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# The adhesion of *Staphylococcus aureus* to epithelial cells: an *in vitro* approach to study interactions within the nasal microbiota

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## Abstract

### Introduction

*Staphylococcus aureus* is a skin and mucous commensal bacterium of warm-blooded animal. In humans, nose is the main ecological niche of *S. aureus*, and nasal carriage is a risk factor to develop an endogenous infection. *S. aureus* nasal colonization is a multifactorial process, involving inter-species interactions among the nasal microbiota.

### Aims

The objectives of this study were to characterize the microbiota of carriers and non-carriers of *S. aureus* and to demonstrate the importance of inter-species relations in the adhesion of *S. aureus*, a key step in nasal colonization.

### Methodology

Firstly, we have characterized the nasal microbiota from thirty *S. aureus* carriers and non-carriers by a culturomic approach. Then, we have evaluated the adhesion of *S. aureus*, first alone and then along with other bacteria of the nasal microbiota. To do that, we used an *in vitro* model to measure the interactions among bacteria in the presence of epithelial cells.

### Results

The analysis of the nasal microbiota of the carriers and non-carriers of *S. aureus* made possible to observe that each microbiota has specific features in terms of composition. However, this composition differs significantly between carriers and non-carriers mainly through two bacterial groups: coagulase-negative staphylococci and corynebacteria. In a second part, adhesion of *S. aureus* to epithelial cells showed competitions between *S. aureus* and these bacteria, suggesting a limitation of nasal colonization by *S. aureus*.

### Conclusion

These findings demonstrate the existence of a negative correlation between *S. aureus* and these species which inhibits adhesion and could limit nasal colonization.

## 1. Introduction

*Staphylococcus aureus* is both a frequent member of nasal microbiota and a major human pathogen [1]. *S. aureus* asymptomatic nasal carriage is known to be a major risk factor for subsequent infections [2, 3], which can be prevented by nasal decolonization using an antibacterial agent [4]. Nasal carriage is the key for colonization of other anatomical sites [1] and has to be considered for control of staphylococcal diseases. Consequently, anterior nares are a target for the detection of *S. aureus* and prevention of infections [5].

In the discovery of human microbiota, the nose has been far less studied than the gut. However, recent works have focused on interactions between bacteria in this ecosystem, most of them using molecular methods [6] or alternative culturomic approach [7]. A large proportion of bacteria found in nasal microbiota belong to Firmicutes and Actinobacteria phyla, and the most abundant genus found are *Cutibacterium*, *Corynebacterium* and *Staphylococcus* [7-10]. However, differences in nasal flora composition exist from one individual to another, depending on various factors: ethnic origin, age, genetics and environmental factors [1, 9, 11-13]. Moreover, about one half of the human population is not colonized by *S. aureus* and this suggests that differences in nasal microbiota composition could have direct influence on colonization [9, 10, 14], and that non-carriers might have a different nasal microbiota. Some authors have reported previously negative correlations for *S. aureus* carriage with *Finegoldia magna*, *Corynebacterium accolens* [14], as well as for coagulase-negative staphylococci: mainly *Staphylococcus epidermidis* [15], *Staphylococcus hominis* [16] and *Staphylococcus lugdunensis* [17]. By contrast, a positive correlation was reported for *Corynebacterium pseudodiphthericum* [14]. This study aims to better understand inter-species interactions in this complex ecosystem, in regard to composition of the nose microbiota. Firstly, we have characterized the nasal microbiota from *S. aureus* carriers and

non-carriers. Then, we have evaluated the adhesion of *S. aureus*, first alone and then along with other bacteria. To do that, we used an *in vitro* model to measure the interactions among bacteria in the presence of epithelial cells. To our knowledge, this the first study that attempted to examine *S. aureus* adhesion on epithelial cells to understand the interactions between species that frequently colonize the nose.

## 2. Materials and methods

### 2.1. Microbiological methods

The volunteers were between 20 and 40 years old, were of European origin and lived in the city of Rennes area, France. Volunteers have been questioned and were not retained if they had a chronic respiratory disease or acute respiratory infection within 7 days before sampling. No laboratory test was performed.

