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# Evaluation of the penetration capacity of bacteria through layers of different face mask types and wearing conditions

Alice Kheng[1], Lara Leclerc[1], Martin Fayolle[2,3], Florence Grattard[2,3], Paul O. Verhoeven[2,3,4]✉ & Jérémie Pourchez[1]

**The aim of this study was to quantify the number of non-airborne bacteria that can passively penetrate the layers of four mask types (surgical mask, community face mask type 1 (CFM1), biocidal CFM1 and CFM2) and to determine the influence of wearing conditions for the surgical type. A mask wearer simulator consisting of a 3D anatomical replica of the upper airway connected to a breathing pump was used. Wearing time, filtration quality of the mask, fit (loose vs. tight) and breathing parameters (tidal volume, respiratory rate) were tested. A *Staphylococcus epidermidis* inoculum was applied to the inner layer. After the wearing simulation, the layers were separated and the bacteria counted. After four hours, no or only a few bacteria were present in the middle and outer layers. Most remained in the inner layer. Surgical mask and CFM1 retained more bacteria and provided a breeding ground for germs. The biocidal CFM1 rapidly reduced the number in the inner layer. The breathing parameters had no influence, in contrast to fit and wearing time. These results confirm that the standard test for bacterial filtration efficiency, which includes the active penetration of airborne bacteria into aerosol droplets, is the most objective measure of the ability of bacteria to penetrate through the mask layers, as the passive penetration ability of non-airborne bacteria is insignificant.**

**Keywords** Face mask layers, Non-airborne bacteria passive penetration, Wearing conditions, Mask wearer simulator, Biocidal mask, Bacterial load

With the emergence of COVID-19, the use of masks as a protective device has become widespread among the general population and has become one of the most important recommendations during the pandemic[1,2]. A breathing mask is a device that reduces the spread of splashes to the wearer, while protecting him or her from potential splashes in the environment[3]. Indeed, surgical masks are not primarily designed to protect the wearer from airborne particles but are initially used to reduce bacterial spread from the mouth, nose, and face. Surgeons, for example, use medical masks to prevent contamination of the patient in the operating room. However, this measure is only effective if they change their mask regularly[4]. Besides, the same masks are also used by the general population to reduce the risk of transmission and spread of disease through their bioaerosol filtering capacity. They are regulated by the European standard EN14683 + AC:2019, which classifies them into three categories according to their airborne bacterial filtration efficiency (BFE) and differential pressure (DP)[5]: type I masks (BFE ≥ 95% and a DP ≤ 40 Pa $cm^{-2}$), type II masks (BFE ≥ 98% and a DP ≤ 40 Pa $cm^{-2}$) and type IIR masks (BFE ≥ 98% and a DP ≤ 60 Pa $cm^{-2}$).

In addition, there are other types of masks, such as the Community Face Mask (CFM) type 1, CFM type 2 and the biocidal CFM1[6]. These masks differ in their composition, manufacturing process and filtering effect[7]. Given the wide variety of textile masks, the filtration properties may differ depending on the type of fabric and the manufacturing process[8]. They are not categorized as medical devices but are regulated by the AFNOR SPEC S76-001 standard for example[9]. CFM1 have an expected filtration efficiency of 90% for particles with a size of 3 µm, while CFM2 have an expected filtration efficiency of 70% for the same particles[10,11]. The protection offered

[1]Mines Saint-Etienne, Centre CIS, INSERM, U1059 Sainbiose, Université Jean Monnet, Université de Lyon, Saint-Etienne 42023, France. [2]GIMAP team, INSERM U1111, CNRS UMR5308, ENS de Lyon, UCB Lyon 1, CIRI (Centre International de recherche en Infectiologie), University of St-Etienne, University of Lyon, St- Etienne, France. [3]Department of Infectious Agents and Hygiene, Unit of Bacteriology-Hygiene, University Hospital of St-Etienne, St-Etienne, France. [4]Laboratoire de Bactériologie-Hygiène, Adresse : Avenue Albert Raimond, St-Priest en Jarez 42270, France. ✉email: paul.verhoeven@chu-st-etienne.fr

by the CFM2 mask is therefore lower. CFM or cloth masks have lower filtration requirements and are not used by medical personnel, but by the general population as the level of protection required is not the same.

Bacteria in airborne droplets can be spread in several ways. The infected person can cough, sneeze, speak or breathe, throwing particles of different sizes into the air, from large droplets (with a diameter of about 100 µm) to small droplets (with a diameter of about 0.1 µm)[12,13]. The surgical mask is thus known to provide better protection to reduce the spread of bacteria as it reduces the number of airborne microorganisms excreted by the infected person when the mask is worn. It is even three times more effective at blocking bioaerosol droplets than a cotton mask[14]. As a result, the first mechanism of bacteria penetration capacity through the layers of a mask can be considered as an active passage when the bacteria can be transmitted by aerosol droplets[15]. Of course, the issue of bioaerosols penetrating through the layers of a mask can also be considered from the point of view of the wearer's health. The situation analysed would be the risk that the air inhaled by the wearer through a mask is contaminated by microorganisms suspended in the ambient air. In this case, the risk would arise from the active passage of an airborne bacterium through the different layers of the mask from the outside layer, and eventually penetrating the mucous membranes of the respiratory tract. In these two exposure scenarios, the active passage of bacteria through the layers of a mask is tested with the BFE standard test, in which a bioaerosol of *Staphylococcus aureus* is blown into the inner layer of a mask. The BFE is performed to determine the filtration efficiency of the masks by comparing the number of colonies of a standardized inoculum before and after filtration through the mask. A sample of the mask is clamped between an aerosol chamber and a six-stage Andersen cascade impactor. This impactor allows the particle size to be analyzed step by step, as the six stages do not allow particles of the same size to pass through[16].

