ORIGINAL ARTICLE

In vitro effectiveness of the filtration in three and four layered surgical masks after a few hours exposure of *S. aureus*: experiment study

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ABSTRACT

Introduction: Surgical masks commonly have many layers. The outer layer acts as the initial filtration and the protection for the middle layer, the middle layer acts as the primary filtration, and the innermost layer absorbs liquid to prevent the spread of droplets from the mask's wearer. The recommended duration of wearing medical and fabric masks is 4–6 hours, while some studies stated that the maximum duration is 8 hours. The use of *S. aureus* in this study was based on its clinical relevance as a major cause of nosocomial infections. This study aimed to analyze the effectiveness of the filtration in three-layer and four-layer masks against *S. aureus*. **Methods:** This experimental study followed the methods of previous research, and followed the Standard Test Method from SNI 8489 2018 with several adjustments consisting of several steps, *i.e.*, preparation of tested masks, bacterial culture media, and tested bacteria; determination of masks' filtration efficacy; confirmation of the efficacy. All steps were repeated 15 times with exposure times of 2, 4, 6, and 8 hours and analysis using the chi-square test. **Results:** The percentage of surgical masks filtration effectiveness was indicated by the growth of *S. aureus* in Nutrient Agar media. The four-layer surgical masks showed the highest percentage (80%) of 2 hours and 4 hours of usage. Both types showed the lowest percentage of 8 hours of usage. Based on the duration of wearing, the mask's filtration effectiveness from the highest and lowest score was the *S. aureus*-exposed media for 2, 4, 6, and 8 hours, respectively $p<0.050$. The comparison value for the effectiveness of mask filtration between the type of masks and the length of use was 0.003. **Conclusion:** The filtration effectiveness of the four-layer masks is higher than the three-layer masks according to the duration of wearing the masks.

KEYWORDS
surgical masks, filtration, duration, *S. aureus*

INTRODUCTION

At the end of 2019, Wuhan, a new business centre in China, encountered a novel Coronavirus plague that killed over 1.800 individuals and infected over 70.000 individuals in the first fifty days of the epidemic.[1] This condition indicated that this virus transmission was very high and caused the World Health Organization (WHO) to declare the COVID-19 plague as a global pandemic on 11 March 2020.[2,3] WHO also advised the infection prevention and control (IPC) that involved hand hygiene, distance restrictions of at least 1 meter, avoiding touching the face, coughing and sneezing etiquette, adequate ventilation in a closed space, COVID-19 test, contact tracing, quarantine, and isolation.[4] WHO also recommended face masks wearing as part of the complete sequence of prevention and control to restrict the virus transmission.[5]

The human-to-human transmission of aerosol-containing viruses or bacteria might occur due to close contact with infected individuals, e.g., coughing, sneezing, and talking.[6,7] The aerosol can penetrate the body through nasal and oral inhalation, spreading until it reaches the lungs.[8] Face masks wearing is one of the essential health protection as preventive efforts.[9] Reusing the disposable face masks to save up masks can decrease the filtration effectiveness because the masks might become damp or the filter's porosity might become more extensive, resulting in the contaminants aerosol containing viruses or bacteria being inhaled by the wearer.[10,11]

The effectiveness of face masks in preventing pathogen transmission is attributed to the type of masks, the materials, and the duration of wearing. The recommended types of masks by WHO are fabric, surgical, medical, and respiratory masks.[12] The fabric masks are advised to be made of cotton and have at least three layers. The surgical and respiratory masks (*e.g.*, N95) are advised to be skin-friendly and have absorbent materials for the innermost layer that contacts the skin. The absorbent materials are utilized to avert the droplet transmission of the wearer to others. The Meltblown Polypropylene is used as the middle layer of the primary filtration. The spun-bonded hydrophobic nonwoven polypropylene is the outer layer for the initial filtration and the middle layer's shield.[13] The maximum recommended duration of surgical and fabric masks wearing is 4–6 hours, but some studies reported 8 hours was the maximum duration. A Massachusetts Lowell University and California Baptist University United States study reported that masks reused, damp, ripped, unclean, and used for prolonged periods were not feasible to wear. Those conditions will cause airflow restriction, the wearer prone to inhaling particles, and reduced filtration capability.[14]