Thirty healthy adult volunteers were swabbed from both nostrils. The samples were taken in the lower third of the nostrils, 1-2 cm deep using a single dry swab for both nostrils.

The samples were suspended in 1 ml of sterile water and two dilutions were made ( $10^{-1}$  and  $10^{-2}$ ). Ten microliters of each dilution were seeded on two Colombia agars supplemented with 5% sheep blood and a blood agar cooked with 10% sheep blood (Oxoid, France). The blood agar plates were incubated at 37 ° C under aerobic and anaerobic using Anaerogen bags (Oxoid, France) conditions, respectively. The chocolate agar plates were incubated at 37 ° C, under 5% CO<sub>2</sub>. All agar plates were observed after two and five or seven days depending on their culture conditions. Some colonies were picked out plates for further characterization, and then were stored on beads in cryopreservative solution at -80 ° C (Cryobank®, Lustiner, France).

After incubation, colonies with different morphotypes were quantified and characterized by identification via the Matrix-Assisted Laser Desorption / ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technique. Briefly, bacteria from pure cultures were deposited on a 96-site plate and treated with 70% formic acid. MALDI-TOF identification was performed using the alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix and the Bruker Microflex (Microflex®, Bruker Daltonics, Germany) coupled to the FlexControl

software as recommended by the manufacturer. Each identification was done twice. For the bacterial species, identification was considered correct if the score was equal to or greater than 2. Otherwise, identification at the genus level was retained if the score was greater than 1.8.

## 2.2. *In vitro* adhesion model

### *Bacterial strains and culture conditions*

Two strains derived from carriers were used to study the adhesion and the internalization of *S. aureus* alone on epithelial cells (*S. aureus* II-6 and II-9). Genes coding for virulence factors were determined using the *S. aureus* genotyping kit 2.0 Alere®. For adhesion with the other bacteria of the nasal microbiota (hereinafter referred to as "Community model adhesion"), one *S. aureus* strain (strain II-9) and two strains of the following species: *S. epidermidis*, *C. acnes* and *C. accolens* were selected. The latter six strains came from non-carriers of *S. aureus*. The isolates were grown in Luria-Bertani (LB) medium at 37 ° C with shaking (150 rpm) for *S. aureus* and *S. epidermidis*, or in Brain Heart Infusion (BHI) medium at 37 ° C with shaking (150 rpm) under anaerobic conditions for *C. acnes* and *C. accolens*.

Cultures were centrifuged for 10 minutes at 5000 g at room temperature and washed twice with a solution of Phosphate Buffered Saline (PBS) (D. Dutscher, France). Then, bacteria were resuspended in Dubelcco's modified Eagle's medium (DMEM) (D. Dutscher, France) and the concentrations were estimated by measuring the optical density (OD) at 600 nm.

### *Cell cultures*

The Ca9-22 cell line (reference JCRB0625, Japanese Cancer Research Resource Bank, Japan) from human gingival carcinoma was used for the adhesion and internalization assays. Cells were cultured in DMEM supplemented with 10% inactivated fetal calf serum (FCS), 100 IU / ml penicillin, 10 mg / ml streptomycin and 5% glutamine (D. Dutscher). Cultures were carried out in cell culture flasks (T75 Falcon®, Corning Incorporated), incubated at 37 ° C, under 5% CO<sub>2</sub> until cells confluence, treated with 0.05% trypsin (D. Dutscher) and then re-suspended in DMEM. For adhesion assay,  $5 \times 10^5$  cells were placed per well in a 12-well plate (Cellstar®, greiner bio-one) and incubated overnight at 37 ° C, under 5% CO<sub>2</sub>.