The second mechanism of bacterial penetration through the layers of a mask can be considered a passive passage, as it does not involve active passage *via* airborne droplets. Indeed, in this study we consider the case where the air exhaled by the mask wearer can contaminate the inner layer of the mask. The non-airborne bacteria in the inner layer of the mask can then migrate through the different layers of the mask and eventually contribute to the spread of microorganisms in the environment (e.g. the air in an operating room). Of course, the issue of the bacterial penetration through the layers of a mask can also be seen from the point of view of the wearer's health. In this case, the study situation would be the risk of the air inhaled by the wearer through a mask contaminated on its outer layer with microorganisms from the environment. In this case, the risk would come from the migration of a non-airborne bacterium that is deposited on the outer layer of the mask, passes to the inner layer of the mask and possibly penetrates the mucous membranes of the respiratory tract[17]. In these two exposure scenarios, this passive mechanism can extend over a long period of time, depending on the duration of the migration of non-airborne bacteria through the mask layers and the conditions under which the mask is worn (mask fit, wearing time, respiratory parameters)[7,18]. A description of the two mechanisms is shown in Fig. 1.

Our study focused exclusively on the passive mechanism of the penetration capacity of non-airborne bacteria through the layers of a mask. We investigated the extent to which bacteria penetrate the different layers of the

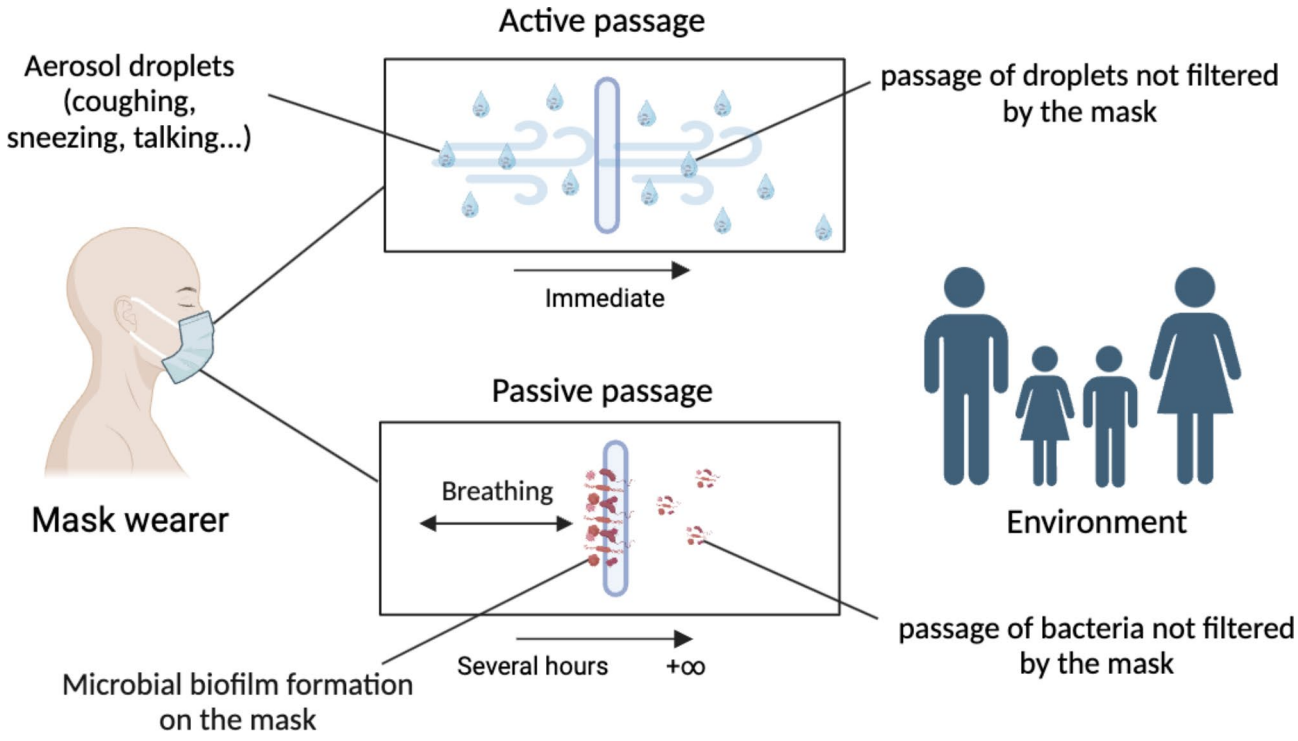



**Fig. 1**. Description of the active (*via* airborne droplets) and passive (*via* non-airborne droplets) mechanisms of bacteria penetration capacity through the layers of a mask. Image created with BioRender.com.

mask or not, depending on the type of mask and the conditions under which it is worn, such as the fit of the mask (loose or tight), the wearing time (from four to six hours) and the breathing cycle (simulation of normal breathing at rest or during exercise). We designate by the term "inner layer" the layer of the mask in contact with the wearer's mouth, "middle layer" the intermediate layer if there is one and "outer layer" the outermost layer in contact with the environment. Figure 2 illustrates this structure.

## Materials and methods

### Face masks

Four types of masks were used in this study: surgical mask type IIR, CFM type 1, CFM type 2 and a biocidal CFM type 1. The surgical mask (Bioserenity company, type IIR, France) consists of three layers of non-woven polypropylene SMS fibers (SMS for spunbound, meltbound, spunbound). The CFM consists of two layers (Oriol & Fontanel, CFM type 1, France; CJ Textile, CFM type 2, France) and the biocidal mask consists of three identical layers of cotton treated with silver and copper zeolite and silver zeolite (DIM company, CFM type 1, France). The experiments were performed with five samples for each mask type and condition. The surgical mask, CFM type 1 and 2 were previously characterized[7]. The measures and the microscopic images of these masks are shown from column two to four of Table 1.