The National Standardization Agency of Indonesia (Badan Standardisasi Nasional) and the American Society for Testing and Materials (ASTM) have conducted mask filtration efficiency tests using *S. aureus* as the biological agent. *S. aureus* was utilized as the test reagent due to its size being more significant than the virus', its pathogenicity to humans, and the primary etiology of nosocomial infection. The bigger size of the cell vis-à-vis the virus indicates the mask's filtration efficiency; if a bacteria cannot be filtered, a virus whose size is smaller will likely pass the mask's filtration. If a mask's filtration cannot restrain bacteria or viruses, the mask's filtration performance is low. The filtration performance is also affected by other factors, e.g., the duration of wearing.[15] Most people tended to use cheaper masks and used them for more than 8 hours.[16] For example, in the non-shift work system, employees usually wear masks for a duration of up to 8 hours, namely when going to work before 7 am and removing or changing the mask when they are at home at 4 pm. Another study also showed that students and health workers were required to wear masks chronically for more than 8 hours, which is the average duration of school learning.[17,18]

The information regarding the effectiveness rate of mask filtration in 8 hours of use still requires more detailed research. The aim of this study about the In Vitro effectiveness of the filtration in three and four layered surgical masks after several hours exposure *to S. aureus* was to determine the filtration effectiveness rate of each type of masks against *S. aureus*. It was based on the rapid mutation of the SARS-COVID 19 virus, the high risk of contamination in reused masks, and a prolonged time of masks-wearing. This present study was expected to generate information regarding the correct and safe duration of wearing three- and four-layered surgical masks at the high risk of contamination. This study hypothesized that four-layer masks have better filtration than three-layer masks. This study aimed to analyze the effectiveness of the filtration in three-layer and four-layer masks against *S. aureus*.

METHODS

The population in this experimental study was surgical masks recommended by WHO and worn by the community and the healthcare providers during the pandemic from 2019 to 2022. The samples were three- and four-layered surgical masks with the same model and brand and were given a repetition procedure from stage one to stage 6, 15 times in exposure duration of 2, 4, 6, and 8 hours. The data retrieval and research process were conducted from 30 January to 5 April 2023 at The Microbiological Laboratory Faculty of Dentistry Universitas Padjadjaran.

The instruments utilized in this study were autoclave (All American Electric Pressure Steam Sterilizer Model no. 25x 121oC/250oF), autoclave (Melag), microscope (Olympus binocular XSZ-107BN, magnification of 10×100), pipette, incubator (Memmert BE500), UV Rays, ruler, inoculating loops, plastic wrap, Bunsen burner, object glass, flashlight, and tripod. The materials utilized in this study were three- and four-layered surgical masks, Aquades, Petri dish, frozen *S. aureus* isolates (ATCC 6538), nutrient agar media, nutrient broth, and NaCl.

The research procedure followed Aruan et al. with some modifications.[19.] Researchers studied the difference between the results of bacteria filtration in the buff and the surgical masks on the exact duration; the sample retrieval process was conducted directly in an open space, and the incubation, isolation, and observation process were conducted at the laboratory. The research procedure involved the preparation of the tested masks, the preparation of the growth media, the preparation of the tested bacteria, the determination of the masks filtration effectiveness (masks testing against bacteria) the confirmation of the masks filtration effectiveness, analysis and data presentation. The modifications in this context include modification of the object of the masks, the previous study used buff type masks and surgical masks, while this study used only surgical masks but with a different number of layers. The previous study also took bacterial samples directly from the open space, while this study used cultured bacteria in the laboratory by dripping them without touching the surgical mask using a pipette and flashlight as a benchmark for drops.

The sample masks utilized in this study were mask products certified by ISO 9001 and ISO 13485 standards. ISO 13485 is the standard for quality management for health instruments, originating from the ISO 9001 quality management system. The masks also had acquired marketing authorization from the Ministry of Health Republic of Indonesia, proven by a code of Domestic Health Instrument (Alat Kesehatan Dalam Negeri, AKD) printed on the packaging and on the official website of the Ministry of Health Indonesia. The marketing authorization of the masks had fulfilled the regional regulation criteria from Indonesian National Standardization Institute (Badan Standardisasi Nasional, BSN). BSN has established 3 Indonesian National Standards (Standar Nasional Indonesia, SNI) regarding medical masks, i.e., SNI 8488, SNI 8489, and SNI EN 14683.[20] SNI 8488 is the standard specification for the composer materials of medical masks. SNI 8489 is the bacterial filtration efficiency standard testing method for medical masks, using the biological *S. aureus* aerosol. SNI EN 14683 is the medical masks requirements and testing method.