#### *Transmission electron microscopy*

In order to determine optimal time to avoid internalization, we performed transmission electron microscopy (TEM). Three conditions were retained: pellets of bacteria, Ca9-22 cells alone, and a mixture of Ca9-22 / *S. aureus*. Three contact times were tested: 30, 60 and 90 minutes. Succinctly, Ca9-22 cells were incubated at 37°C under 5% CO<sub>2</sub> for 24 hours in wells (Nunclon tm <sup>Δ</sup> Surface, ThermoFisher Scientific) at a rate of  $5 \times 10^5$  cells / well. Confluent cells were washed twice with PBS prior to contact with *S. aureus* in exponential phase at the MOI 50. After incubation, the samples were prepared as described elsewhere [18]. Briefly, infected and control Ca9-22 cells were washed with 0.15 M cacodylate buffer, then fixed in 2.5% glutaraldehyde in cacodylate buffer and stained with 1% osmium in cacodylate buffer. Following dehydration in an alcohol gradient, cells were embedded in resin (mix of Epon and DMP30). Finally, ultrathin sections were observed by TEM. The process was similar for bacteria used alone but the dehydration was realized in an acetone gradient. TEM was also performed with another protocol. After a contact time of 60 minutes, Ca9-22 cells with *S. aureus* at the MOI 50 were washed twice with PBS and treated 2h at 37°C with DMEM

supplemented with 100 µg.ml<sup>-1</sup> gentamicin to kill extracellular bacteria. Samples were prepared as described before.

#### *Adhesion assays*

Adhesion of *S. aureus* alone was achieved in both exponential and stationary phases. Three different Multiplicity of Infection (MOI, *e.g.* ratio of *S. aureus* cells to epithelial cells) were tested: 10: 1, 50: 1, 100: 1. Confluent Ca9-22 cells were washed twice and then incubated during one hour at 37 ° C, under 5% CO<sub>2</sub> with *S. aureus* suspension in DMEM (not supplemented). After incubation, Ca9-22 cells were washed softly three times with PBS to remove non-adherent bacteria, and then lysed using 200 µl of a 0.05% Triton solution in sterile water. Remaining bacteria, considered to be adherent but not internalized in cells, were counted by spreading on Trypticase Soja agar (TSA) plate and incubation at 37 ° C for 24h. For each MOI tested, inocula were controlled by spreading on TSA and incubated at 37°C during 24h and counting. The ratios were calculated as follows: adhered bacteria / bacteria in contact with cells

For the community model, one *S. aureus* isolate in exponential growth was mixed with one isolate of another species (*S. epidermidis*, *C. acnes* or *C. accolens*) in stationary phase. The *S. aureus* strain was used at the MOI 10: 1 constantly, but the other bacteria were tested at 70:30, 50:50 and 30:70 ratios with *S. aureus* (*e.g.* suspension corresponding to a 70:30 ratio contained 70% *S. aureus* and 30% of other bacteria).

*C. acnes* being an anaerobic bacterium, its viability was verified after contact with *S. aureus* on Ca9-22 cells by seeding on blood agar and incubation for 48 h at 37 ° C in an anaerobic atmosphere. To differentiate *S. aureus* and *S. epidermidis*, TSA boxes containing

deferoxamine mesilate have been manufactured to inhibit growth of *S. epidermidis* (species susceptible to deferoxamine whereas *S. aureus* is not) [19].

### *Internalization assays*

Internalization of *S. aureus* alone was achieved in exponential phase. Confluent Ca9-22 cells were washed twice and then incubated during one hour at 37 ° C, under 5% CO<sub>2</sub> with *S. aureus* suspension in DMEM (not supplemented) at MOI 10:1. After incubation, Ca9-22 cells were washed softly three times with PBS to remove non-adherent bacteria and treated 2h at 37°C with DMEM supplemented with 100 µg.ml<sup>-1</sup> gentamicin to kill adhered and extracellular bacteria. After incubation, Ca9-22 cells were washed softly three times with PBS and then lysed using 200 µl of a 0.05% Triton solution in sterile water. Remaining bacteria, considered to be internalized in cells, were counted by spreading on Trypticase Soja agar (TSA) plate and incubation at 37 ° C for 24h.

