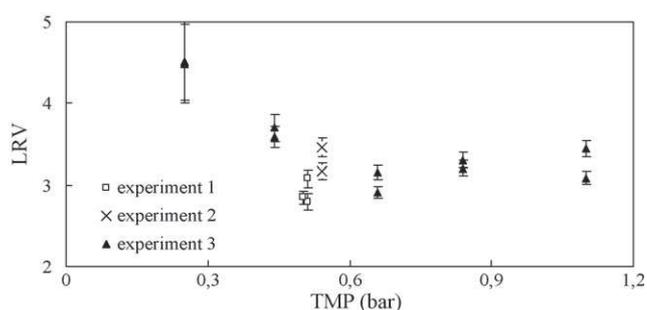


Fig. 4. Evolution of the log reduction value (LRV) during filtration of 10^4 CFU/mL *E. coli* suspension on $0.4\ \mu\text{m}$ membranes versus the transmembrane pressure (TMP).

however this assumption remains to be confirmed for instance by light scattering measurements. Moreover, another mechanism is likely to explain these experimental results. Indeed, when the feed concentration increases, the bacteria probability of collision is enhanced and collisions near to the entrance of the pore could lead to pore blockage due to particles bridging over the pore opening [27,28]. In these conditions the higher is the feed concentration, the more the bacteria transfer through the membrane is limited.

Through the examination of those results, a feed concentration of 10^4 CFU/mL is high enough to lead, when leakage are likely to occur, to bacterial permeate concentrations higher than the detection limit. On the other hand this concentration is low enough to assess the membrane removal efficiency in conditions near to the “worst-case conditions” i.e. conditions leading to the maximum transfer. Indeed, the higher the feed concentration is, the more the membrane rejection will be overestimated due to the effect of the concentration upon the membrane efficiency.

In the dead-end configuration used for our experiments, the bacterial concentration increases in the filtration cell during the run. The log reduction value as a function of bacterial concentration (displayed in Fig. 3) exhibits a non-linear evolution: below 10^5 CFU/mL, the log reduction value reaches a plateau value around 2.5. By using an initial feed concentration near to 10^4 CFU/mL, and knowing that the final concentration in the retentate is at most 10^5 CFU/mL, the effect of the change in retentate concentration during the experiment (corresponding to the area delimited by the dotted line in Fig. 3) should not be significant.

The experiments described below have thus been operated at a bacterial feed concentration of 10^4 CFU/mL.

The increase in bacterial concentration in the retentate side during the filtration is one of the reasons of the observed flux decline. In spite of the very small amount of bacteria brought to the membrane surface, which is equivalent to less than one layer of microorganisms, fouling mechanisms seem to be non-negligible. In these conditions, we decided to present the log reduction value results versus filtered volume instead of time filtration, as fouling is meant to be linked to the integral amount of bacteria brought to the membrane.

To assess the effect of the transmembrane pressure, several experiments were performed on the same “bacteria/membrane pore size” system (a feed suspension of *E. coli* at around 10^4 CFU/mL and $0.4\ \mu\text{m}$ polycarbonate track-etched membrane) under various transmembrane pressures in the range 0.2–1.0 bar (Fig. 4). In the range between 0.2 and 0.5 bar, an increase in applied pressure leads to a decrease of the bacterial removal whereas beyond 0.5 bar the log reduction value reaches a plateau value at 3 (Fig. 4).

Considering these results and in order to experiment the “worst-case conditions”, the rest of our trials was operated under a transmembrane pressure set to 0.5 bar.

To summarize, the transfer of bacteria through membrane pores of smaller size is maximum for some values of microorganisms concentration and transmembrane pressure. In order to check to which extend the behaviour of *E. coli* is specific, additional experiments involving different strains were performed on the same polycarbonate track-etched membranes.

3.3. Effect of the bacteria characteristics

Six different bacterial strains (including *E. coli*) were filtered under the same operating conditions (0.5 bar and around 10^4 CFU/mL) on polycarbonate track-etched membrane of $0.4\ \mu\text{m}$ pore size. Note that the ratio of the particles size to the nominal pore size was in each case unfavourable to the transfer and that a new membrane coupon was used for each experiment in order to avoid cross-contamination.

The results reported in Table 1 (last column) correspond to the experimental LRV measured after 30 min of filtration. Note that when no bacteria was detected in the permeate samples, the LRV is estimated to be higher than 7. They indicate that three strains out of six are fully rejected whereas the three others leaked through the membrane to some extent.