The images of the masks were taken using an upright microscope (DM750 LED, Leica Microsystems) with a 4x magnification C Plan objective (ref 506226) and a Leica ICC50 HD camera. The images of the scanning electron microscope were taken with a JEOL JSM-6500 F. A surface area of approximately 1 $cm^2$ was placed on a brass support. The samples were fixed with a double-sided carbon tape and coated with a 14 nm thick gold layer (Quorom Q 150R ES). The accelerating voltage of the beam was 5 keV. The observed fibers of the biocidal CFM1 were not round, but rather flat. They were intertwined, but some of them were disordered. The measures and the microscopic images of this mask are shown in the last column of Table 1.

### Simulator of mask wearing

To simulate wearing conditions as realistic as possible, a simulator was previously developed, validated and used to assess the variation of BFE of surgical masks as a function of wearing time[19]. An anatomical replica of the upper airway of an adult was used. All masks were attached to the replica with a knot at the back of the head. The trachea was represented by a 15 cm long, ring-shaped tube connected to a medical heated humidifier (Fisher&Paykel MR410). The humidifier is also connected to a respiratory pump (Pari Compas II, Pari GmbH, Starnberg, Germany). The work plan is shown in Fig. 3a and a focus on the replica is shown in Fig. 3b. The parameters of the pump are varied, such as the tidal volume, the duration of exhalation and inhalation, or the number of respiratory cycles per minute.

### Microbiological procedures

*S. epidermidis* RP62a strain was transformed with the pSK265::DsRed plasmid as previously described[20]. DsRed-expressing *S. epidermidis* RP62a (designated as *S. epidermidis* STAH43) emit red fluorescence and turn red on agar plate after 48 h, which make them easier to recognize. *S. epidermidis* STAH43 was grown in Tryptic soy agar

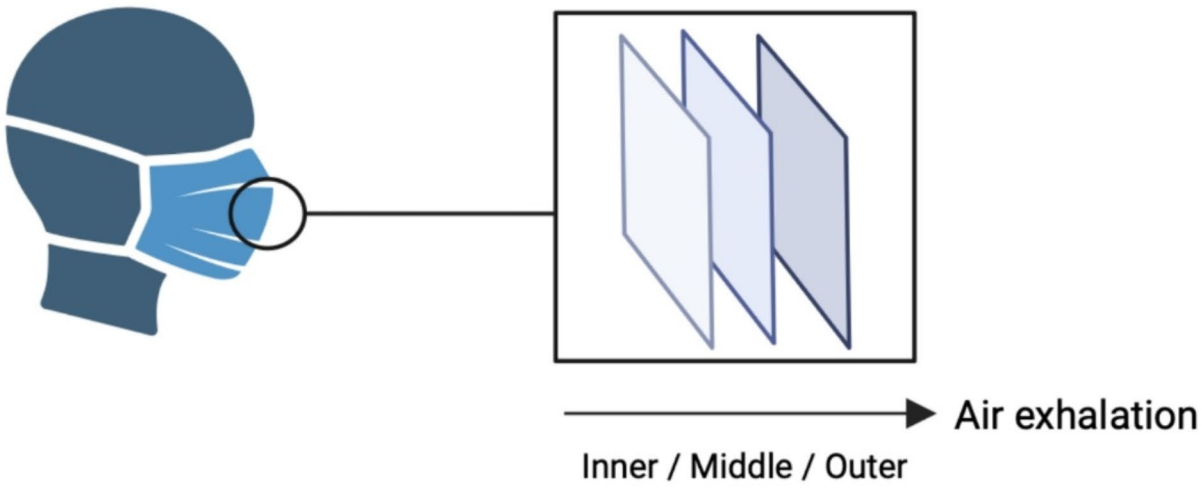



**Fig. 2**. Designation of the different layers of the mask. Here we take the example of the surgical mask which has three layers. The inner layer is the one in contact with the mouth, the outer layer is the one exposed to ambient air, and the middle layer is enclosed inside the mask. The arrow indicates the direction of air exhalation Image created with BioRender.com.