### *2.3. Statistical analysis*

Statistical analyses were performed using Graphpad Prism5® software. The Chi square and Fischer tests were undertaken to analyze the distribution of bacterial species within the microbiota of *S. aureus* carriers and non-carriers. The Fisher test was used when the expected frequencies were less than 5. The Student test was performed to compare the bacterial population density of carriers and non-carriers as well as the number of isolated bacterial species. Beforehand, normality was verified using the Kolmogorov-Smirnov normality test and a Fisher test was used to compare variances. For comparison of the bacterial population density, values were also normalized using logarithmic data. For adhesion and internalization

capacities of *S. aureus* strains, each experiment was carried out in triplicate. For each strain and *MOI*, adhesion and internalization capacities were compared between exponential and stationary phases with a student test. A Fisher test was used to compare variances. When variances were unequal, a Welch corrected test was done. For adhesion in community models, each experiment was carried out in triplicate. The adhesion of STAU II-9 strain in exponential phase at the *MOI* 10 was used as a reference and adhesion of *S. aureus* in community models for each ratio was compared to the reference using the Student test. A Fisher test was used to compare variances in order to verify equality of variances. When variances were unequal, a Welch corrected test was done. The effect of the three ratio tested was verify using a one way Anova test. The behavior of two strains of the same inhibitory bacterium (*S. epidermidis*, *C. accolens* or *C. ances*) on the adhesion of *S. aureus* was also verified by a one way Anova test.

### 3. Results

#### 3.1.Characteristics of the bacteria constituting the nasal microbiota

Each sample was positive for at least one species, with identification of 27 different species at all (Fig. 1). By mass spectrometry 96% of taxa were identified at the species level and in the remaining 4%, identification was obtained at the genus level for *Cutibacterium*. On average, 4 different species were found per individual at a bacterial count of  $3 \times 10^4$  CFU. Among all identified bacteria, three *phyla* have been found: Firmicutes, Actinobacteria and Proteobacteria (37%, 48% and 18% respectively).

Among Firmicutes, the predominant genus was *Staphylococcus* and it accounted for 26% of all species. Coagulase-negative Staphylococci (CoNS) were frequently isolated mainly *S. epidermidis* but also other CoNS such as *Staphylococcus capitis*, *Staphylococcus warneri*, *Staphylococcus haemolyticus*, *S. hominis*, *S. lugdunensis*.

Within Actinobacteria, *Cutibacterium* and *Corynebacterium* were the main genus encountered. Five species of *Corynebacterium spp* were recovered: *C. accolens*, *C. pseudodiphthericum*, *Corynebacterium striatum*, *Corynebacterium propinquum* and *Corynebacterium aurimucosum* i.e.18% of the detected species. Three species of *Cutibacterium spp* were identified: *C. acnes*, *Cutibacterium granulosum*, *Cutibacterium avidum*. They represented 11% of all species. Among Proteobacteria, Enterobacteriaceae were the most commonly encountered family (80% of Proteobacteria) including *Escherichia coli*, *Citrobacter koseri*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*. Seven anaerobic species were isolated and identified. The genus *Cutibacterium* was the most represented. Other strict anaerobic species were *Finegoldia magna*, *Anaerococcus octavius*, *Veillonella dispar* and *Dermacoccus nishinomiyaensis*. It was noteworthy that no *Streptococcus spp* was isolated

during this work. When results were expressed by individuals, the most represented species were *S. epidermidis* (86% of individuals), *C. acnes* (73%) and *C. accolens* (53%).

### 3.2. Differences between *S. aureus* carriers and non-carriers

*S. aureus* was isolated from seven individuals, *e.g.* 23% (Fig. 1). According to colony counts and species, there was not significant difference either quantitative or qualitative, between the carriers and non-carriers group ( $p= 0.31$  and  $p= 0.50$ ). Ten different species were isolated from carriers group including, Firmicutes (50%) and Actinobacteria (50%), but not Proteobacteria. In contrast, 23 species were found in the non-carriers group: Firmicutes (35%), Actinobacteria (44%) and Proteobacteria (21%) (Fig. 2A). Enterobacteriaceae accounted for 17% of bacterial species and constituted the majority of Proteobacteria (80%). There was not significant difference between the two groups ( $p= 0.32$ ) but a trend for decrease of Actinobacteria and Firmicutes in the non-carriers group. In addition, *S. aureus* carriage was negatively associated with two bacterial groups: CoNS and corynebacteria (respectively  $p= 0.04$  and  $p= 0.003$ ) (Fig. 2B).