The first step in preparing the tested masks was to modify the masks by cutting the stretched masks into a round shape with dimensions of 12 cm diameters and the center point in the middle of the masks. The dimension was selected according to the general width of the mouth (10 cm) and for being closer to the respiratory tract when the mask was used (Figure 1). A 2 cm addition was applied to provide a gap for dropping bacterial suspension onto the masks. The modified masks were put into the petri dish and sealed tightly using plastic wrap. The masks in the petri dish were sterilized to create zero contamination to minimize research bias; the sterilization process used the autoclave at 121°C for 15 minutes. The sterilized masks were stored in a sterile storage room before being used.[17] A mask from each test group was implanted in the agar nutrient solid media, then incubated at 37°C for 18–24 hours. The following day, the media that contacted the masks was assessed to see if there was bacterial colony growth. If a bacteria colony was found, the mask was not sterile and must be re-sterilized using the UV rays.

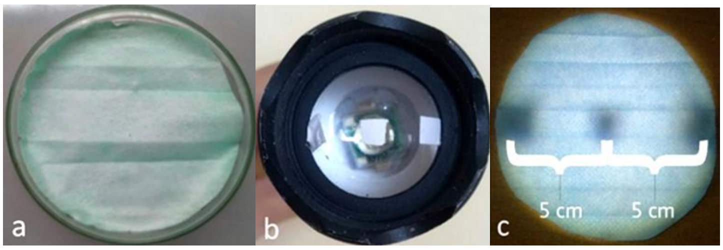

Figure 1. Masks modifications: a. The display of masks positioning in the petri dish; b. The flashlight modification as the reference point of *S. aureus suspension* dropping; c. The reference points from the flashlight for *S. aureus suspension* dropping onto the masks

The bacterial growth media in this study was the solid agar nutrient (AN). The AN powder was dissolved into sterile Aquadest in an Erlenmeyer tube with the ratio printed on the packaging, and then the solution was heated until boiling. The liquid media tube was sterilized in an autoclave at 121°C for 15 minutes. The sterilized liquid media was poured into a petri dish and sealed until it solidified. The petri dish was wrapped using plastic wrap and put in storage. The pouring was conducted under sterile conditions.

The tested bacteria in this study were frozen *S. aureus isolates* (ATCC 6538) from the Microbiological Laboratory Faculty of Dentistry Universitas Padjadjaran. The bacteria were collected using inoculating loops, inoculated in the nutrient broth, and then incubated for 18 hours. A 0.5 mL of liquid bacterial suspension was resuspended into 5 mL of 0.9% NaCl, and the turbidity matched the 0.5 McFarland standard or was proportional to the number of bacteria of 108 (CFU)/mL. The bacteria suspension in NaCl was used as the tested bacteria in the determination of masks filtration effectiveness.

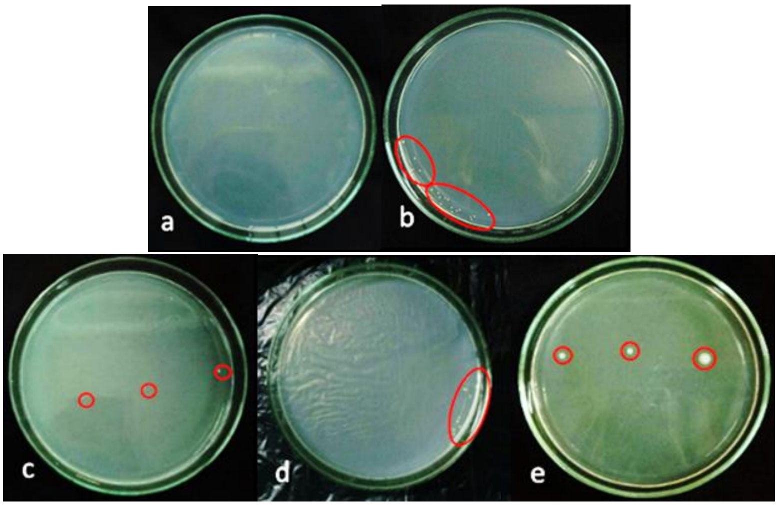

Figure 2. The sterile media (control) and the exposure media (test) incubation results: a. The sterile media after incubation showed no growth of *S. aureus colony*; b. The sterile media after the implantation of three-layered surgical masks exposed by *S. aureus for* 2 hours, bacterial colonies (red circle); c. The sterile media after the implantation of three-layered surgical masks exposed by *S. aureus for* 4 hours, bacterial colonies (red circle); d. The sterile media after the implantation of three-layered surgical masks exposed by *S. aureus for* 6 hours, bacterial colonies (red circle); e. The sterile media after the implantation of three-layered masks exposed by *S. aureus for* 8 hours, bacterial colonies (red circle).