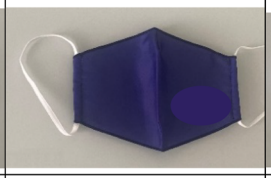
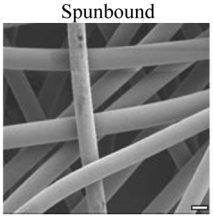
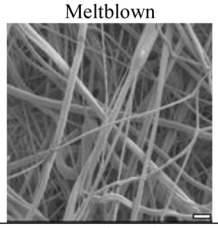
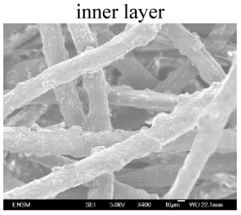
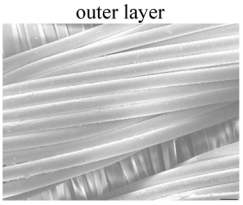
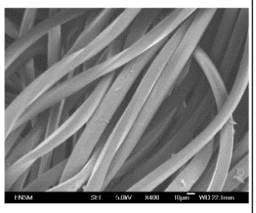
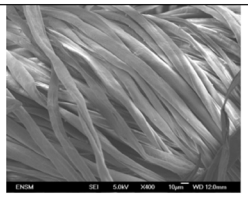
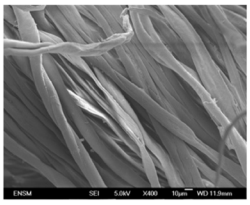
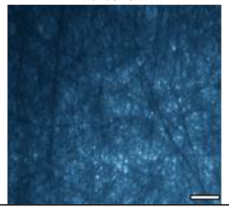
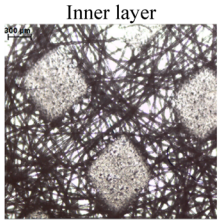
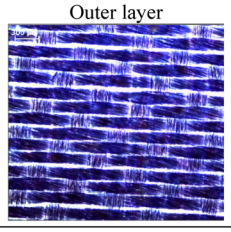
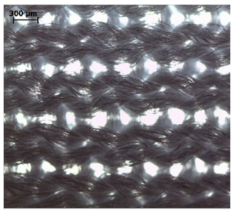
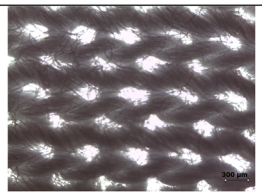
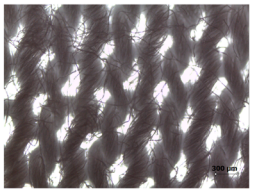
| | Medical Face Mask Type IIR (MFM) | Community Face Mask Type I (CFM1) | Community Face Mask Type II (CFM2) | Community Face Mask Type I Biocid |
|---|---|---|---|---|
| Type of mask |  |  |  |  |
| Key features of the mask structure | 3 layers of non-woven polypropylene fibres (spunbond, meltblown and spunbond layers)<br>Pore size of the spunbonded layer ≈ 100 µm<br>Pore size of the meltblown layer ≈ 20 µm<br>Fibre diameter of the spunbonded layer = 25 ± 1 µm Fibre diameter of the meltblown layer = 6 ± 3 µm | CFM is composed of 2 different layers<br>Pore size of the inner layer ≈ 300 µm<br>Pore size of the outer layer ≈ 50 µm<br>Fibre diameter of the inner layer = 31± 5 µm<br>Fibre diameter of the outer layer = 18± 1 µm | CFM is composed of 2 identical layers<br>Pore size ≈ 200 µm<br>Fibre diameter = 18 ± 0 µm | The CFM consists of 3 identical layers<br>Pore size :<br>Width ≈ 174 µm<br>Length ≈ 357 µm<br>Size of the fibres = 17 µm ± 3 of the width |
| Electron micrographs of the microscopic structure Scale bar corresponds to 30 µm (MFM) or 10 µm (CFM) 400× magnification | Spunbound<br>  <br>Meltblown<br>  | inner layer<br>  <br>outer layer<br>  |  |  <br>  |
| Light microscopic images of the microscopic structure Scale bar corresponds to 300 µm 4× magnification | Spunbound<br>  <br>Meltblown<br>  | Inner layer<br>  <br>Outer layer<br>  |  |  <br>  |
| Expected conformity in terms of filtration efficiency by the manufacturer | >98% of BFE | >90% of PFE | >70% of PFE | >90% of PFE |
| | EN14683:2019 standard | AFNOR SPEC S76-001 requirement | | |

**Table 1**. Key features of the masks tested in this study. PFE refers to particle filtration efficiency and BFE refers to bacterial filtration efficiency. The measurements and images for the surgical mask, CFM1 and CFM2 were taken from reference[7]. The information in the last column was obtained during our study.

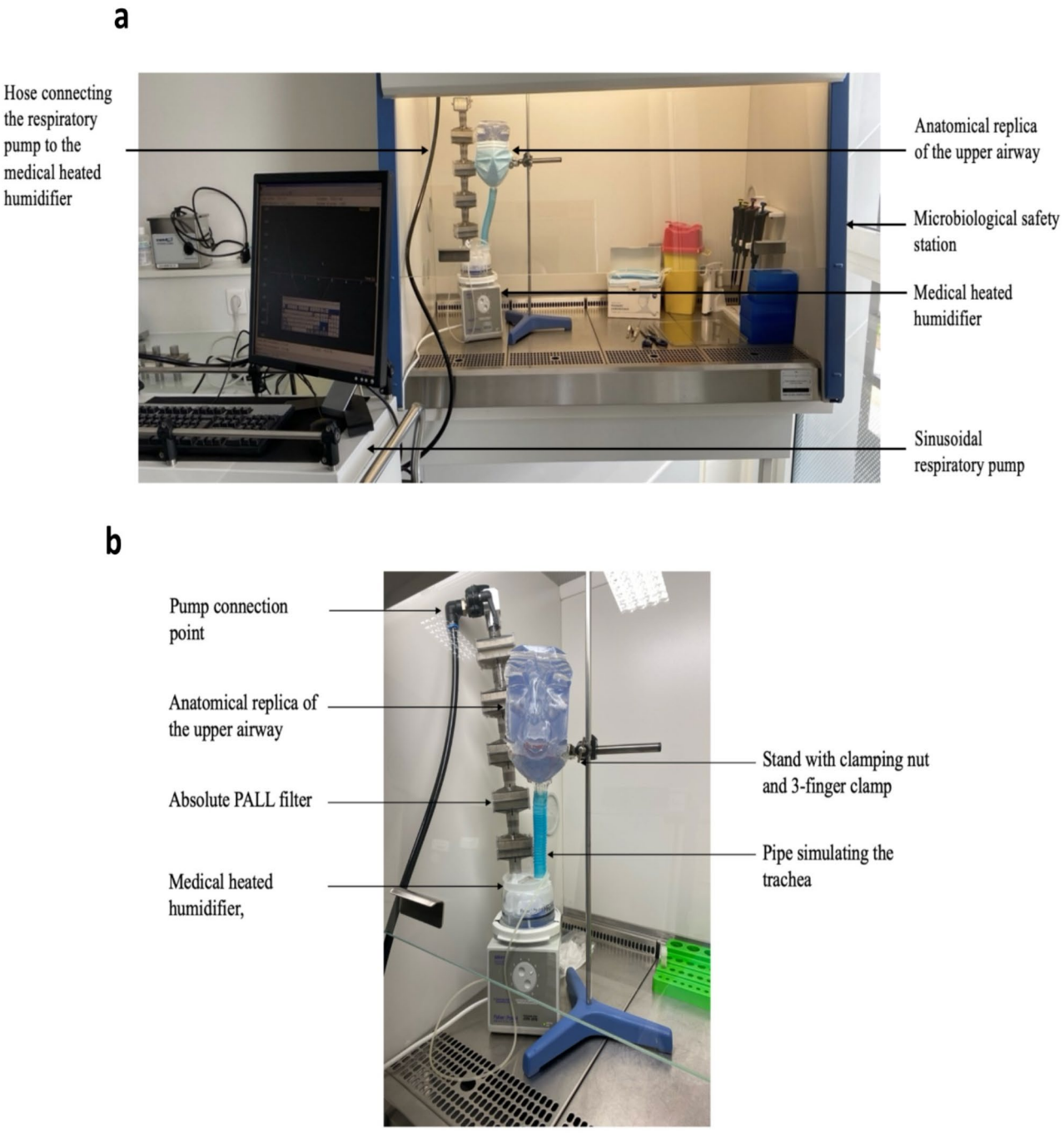



**Fig. 3**. Photographs of the working environment and the simulation bench (**a**) Mask-wearing simulation set-up (**b**) Experimental set-up showing of the anatomical replica of the upper airway.