### 3.3. Transmission electron microscopy

TEM was achieved in order to check out internalization time of bacteria by epithelial cells. Internalized bacteria were not present within Ca9-22 cells after 30, 60 and 90 minutes of contact, but internalized bacteria were observed only after 180 minutes (60 plus 120 minutes). At this time, the cells remained intact (Fig. 3). These results confirmed that internalization did not occur during time less or equal to 90 minutes, and we have then considered that our findings only took into account adhered bacteria.

### 3.4. Adhesion and internalization capacities of *S. aureus* strain

Two strains of *S. aureus* (strains II-9 and II-6) were tested for *in vitro* adhesion and internalization to Ca9-22 cells. Overall, they had the same virulence profile and belonged to the clonal complex 30 lineage. *S. aureus* II-6 differed from II-9 by the presence of *tstI* (toxic shock syndrome toxin 1) and *sea* (enterotoxin A). The main genes involved in adhesion including MSCRAMMs (Microbial surface components recognizing adhesive matrix molecules) were present except *fnbB* (fibronectin-binding protein B) and the profile was also similar (table S1). For both, adhesion was significantly higher in exponential growth phase than stationary growth phase (at *MOI* 10,  $p < 0.0001$ , *MOI* 50,  $p = 0.0009$ , *MOI* 100,  $p < 0.0001$  for strain II-9; and at *MOI* 10,  $p = 0.0044$ , *MOI* 100,  $p = 0.0104$  for strain II-6) (Fig. 4A and 4B). When the two strains were compared to each other, no significant difference was found for both exponential and stationary phases. We also found that internalization was significantly lower than adhesion ( $p < 0.0001$  for strain II-9 and  $p < 0.0001$  for strain II-6) (Fig. 4C). The internalization abilities did not differ significantly between the two strains. Consequently, we considered that their adhesion and internalization capacities were analogous.

### 3.5. Adhesion in community models

As *S. epidermidis*, *C. accolens* and *C. acnes* were the three most frequent species found in the nasal microbiota, we therefore tested the adhesion capacities of *S. aureus* II-9 in the presence of these species on the Ca9-22 cells. We observed that the adhesion capacities of *S. aureus* were decreased with all of them.

With *S. epidermidis*, *S. aureus* adhesion was lower compared to that observed in the single model (*S. aureus* alone):  $p= 0.001$  for the ratio 70/30 and  $p< 0.0001$  for the ratio 30/70 with the strain II-4;  $p= 0.002$  for the ratio 70/30 and  $p= 0.0005$  for the ratio 30/70 with the strain I-6) (Fig. 5A and 5B). The adhesion of *S. aureus* II-9 was similar with the 2 strains of *S. epidermidis*. Indeed, no significant difference was showed. Nevertheless, there was a slight difference since the change in ratios with the *S. epidermidis* II-4 strain did not modify the adhesion of *S. aureus*, whereas it was observed with the *S. epidermidis* I-6 strain ( $p= 0.0297$ ).

With *C. accolens*, for each ratio tested, adhesion was significantly different in the community model compared to the single-model (respectively  $p< 0.0001$  for the three ratios with *C. accolens* III-5 and  $p= 0.0023$ ,  $p= 0.0065$  and  $p= 0.0047$  with *C. accolens* III-7). In the same manner that observed with *S. epidermidis*, there was a variation inhibition of adhesion capacities from *C. accolens* strain to another strain (Fig. 5C and 5D). The inhibition of adhesion was strongly decreased with *C. accolens* III-5 ( $p= 0.002$ ). The use of different ratios did not modify *S. aureus* adhesion for the two *C. accolens* strains.

In the presence of *C. acnes* (Fig. 5E and 5F), *S. aureus* adhesion was reduced compared to the single model. There was a significant difference between the single model and the community model for each ratio tested *i.e.* 70/30, 50/50 and 30/70 respectively ( $p= 0.0007$ ,  $p<0.0001$  and  $p= 0.0002$  with *C. acnes* II-5 and  $p= 0.018$ ,  $p= 0.0002$  and  $p= 0.0002$  with *C. acnes* III-1). The behaviour of the two *C. acnes* strains on *S. aureus* adhesion was similar. The different ratios tested did not modify the adhesion.