The masks were placed in the petri dish containing the AN media. The mask surface where the suspension was dropped faced upwards (Figure 2a). A 0.5 mL bacterial suspension with a turbidity of 0.5 McFarland was dropped using a pipette on three spots (middle, left, and right side of the masks).

Each spot was spaced 5 cm using a flashlight placed above the masks as the reference point for spacing (Figure 2.b and 2.c). The determination of the spots depicted the mouth form while wearing a mask (left corner, right corner, and middle of the mouth). The masks were exposed to the bacteria for 2, 4, 6, and 8 hours. Following the exposure, the masks were removed from the petri dish and then incubated at 37ºC for 18–24 hours under aerobic conditions.[21]

The success of the determination of the masks filtration effectiveness test was confirmed by observing the growth of *S. aureus colonies*. The treated media, after incubation, was observed. The *S. aureus colonies* on the agar nutrient were round-shaped with 0.5–1.5 mm dimensions and yellowish-white-coloured. If colonies were found in a media, a confirmation was conducted by creating a bacterial smear for the particular colonies.

A small amount of 0.9% NaCl was dropped onto the object glass. A small quantity of the colony was collected from each colony using an inoculating needle and then spread on the object glass to form a thin film. The object glass containing the bacteria was fixed by surpassing the glass over a Bunsen burner three times. The dried bacterial smear was dyed using the Gram staining, added a drop of immersion oil, and observed under the microscope with a magnification of 10×100.[22] The objective of the bacterial confirmation was to see *S. aureus on* the object glass, as seen by a purple-coloured (positive Gram) coccus cell-sized 0.8–1.0 micron with grape-like clusters. The masks filtration effectiveness was calculated using the percentage formulation.[23]

The masks filtration effectiveness was calculated using the percentage formulation; number of no exposure to bacteria/number of no repetitions (15)x100%.[23] The data was statistically analyzed using the Chi-squared test with a p-value of 0.05 to compare the filtration effectiveness between both tested masks and generate information to determine the risk of wearing the three- and four-layered masks using the relative risk score.

RESULTS

The observation results of the surgical masks filtration effectiveness towards the growth of *S. aureus colonies* are presented in Figure 3. A round-shaped and yellowish-white-coloured colony marks the existence of the bacteria with a diameter of 0.5–1 mm on the media. No bacterial colony was found on the sterile control media; this proved that the surgical masks were sterilized and had zero contamination. On the media exposed for 2 hours, a few bacterial colonies were found on the left side of the media. On the media exposed for 4 hours, bacterial colonies were found on all sides of the media. On the media exposed for 6 hours, bacterial colonies were filled on the right side of the media. On the media exposed for 8 hours, bacterial colonies were found on all sides of the media. The bacterial colony confirmation asserted that the particular colonies were *S. aureus*. The bacterial cell seemed to be round with a diameter of 0.8--1.0 µm, purple-stained, and formation of grape-like.

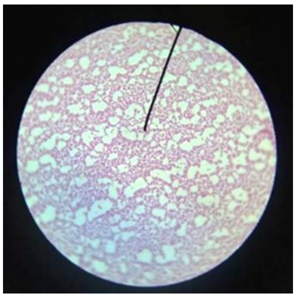

Figure 3. The microscopic view of the Gram-stained bacteria from a sterile media after the implantation of three-layered masks exposed by *S. aureus for* 2 hours, magnification of 10×100 with immersion oil

Table 1 shows the test results that at 2 - 6 hours there was less bacterial growth on the 4-ply surgical masks compared to the 3-ply surgical masks, and at 8 hours there was already a lot of bacterial growth on both the 4-ply and 3-ply surgical masks. This shows that a four-layer mask has higher filtration effectiveness than a three-layer mask, provided that the duration of use is limited to 2-6 hours.