Since no bacterial lysis was observed by scanning electronic microscopy and mass balance performed by the method described in Section 2.3 is correct, we could not either explain these observations by assuming that some bacteria were disrupted (e.g. by shear forces) whereas others were not altered.

We hence note that *E. coli* is not the only one to pass through membrane pores smaller than their own size but also that this unexpected transfer turns out to be selective. Indeed, one strain (*Corynebacterium xerosis*) having the same size and shape as *E. coli* does not leak.

Considering those results and the morphological properties of bacteria, it appears clearly that the nominal cell size and shape are not the determining parameters for bacteria transfer. As a consequence, the selectivity appears to be not directly governed by physical sieving mechanisms. Moreover, the hydrodynamic alignment near a pore entrance [29] does not either play a role in the leakage phenomenon, since the $2\ \mu\text{m} \times 1\ \mu\text{m}$ *E. coli* leaks while the $2\ \mu\text{m} \times 1\ \mu\text{m}$ *C. xerosis* does not.

Various assumptions could be considered to explain this phenomenon. The first one is the presence of a small number of defects of the homoporous membrane structure such as pores doublets (large pore resulting from two joined impacts). Nevertheless, this hypothesis is insufficient since the membranes exhibit different rejections depending on the bacterial strain. For instance, reproducible results show that *E. coli* ($2\ \mu\text{m} \times 1\ \mu\text{m}$) passes through the membrane whereas *C. xerosis*, a bacterial strain which presents the same morphological properties (shape and size) is fully rejected, as *S. aureus* ($0.8\ \mu\text{m}$), even in spite of its smaller size.

Physico-chemical properties of the microorganisms such as surface charge and hydrophobicity are often considered to justify rejections unexplained by mechanisms based on size exclusion. However, in our cases, these properties are not either responsible for the observed selectivity as the effects of physico-chemical properties which are generally accepted in cases of viruses and bacteriophages filtration [9], are more debatable in cases of bacteria filtration. In addition, the bacterial suspensions being prepared in NaCl aqueous solution (9 g/L), the high ionic strength (150 mmol/L) severely screens electrostatic interactions out.

In order to further investigate the effect of bacteria surface properties, we have measured their zeta potential and the percentages of microbial adhesion to solvents. The values are displayed in Table 2. We note an absence of correlation between such properties and the leaks through the $0.4\ \mu\text{m}$ membrane. For instance, *E. coli* and *C.*

Table 2

Zeta potential (measured in NaCl 9 g/L as support electrolyte where ionic strength and pH are 150 mmol/L and 5.5, respectively) and MATS index of bacterial suspensions.

Bacterial strain	ζ (mV)	Adherence degree to solvents (%)			
		Chloroform	Hexadecan	Ethyl acetate	Decan
<i>B. diminuta</i>	-3.8	100	81	35	80
<i>P. aeruginosa</i>	-17.4	100	58	52	61
<i>E. coli</i>	-16.2	100	13	44	18
<i>S. aureus</i>	-21.5	99	75	29	98
<i>C. xerosis</i>	-16.5	100	100	96	11
<i>M. luteus</i>	-32.9	87	8	24	100

xerosis, the two former bacteria of same size and shape, present identical zeta potential whereas they behave differently when filtered with the same membrane. Concerning hydrophobicity values, high percentages of adherence to hexadecan were observed for *B. diminuta* and *S. aureus* while *E. coli* and *Micrococcus luteus* show the lowest values. These variations do not correlate to the observed leakage. To conclude, the propensity of bacteria to pass through pores smaller than their own size is primarily not related to their surface physicochemical properties.

In this context, the physiological behaviour of microorganisms during filtration was investigated and a transfer mechanism depending on the bacteria deformation and volume reduction was suggested.

3.4. Bacteria transfer mechanisms and selectivity

Since it is established that biological particles such as red blood cells or yeasts are deformable under mechanical stress, we wanted to check if similar modifications can occur during the filtration of bacteria due to the applied transmembrane pressure. When approaching the entrance of a pore, bacteria are submitted to the shear and drag forces created by the permeation flux and to the transmembrane pressure applied during the filtration step. Such stress could lead to their volume reduction (related to the osmotic equilibrium) and surface deformation (governed by the cell-wall Young modulus value) which would allow the cell to penetrate into the membrane pore. The bacteria may pass the pore with or without disruption of the cell membrane. If the bacteria penetrating the membrane retains its integrity, this bacteria keeps its potential pathogenicity, and the permeate could then present an infectious risk towards the consumer. In these conditions, there is a constraint threshold beyond which the bacteria do not withstand higher sur-

face deformation or volume reduction, leading to the plateau value at 3 observed in Fig. 4.