#### 4. Discussion

According to the literature, the nasal microbiota is dominated by species of the genera *Cutibacterium*, *Corynebacterium* and *Staphylococcus* [7-10] which is confirmed by our results. Species of these three genera represent the core of this bacterial community (55% of the species). At a higher taxonomic level, the three phyla described as the majority - Actinobacteria, Firmicutes and Proteobacteria – which are also predominant in our study.

We could identify 27 different species. These results are lower in number than those previously reported with a culturomic approach [7, 20]. This difference is explained by the fact that we did not use enrichment broth prior to culturing on a solid medium. Nonetheless, we were able to quantify from the outset of the cultivable nasal microbiota. It is a fairly poor microbiota (about  $3 \times 10^4$  CFU) as compared to the intestinal microbiota [21].

Few anaerobic species have been isolated and identified. Several physiological and technical reasons can explain these findings: the nasal cavity is ventilated, the swabs have not been transported in a transport medium adapted to strict anaerobic bacteria, and finally we did not perform culture enrichment beforehand. According to some authors, the nasal microbiota contains many anaerobic species [14]. Using a different approach, it is possible to isolate and identify them appropriately [7]. This approach involves removing the nasal cavity in its entirety, using specific transport media for anaerobic bacteria and thioglycolate enrichment broths.

Similarly, no *Streptococcus spp* was found. Samples were taken from adult volunteers, and adults with a low prevalence of this bacterial genus are described predominantly for *Streptococcus pneumoniae* [22]. Moreover, species of *Streptococcus spp* colonize more the pharynx than the nose [23]. This might also explain the absence of this genus in our work.

371 In this study, seven individuals were positive for *S. aureus*, i.e. 23% of 30 individuals. These  
372 results are consistent with those previously described [24]. The ecological niche of this  
373 bacterium in humans is the nose. It is found mainly in the anterior part of the nasal cavity  
374 (nasal vestibulum) [25]. Most of the research of *S. aureus* at the nasal level is therefore done  
375 by simple swabbing of the anterior nasal passages [26]. Recently, two studies [7, 10] showed  
376 higher-than-average nasal carriage rates. These results can be explained by a new procedure  
377 for the analysis of the nasal microbiota: surgical approach with multiple anatomical sites  
378 investigated (anterior and posterior vestibule, meatus). This work resulted in redefining the  
379 main habitat of *S. aureus*: the entire nasal cavity.

380 We have not looked for differentiate carriage status, intermittent or permanent, since nasal  
381 samples were taken only once. It was therefore not possible to meet the criteria established for  
382 the different types of carriage [27], even though these were recently modified [28].

383 Interestingly, all Enterobacteriaceae were isolated in non-*S. aureus* carriers. Despite these  
384 variations, no significant difference between the two groups has been observed. The low  
385 power of our study (30 volunteers) may explain the absence of significant difference. Köck  
386 [31] showed similar results with a high prevalence of Enterobacteriaceae in the German  
387 population (30%). According to their results, nasal colonization by Enterobacteriaceae would  
388 prevent nasal carriage of *S. aureus*, but the mechanism is still unexplained.

389 In the same manner, *M. catarrhalis* belongs to Proteobacteria phylum and was found only in  
390 non-carriers. Proteobacteria are therefore be negatively correlated with the presence of *S.*  
391 *aureus*. It was previously reported that the non-carrier microbiota was enriched in  
392 Proteobacteria [10].

393 Our results suggest a competition between *S. aureus* and CoNS and corynebacteria. Indeed, a  
394 negative correlation is highlighted between these bacteria. Currently, not much is known  
395 about the dysbiosis in *S. aureus* nasal carriers. Some studies show a negative correlation

396 between *S. aureus* and *S. epidermidis* [9, 15, 16], while others do not find this negative  
397 association [7, 10]. A negative correlation was found between *S. aureus* and *Corynebacterium*  
398 *spp* [15, 32]. Another study reported that *C. pseudodiphthericum* is positively associated with  
399 *S. aureus* while *C. accolens* is negatively linked to it [14]. Conversely, others described a  
400 positive association between *C. accolens* and *S. aureus*, while *C. pseudodiphthericum* is  
401 negatively associated with *S. aureus* [10].