Table 2. provides the results of filtration effectiveness in both types of surgical masks after *S. aureus exposure*. The results indicated that the surgical masks filtration effectiveness differed significantly (sig<0.050) according to the number of surgical masks layers and the duration of wearing. Based on the duration of wearing, the surgical masks filtration effectiveness from the highest and lowest score was the *S. aureus exposed* media for 2, 4, 6, and 8 hours, respectively (sig<0.050).

The relative risk of the three- and four-layered surgical masks wear is provided in Table 3. The risk of bacterial contamination by wearing three-layered surgical masks for 2, 4, and 6 hours was 2.00, 3.33, and 1.75 times higher than wearing four-layered surgical masks, respectively. These findings showed that the three-layered surgical masks had a higher risk of bacterial contamination compared to the four-layered surgical masks in the duration of wearing for 2–6 hours. Meanwhile, the bacterial exposure for 8 hours on both surgical masks resulted in a constant exposure score, showing that the filtration of both surgical masks was no longer effective during the 8 hours of wearing.

Table 1. The data of *S. aureus colonies* growth from the masks filtration effectiveness test

		***S. aureus exposure* duration (hour)**							
		Three-layered surgical masks				**Four-layered surgical masks**			
	Times of exposure	2	4	6	8	2	4	6	8
S. aureus colonies found per repetition	1	+	-	+	+	+	+	+	+
	2	-	+	+	+	-	+	+	+
	3	+	+	+	+	+	+	+	+
	4	+	-	+	+	-	-	+	+
	5	+	+	+	+	+	-	-	+
	6	+	+	+	+	-	-	+	+
	7	-	-	+	+	-	-	-	+
	8	-	-	+	+	-	-	-	+
	9	-	+	-	+	-	-	-	+
	10	-	+	+	+	-	-	+	+
	11	-	+	+	+	-	-	-	+
	12	-	+	+	+	-	-	-	+
	13	-	+	+	+	-	-	+	+
	14	+	+	+	+	-	-	-	+
	15	-	-	+	+	-	-	+	+
Filtration effectiveness (%)		60.0	33.3	6.7	0	80.0	80.0	46.7	0

+ : *S. aureus* colonies were found**, - :** no *S. aureus* colonies were found

Table 2. The comparison of the surgical masks filtration effectiveness between the type of masks and the duration of wearing

Between the type of surgical masks		**p-value**	**Between the duration of wearing (hour)**		**p-value**
Three-layered surgical masks	Four-layered surgical masks	0.003	2	4	0.284
				6	0.001
				8	0.000
			4	2	0.284
				6	0.018
				8	0.000
			6	2	0.001
				4	0.018
				8	0.005
			8	2	0.000
				4	0.000
				6	0.005

Table 3. The relative risk of the filtration effectiveness of each type of surgical masks according to the duration of wearing

Duration of wearing (hour)	**Type of surgical masks (layer)**	***S. aureus colony* growth**		**Relative risk**
		Found	**Not Found**	
2	3	6	9	2.00
	4	3	12	
4	3	10	5	3.33
	4	3	12	
6	3	14	1	1.75
	4	8	7	
8	3	15	0	-
	4	15	0	

DISCUSSION

Table 1 shows the test results that the four-layered surgical masks had higher filtration effectiveness than the three-layered masks, with a note of duration of wearing limited to 2–6 hours. The results presented in table 2, show that the filtration effectiveness of surgical masks differs significantly ($p<0.050$) according to the number of surgical masks layers and length of use, in line with table 3 which shows that there are differences in the risk of bacterial contamination when using three-layer surgical masks for 2, 4, and 6 hours respectively 2.00, 3.33, and 1.75 times compared to using a four-layer surgical masks. This shows that three-layer surgical masks have a higher risk of bacterial contamination than four-layer surgical masks for a duration of use of 2-6 hours. The four-layered surgical masks were discovered to be more effective because they have two layers acting as the filtration, whereas the three-layered surgical masks only have one layer acting as the filtration. The filtration layer is commonly made of *melt-blown nonwoven fabric* and is responsible for particle filtration. This material can detain a relatively large particle (>0,3 μm) through the sedimentation and impaction process.[24]