A similar mechanism has already been proposed by Suchecka et al. [22] to explain their experimental results observed with a Gram-negative bacteria (*B. diminuta*) upon a microfiltration membrane. They theoretically analysed the penetration of a spherical cell into a cylindrical pore of smaller diameter. Their calculated results suggest that the cell transfer to the other side of the membrane is possible by way of releasing intracellular matter into the environment and can be completed in a time period of the order of a few minutes. However, contrary to the model proposed by Smith et al. in case of yeasts [14–16], these authors do not take the cell-wall elasticity (Young modulus) into account although we believe that the deformation should be made more or less easier depending on this elasticity.

Note that this mechanism is different from the bacteria adaptation during growth suggested by several authors [30]. In our case, this assumption has however to be revoked considering that the filtration duration is shorter than the generation time of tested microorganisms and that the suspension is exempt of nutrients.

Considering all the experimental results, the most obvious correlation is the one between the bacteria external structure and the filtration behaviour. It is remarkable that the tested strains able to pass through the 0.4 μm membrane are the Gram-negative ones whereas the rejected ones are all Gram-positive.

Gram-negative and Gram-positive bacteria are two types of bacteria which can be distinguished according to their cell wall structure. The bacterial cell-wall is composed of a specific layer of a cross-linked polymer, the peptidoglycan, the quantity of which is function of the type of bacteria. In Gram-negative bacteria this layer thickness is around 2 to 6 nm, whereas Gram-positive bacteria present a thicker peptidoglycan layer is around 20 to 80 nm (cf. Fig. 5) [31]. Note that this thickness difference leads to various cell blow-out resistance: in the range of 0.3–0.5 and 25–35 bar, respectively for Gram-negative and Gram-positive bacteria [6].

Knowing that this cross-linked polymer is responsible for the cell-wall mechanical strength and that its elastic properties have been demonstrated by atomic force microscopy experiments [32], we can assume that the bacteria deformation capability is governed by the thickness of its peptidoglycan layer. Thus, the more this layer is thin, the more the cell is deformable, and likely to pass through smaller pores.

Gram-negative bacteria present a thin peptidoglycan layer which allows their deformation and their passage through smaller membrane pores than their own size at rest (“deformable particle”). On the other hand, the large peptidoglycan layer thickness of the

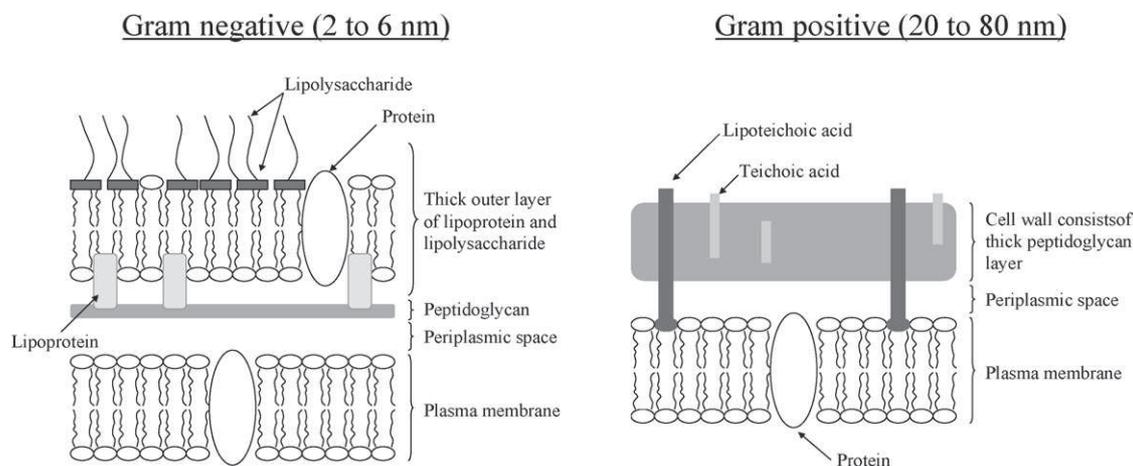


Fig. 5. Diagrammatic representation of the bacterial cell-wall showing the peptidoglycan layer.