(TSA) (PO5012A, Thermo Fisher Scientific) supplemented with 10 mg/ml of chloramphenicol when required. A stock solution was prepared at a concentration of $1.6 \times 10^6$ colony-forming units (CFU)/ml and stored frozen at -80 °C. For each experiment, fresh working solution was prepared by adding 100 µL of the bacteria in a final volume of 2 mL of peptone water (as recommended for BFE analysis in EN14693:2019). The working solution was then stirred for 15 min prior to be used to allow the bacteria recover and the two components to mix.

## Experimental protocol for investigating the influence of wearing conditions

Face masks were first pre-inoculated with the working solution of *S. epidermidis* STAH43. Drops of two microliters of working solution of *S. epidermidis* STAH43 were applied to the inner layer of the different masks (Fig. 4). The position of the drops was chosen to cover the entire surface concerned when transported through the simulator. They have the shape of a rectangle that is 5 drops wide and 10 drops long (Fig. 4). Then, different

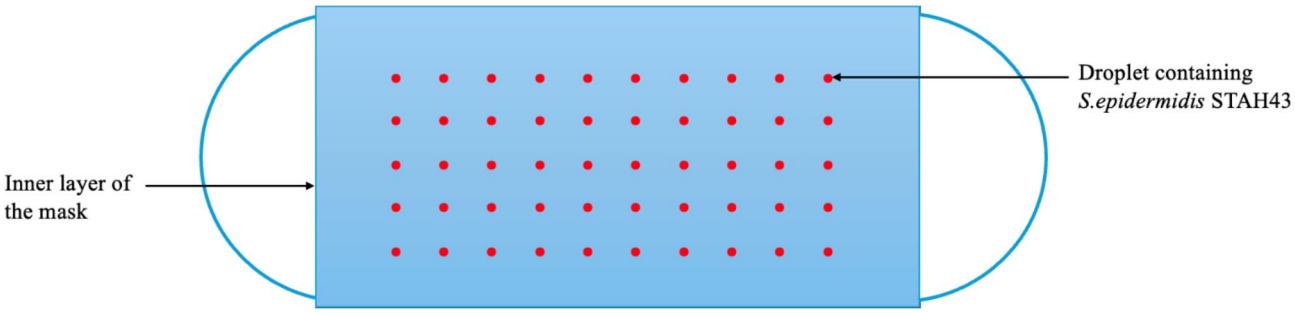



**Fig. 4**. Scheme of the location of droplets on the inner layer of the mask.

| Parameter studied | Mask type | Bacteria deposit on the inner layer | Mask fit | Wearing duration | Respiratory conditions |
|---|---|---|---|---|---|
| Negative control* | Surgical | No | Loose | 4 h | Normal |
| Reference - Recommended mask use | Surgical | Yes | Loose | 4 h | Normal |
| Impact of wearing duration | Surgical | Yes | Loose | 6 h | Normal |
| Impact of mask type | Biocidal CFM1 | Yes | Loose | 4 h | Normal |
| | CFM1 | | | | |
| | CFM2 | | | | |
| Impact of mask fit | Surgical | Yes | Tight | 4 h | Normal |
| Impact of breathing parameters** | Surgical | Yes | Loose | 4 h | Effort |

**Table 2**. Summary of the experimental conditions investigated using the simulator of mask wearing. *Verification of the absence of *S.epidermidis* STAH43 beforehand. **The tidal volume, inhalation and expiration duration were modified (normal/effort: 500mL/ 900 mL, 2 s/1,2 s and 3 s /1,8 s).

conditions for wearing the mask were tested to assess their impacts on the amount of non-airborne bacteria penetration through the different layers of the mask. A check of the viability of the bacteria in the mask without wearing through the simulator was carried out at t = 5 min and t = 240 min. The tests were performed with a sample of five masks for each type. A negative control was performed to ensure that no *S. epidermidis* STAH43 remains in the simulator after the experiments. A brand-new surgical mask was placed on the simulator and loosely secured, i.e. air leaks were possible. The respiratory parameters of the pump were previously modelled and were based on physiological respiratory parameters[21].

To observe the influence of mask type, a biocidal CFM1, CFM1 and CFM2 were used and compared with the performances of a surgical mask considered as a reference. To assess the influence of the fit of the mask, possible air leaks were prevented with adhesive tape. Finally, the respiratory cycle is characterized by the mobilized tidal volume, the duration of exhalation and inhalation and the number of respiratory cycles. For the normal cycle: tidal volume = 500 ml, inhalation time: 2 s, exhalation time: 3 s. For the effort cycle: tidal volume = 900 mL, inspiratory time: 1.2 s, expiratory time: 1.8 s. A summary of the conditions can be found in Table 2. The experiments on the simulator were repeated five times for each condition.

## Masks cutting and filtration

The masks were cut in such a way that only the unbound layers were preserved. The tweezers and scissors were autoclaved prior to use to avoid any external contamination. Each layer of the mask was placed in 200 ml of extraction liquid (as recommended for microbial cleanliness in EN ISO 14683:2019) and stored overnight at + 4 °C. The 200-ml volume of extraction liquid was filtered on 0.45 µm membrane (Millipore, Ref. HAWG047S).