The contaminant used in this study was *S. aureus,* with a diameter of 0.8-1.0 μm. The observation results of the surgical masks filtration effectiveness towards the growth of *S. aureus colonies* can be seen in figure 2 and the microscopic view in figure 3 which is presented in the results section. With its size, *S. aureus should* be able to be filtered by the filtration layer; but due to the presence of aerosol from the respiratory tract, the surgical masks become moist and damp. A study by Indrayanti et al. stated that a damp mask causes a decrease in filtration performance, resulting in a weaker restraint of particles.[25] A damp mask also increases the porosity size in the filtration layer, making the masks a fit area for fungal growth. Fungi are generally more prominent than bacteria and can be restrained by surgical masks. However, an increase in humidity can induce fungal growth, precisely the fungi that cause infection, *e.g.* dermatitis perioral.[26]

The wearing of surgical masks has not been able to protect against the coronavirus due to its diminutive size (60–140 nm). However, the primary coronavirus transmission is through the aerosol droplet size <5 μm that a surgical masks can filter.[14,15] The surgical masks can obstruct droplets from coughing and control airborne infections.[29] The wearing of the surgical masks is also better compared to not wearing a masks at all in preventing respiratory viral infections transmitted through droplets.[20,21] This limitation of masks in preventing the coronavirus infection is the excuse why wearing the masks is not the only way to prevent COVID-19 transmission; hence, other preventions are highly recommended and essential to conduct, i.e., avoiding crowds, a minimal 1 meter of physical distancing, hand hygiene using soap and water, and maintaining the coughing and sneezing etiquette.[32]

The longer the duration of wearing surgical masks, the less the filtration performance effectiveness of surgical masks. The mass amount of unfiltered and growing bacteria on the surgical masks indicates ineffective surgical masks filtration.[33] Bacteria can adhere to many media, including solid media such as fibres in the masks. Some bacteria live in an environment with humidity over 85%.[34] A surgical mask worn for a long duration will increase the humidity and become a medium for bacterial growth. This condition relates to the duration of bacterial growth (doubling time). Doubling time in bacterial growth is the required time for the cells or a mass of the cell to double its amount or mass in one cycle. Doubling time differs in many bacteria based on their development.[35] A study by Putri et al. reported that the Gram-positive bacteria (*S. aureus*) had a doubling time 1.32 times slower than the Gram-negative bacteria (*Escherichia coli*) in the same media.[36] Other masks conditions (*e.g.*, ripped, dirty, and damp) also make the masks not feasible to wear and might restrict the airflow; hence, making the wearer more prone to inhaling particles and decreasing the masks's filtration performance.[14]

The bacterial growth rate relies on the environmental state; if the surrounding environment has few nutrients, the growth rate will be slower than in the rich-nutrition environment.[37] This study's results were in line with a study by Macintyre et al. stating that prolonged masks-wearing while working for 8 hours increased the risk of infection transmission because bacterial growth had already occurred on the surgical masks. The bacterial growth might lead to a decrease in the immune system and eventually initiate other diseases. If an infection's treatment is delayed, the infection will lead to a more severe health issue.[33]

Besides the number of layers and the duration of wearing, the masks' effectiveness is also attributed to the severity of the environmental contamination. WHO has established a guideline for wearing masks for the community and the healthcare providers and suggests wearing surgical masks while being or performing any activities in high-risk or contaminated places.[19] The working environment indicated the contamination level of the virus. Those working as a nurse for a patient with an infectious disease at a hospital will be more exposed to contaminants than those working in a sterile storage room. This condition follows the recommendation of wearing Personal Protective Equipment (PPE) in Indonesia that states that the general public who are showing symptoms or are sick and healthcare providers (including a dentist), as well as non-workers in an aerosol-releasing environment, must use at least the level 1 PPE. Level 1 PPE includes the usage of a three-layered surgical masks.[38]

The limitation of this research was the level of sterility of the laboratory environment which was simple and was not carried out on laminar airflow due to equipment limitations, so for future studies, it is hoped that future research can use environmental laboratories and tools that are better than the previous ones. Clinical recommendations are to suggest using a 4-layer surgical masks for better filtration protection

CONCLUSION

The four-layered surgical masks bear a higher mask filtration effectiveness compared to the three-layered surgical masks in a wearing duration of 2–6 hours.

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Informed Consent Statement: "Not applicable". This studies do not involve humans.
Data Availability Statement: Data supporting reported results can be found in the results section of the article manuscript.
Conflicts of Interest: The authors declare no conflict of interest.

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