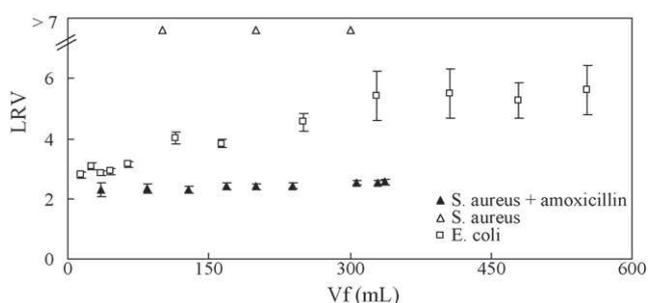


Fig. 6. Evolution of the log reduction value (LRV) during filtration at 0.5 bar of *S. aureus* treated or not by amoxicillin on 0.4 μm membranes versus the filtered volume (Vf).

Gram-positive bacteria limits their flexibility which would prevent their transfer through smaller pores ("stiff particle").

Moreover, we suspect that the peptidoglycan cross-linkage characteristics may have an impact on the bacteria deformability. Depending on the nature and the number of the transversal bridges, peptidoglycan could be more or less elastic as noticed by Bolshakova et al. [23]. However, note that, if the role of the peptidoglycan layer thickness allows to explain the different behaviours observed in filtration from a bacteria type to another one, it is difficult to assess the role of the elasticity of this layer because of the lack of bacterial strains identical in each property excepted the degree of peptidoglycan cross-linkage.

To confirm the leading part played by the peptidoglycan elasticity in the deformation propensity of bacteria, further experiments were performed with *S. aureus* treated with amoxicillin. This treatment was devoted to increase the bacterial cell-wall elasticity without any shrinkage of the cell. According to Lorian [33] an increase in bacterial size would be expected for longer contact times and higher antibiotic concentration than the ones used in the present study. We have controlled by optical microscopic observations (at a magnification of 1000 \times) that no significant difference in terms of size and shape could be noticed between treated and non-treated *S. aureus* (results not shown here).

The results in terms of log reduction value versus the filtered volume are displayed in Fig. 6. First of all, the increase of the log reduction value with the filtered volume is resulting from the combined effect of the concentration factor in the filtration cell and of the membrane fouling which mechanisms are not developed in the present paper.

Then, we note that non-treated *S. aureus*, like Gram-positive bacteria, was fully rejected. After contact with the antibiotic, one observes its transfer through membrane pores twice smaller. The results obtained during the filtration of *E. coli* (Gram-negative) upon the same membrane are also reported. Log reduction values obtained for the modified *S. aureus* are in the same range as those obtained for a Gram-negative bacteria. Those results well illustrate the role of the stiffness of the peptidoglycan layer in the retention mechanism: the amoxicillin treatment induces a transition between a "stiff particle" behaviour specific to Gram-positive bacteria to a "deformable particle" one specific to Gram-negative strains.

Those last results confirm the leading part of the peptidoglycan layer in the retention of bacteria by microfiltration membranes and therefore the proposed transfer mechanism based upon bacteria deformability.

4. Conclusion

This study pointed out that if size is an important parameter for understanding bacteria transfer through porous membranes, the

bacterial cell-wall mechanical properties may explain unexpected leaks through pores which can be as small as half of the smallest dimension of the bacteria at rest. This phenomenon is governed by the structural characteristics of the cell-wall, namely the peptidoglycan layer. The more this layer is thin and elastic, the more the bacteria is deformable, and likely to pass through pores of smaller size than bacteria size. As a consequence, bacteria of equal size can exhibit different behaviours in filtration: Gram-positive bacteria which present a thicker peptidoglycan layer are less deformable and so better rejected than Gram-negative one.

We also obtained experimental evidence that this wall deformability is more determining in the transfer mechanisms than other often invoked properties such as surface zeta potential or hydrophobicity/hydrophilicity balance.

The effects of cells concentration and of pressure on the bacteria transfer when this one may exist, are as expected, but they necessarily complicate the interpretation of curves. It is possible (and necessary in the case of membrane characterization) to select values for these two parameters which would maximize the bacteria transfer, in order to be in "worst case" conditions and therefore allow a conservative characterization of the membrane or the module.

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