The membrane that retained the bacteria was then transferred aseptically onto tryptone soy agar (TSA) plate and incubated up to 72 h at 37°C to let the colonies grow on the filter.

## Statistical analysis

GraphPad Prism 9 (Dotmatics, USA) was used to perform the statistical analyses. They were carried out on the data of the inner layer of each mask type, as the data of the other layers were too small or not available. To determine the effects of the different parameters such as mask fit and respiratory cycle, the results were compared with the reference condition (surgical mask worn for four hours on the simulator). A parametric Student's t-test with a 95% confidence interval was used to compare the effects of mask fit and respiratory cycle. The difference is considered significant if the p-value is less than 0.05. A Student's t-test was used for the effect of mask type, except when comparing CFM2 with other types of masks, where a Mann-Whitney test was used.

## Results

### Viability test

The percentage of bacteria found in the mask after five minutes and 240 min is shown in Fig. 5. The average percentages are given in relation to the theoretical number of colonies deposited at the beginning i.e. 8,000 (CFU). Five minutes after the bacteria were deposited, the percentages of culturable bacteria in the inner layer are close to the number of bacteria previously deposited in the surgical mask, CFM1 and CFM2 ($97.8 \pm 0.9\%$, $93.5 \pm 8.5\%$ and $87.6 \pm 4.0\%$, respectively). With the biocidal CFM1, the percentage of bacteria found in the inner layer drops to $69.2 \pm 2.3\%$ after five minutes. A small percentage of bacteria have crossed the inner layer to remain in the middle layer of the biocide CFM1 ($0.9 \pm 1.3\%$) and in the outer layer for CFM1 ($0.9 \pm 1.0\%$) and CFM2 ($2.6 \pm 0.8\%$).

After 240 min, the percentage of bacteria found in all mask types decreases, both on the test bench and when worn on the simulator. The surgical mask is the mask type that enable the recovery of the highest ($45.0 \pm 13.4\%$ on the test stand and $30.2 \pm 18.3\%$ with the simulator) and the CFM2 the lowest ($0.7 \pm 0.9\%$ on the test stand and $0.15 \pm 0.2\%$ with the simulator) proportion of the initial bacterial load at the inner layer. The number of colonies found on the masks with and without simulator wearers after 240 min showed no significant differences for the individual mask types.

### Influence of mask type and wearing conditions

The results of the influence of the mask type are shown in Fig. 6a. The surgical mask and CFM1 are the ones that retain the most bacteria in the inner layer with $2416 \pm 1466$ CFU and $2104 \pm 923$ CFU, respectively. CFM2 is the mask that retains the least bacteria in its layers with $12 \pm 18$ CFU. No bacteria were found in the other layers of the masks of all types. There is a significant difference concerning the number of CFU found in the inner layer between the surgical mask and biocidal CFM1 ($p < 0.05$), biocidal CFM1 and CFM1 ($p < 0.01$), CFM1 and CFM2 ($p < 0.01$), surgical mask and CFM2 ($p < 0.01$). No significant difference was found between the surgical mask and CFM1 and between biocidal CFM1 and CFM2.

Figure 6b shows the number of bacteria detected on the layers as a function of the fit of the mask. There is a significant difference in the number of bacteria retained in the inner layer between the loose fit and the tight fit. The number of CFU found when air leakage through the mask was prevented was $700 \pm 370$ CFU. No CFUs were found in the other layers.

There is no significant difference in the number of CFU found in the inner layer between a normal and effort breathing cycle, as shown in Fig. 6c. The number of bacteria in the inner layer of the mask where the breathing parameters take place during effort is $1008 \pm 960$ CFU. No CFUs were found in the other layers.

A difference in the number of CFU can be observed in relation to the influence of the gestation period (Fig. 6d). Indeed, $432 \pm 203$ CFU are present after six hours instead of $2416 \pm 1466$ CFU after four hours of wear. No CFUs were found in the other layers.

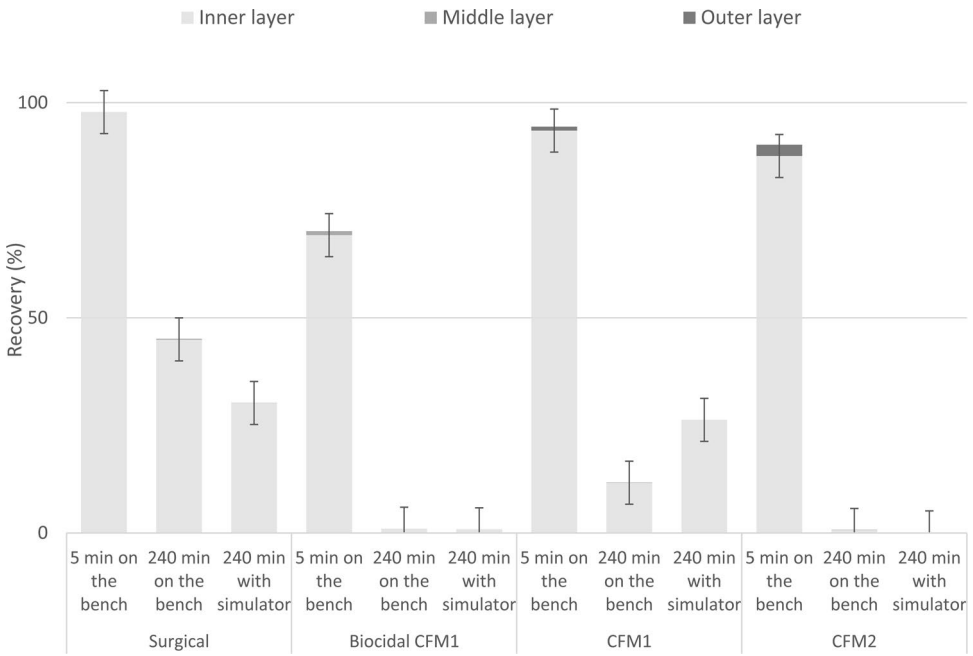



**Fig. 5**. Viability rate of bacteria on masks with or without wearing through the simulator. A rate of 100% corresponds to the entire dose of inoculum initially deposited on the inner layer of the mask.

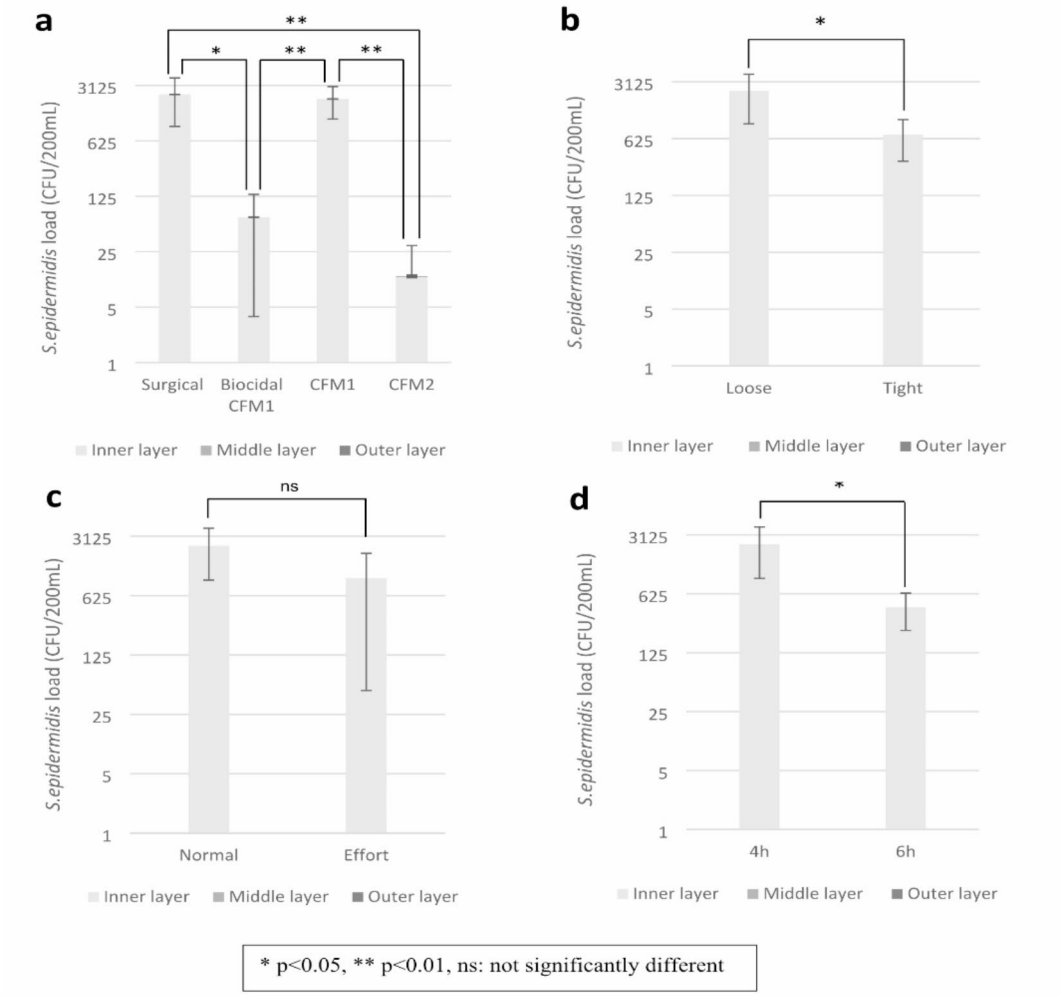



**Fig. 6**. Graphs showing the number of CFU according to the layers of the mask during 4 h (240 min) of wear (**a**) Impact of mask types. These results are the same as Fig. 4 concerning the column "240 min with simulator" but quantified in number of colonies formed. (**b**) Impact of mask fit (**c**) Impact of breathing cycle. (**d**) Impact of wearing time. Results of (**b**), (**c**) and (**d**) were collected on the surgical mask type.

## Discussion

The passive migration of non-airborne bacteria in the mask caused by breathing is an unknown phenomenon in contrast to the active passage of airborne bacteria, which is assessed using the BFE standard method. Interestingly, our study is one of the first to investigate the distribution of non-airborne bacteria through the layers of masks over time for different mask types and wearing conditions. The decision to use *S. epidermidis* as bacteria is explained by the fact that this species ranks among the most frequently encountered on masks and its concentration in the experimental condition is similar to that observed under actual wearing conditions[22–24].

Our results demonstrate that the non-airborne bacteria remain mainly in the inner layer showing no or very low penetration capacity. After five minutes without the use of the simulator, the percentage of bacteria in the inner layer of all types of masks is close to the quantity initially deposited on the inner layer for each mask, except for the biocidal CFM1, which shows a significant decrease. The biocidal effect of the CFM1 can be easily explained by the silver and copper zeolite molecules and the silver zeolite already present on the fabric, which enable the bactericidal effect[25]. While the exact mechanism of deactivation remains unknown, most theories postulate that positively charged silver ions disrupt the wall and membrane of bacterial cells, leading to a metabolic pathway alteration that results in cell death[26–28]. In addition, *S. epidermidis* have been shown to be

sensitive to the bactericidal effect of silver nanoparticles as their quantity increases[29]. We can therefore observe an efficient action of these molecules after five minutes which is a short period of time. However, the fact that a few colonies can pass through the middle (for the biocidal CFM1) or the outer (for CFM1 and CFM2) layer shows that the efficiency of filtration depends on the type (polypropylene or textile fibers) and manufacture of the masks. Polypropylene is known to have a hydrophobic surface and does not absorb liquid, whereas textile fibers such as cotton are known to be hydrophilic materials[30,31].

Unlike the BFE test, our study focuses on the migration of bacteria already present within the mask and not aerosolized. The filtration mechanism depends on mechanical and electrostatic phenomena[32,33]. For particles in the size range of 1 to 10 µm, mechanical filtration in the form of gravitational sedimentation and inertial impaction are the most involved in the capture of particles. Particles of larger size exhibit greater inertia, thus moving in a more linear trajectory and failing to circumvent the mask fibers. Consequently, they adhere to the fibers and are incapable of passing through the filtration barrier. For small particles ranging in size from 0.1 to 1 µm, interception and Brownian motion represent the most prevalent mechanical filtration mechanisms[34,35]. Then, electrostatic filtration utilizes charged fibers, referred to as electrets, endowed with a quasi-permanent electric field, thereby employing electrostatic attraction to filter particles. The more charged a material is, the more its filtration performance increases[36]. The combination of mechanical and electrostatic filtration enhances the filtration efficiency of masks, since a higher number of particles will be retained. However, cotton is considered to be less charged compared to other fabrics and retain less particles[34]. CFM2, which is made of cotton, is the mask where the number of CFU found in the outer layer was the higher after five minutes on the bench. Bacteria were thus less retained and pass through the fibers. Moreover, concerning textile masks, the more layers there are, the greater the filtration efficiency is, since the volume of droplets transmitted through the layers decreases[37]. This fact can also explain why more bacteria can pass through the inner layer of CFM2 compared to the biocidal CFM1 after a short period of time since the last one is composed of three layers instead of two.

After the masks were left on the bench or worn in the simulator for 240 min, two main behaviors can be observed:

- The hydrophobic nature of the inner layer of surgical and CFM1 masks can keep the bacterial droplets relatively intact and keep them alive under good conditions. Therefore, the bacteria do not migrate and a high proportion of culturable bacteria remain on the inner layer.
- CFM2 masks have a high moisture absorption capacity, which makes the bacteria susceptible and more likely to die due to lack of moisture and exposure to dryness. This behaviour leads to a very similar bactericidal effect as the molecules added to the biocidal CFM1. As a result, the bacteria cannot migrate and a small number of culturable bacteria remain on the inner layer.

It has also been shown that respiratory droplets have a very low survival capacity in masks with a hydrophilic surface where absorption occurs, resulting in less droplet residue after evaporation at the mask surface compared to a hydrophobic surface[38]. This therefore supports the theory of a «natural bactericidal» action of textile fibers.

In addition, the lack of nutrients over time could also explain the absence of bacteria, as there are molecules in saliva that allow bacteria to stay alive. Indeed, the salivary pellicle which is formed inside the oral hard and soft tissues determines the initial adhesion and proliferation of micro-organisms[39,40]. Proteins, glycoproteins and gingival crevicular fluid that are present in the saliva offer a favorable environment for bacteria. Thus, saliva plays a key role in the formation and maintenance of the ecological balance of the resident oral microbiota[18,41]. This could explain why the number of colonies in the inner layer of the mask decreases on the inner layer after six hours of wear since there was no bacterial renewal within our experimental design over time.

Besides, the fit of the mask plays a role in the amount of CFUs found. In the general population, the mask is worn with a degree of laxity, permitting the potential release of air through the mask's lateral openings. By eliminating any possibility of air leakages, the mask retains moisture of the breath and keeps it inside the different layers. By absorbing moisture, the mask deprives bacteria of the environment they need, making it difficult for them to thrive. This would explain why the results showed a decrease in the number of CFUs.

Although it might be thought that with a higher respiratory frequency and tidal volume, the mask fibers would allow more bacteria to pass through, we observed no significant difference between a breathing cycle at rest and at exertion. This demonstrates that it is not a parameter influencing the passage of bacteria between the layers of the mask.

It would also be interesting in routine clinical practice to analyze the mortality of the bacteria by placing the layers of the mask under investigation in broth and seeing whether the bacteria are dead or just unable to be cultivated. Moreover, the reuse of textile mask can impact the filtration efficiency of the mask and may facilitate the passage of bacteria through the layers after a short period of time. Finally, a study focusing on viral particles would also be interesting in the future to see whether the type of microorganisms has an impact on their distribution within the different layers of the mask.

## Conclusion

The results of the study show that the non-airborne bacteria remain mainly on the surface of the mask and do not easily pass through the layers over time, especially in the surgical mask and CFM1. However, the number of bacteria found in the layer decreases very significantly after four hours, especially for the biocidal CFM1 and CFM2. Surgical masks and CFM1 are more capable of binding bacteria and creating a breeding ground for germs. The biocidal nature of the biocide CFM1 proved to be effective, especially within a short period of time. The breathing parameters have no influence, but the fit of the mask and the duration of wearing could impact the number of bacteria found in the inner layer. These results confirm that the standard test for bacterial filtration efficiency (using the penetration of airborne bacteria in aerosol droplets) is the best test that can objectively

measure the penetration ability of bacteria through the layers of a mask, as we have shown that under our experimental conditions the penetration ability of non-airborne bacteria is insignificant.

## Data availability

The data that support the findings of this study are available on request from the corresponding authors.

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## Author contributions
A.K. conducted the experiments and worked on formal analyses, wrote the original draft and contributed the review of the manuscript. L.L. helped for the experiments, methodology, conceptualization, data curation and contributed the review of the manuscript. M.F. supervised the work and contributed the review of the manuscript. F.G. designed the experiments, helped for the methodology, conceptualization, supervised the work and contributed the review of the manuscript. P.V. designed the experiments, conceptualization, supervised the work and contributed the review of the manuscript. J.P. designed the experiments, conceptualization, supervised the work and contributed the review of the manuscript.

## Declarations

## Competing interests
The authors declare no competing interests.

## Additional information
**Correspondence** and requests for materials should be addressed to P.O.V.

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