402 We then focused to the adhesion and internalization of *S. aureus* on epithelial cells. *S.*  
403 *aureus* isolates II-6 and II-9 were selected for *in vitro* adhesion tests because they were  
404 recently recovered from nose samples and we consider them suitable for studies of nasal  
405 colonization. Furthermore, these isolates belong to the MLST Clonal Complex 30 which is  
406 one CC found predominantly in longitudinal studies on nasal carriage. [33, 34]. Our results  
407 showed a statistically lower rate of internalization than adhesion. As described elsewhere with  
408 the same approach the percentage of internalized *S. aureus* after adhesion is very low [35].  
409 These findings were confirmed by TEM: we do not observed internalization at 60 and 90  
410 minutes. These results are consistent with that were described previously in the literature *i.e.*  
411 adhesion observed after a time of contact of 60 minutes [36, 37] or 90 minutes [35]. We can  
412 therefore consider that our findings only take into account adhered bacteria.

413 During nasal colonization, *S. aureus* is metabolically active with a high expression of  
414 adhesion factors including MSCRAMMs and the two component system *agr* is not effective  
415 [38] explaining our choice to work subsequently with *S. aureus* in exponential phase. *S.*  
416 *aureus* adhesion to Ca9-22 cells was greater in the exponential phase than the stationary phase  
417 for the two strains tested. These results can be explained by a preferential expression of  
418 adhesion factors in an exponential phase, particularly MSCRAMMs. This set of proteins is the  
419 major actor of *S. aureus* adhesion [39]. Their expression is mainly controlled by global  
420 regulators; the most characterized is the *agr* system [40]. This system is not active in

exponential phase of growth but is activated in stationary phase, repressing expression of MSCRAMMs [41]. In stationary phase, we observed a significant decrease in adhesion with a low percentage of bacteria adhered. It could be explain through the action of other factors like SasG, another surface protein of *S. aureus* which is not part of the MSCRAMMs group. SasG is expressed in stationary phase and promotes adhesion of *S. aureus* to nasal epithelial cells [42].

A limitation of this work is that Ca9-22 cell line is not derived from nasal epithelial cells but from buccal cells. Nonetheless, we have observed percentages of adhered bacteria similar to that reported with human keratinocyte cell line or human nasal carcinoma cell line [35, 37].

Our objective was to seek an inhibition of the implantation of *S. aureus* in the nasal area by the other key bacteria constituting the nasal microbiota. Our hypothesis assumed a decrease of adhesion in a community model. We confirmed that *S. epidermidis*, *C. acnes* and *C. accolens* significantly reduce *S. aureus* adhesion. There are competition/cooperation phenomena between *S. aureus* and these three species. The literature deals little with inhibition of *S. aureus* adhesion. *Corynebacterium spp* may compete at the same sites of *S. aureus* adhesion to epithelial cells [15]. *S. epidermidis* binds to the receptors of keratinocytes and thus reduces *S. aureus* adhesion through a competition phenomenon [43]. *S. epidermidis* is also able to secrete a serine protease, Esp [44], which degrades surface proteins of *S. aureus* such as adhesins [45]. These data may partially explain our data. Results of *S. aureus* adhesion in a community model with *C. acnes* are more surprising: co-operation phenomena are more often described by others, in particular, by promoting the virulence of *S. aureus* [46, 47]. In contrast, some strains of *Propionibacterium freundenreichii sp. shermanii* have the ability to inhibit adhesion of *S. aureus* to intestinal epithelial cells, particularly through competition events [48], perhaps by production of organic acid and H<sub>2</sub>O<sub>2</sub> [49].

We could link “culturomic” data and adhesion tests. Indeed, CoNS and corynebacteria are significantly less isolated from *S. aureus* carriers than from non-carriers. Inhibition of *S. aureus* adhesion in community model (with *S. epidermidis* and *C. accolens* respectively) accentuates the hypothesis of competitive inhibition between these bacteria. So, as these two bacteria inhibit the adhesion of *S. aureus*, it makes sense to find them less frequently in a nasal microbiota of *S. aureus* carriers. Nasal colonization of *S. aureus* would involve a stage of adhesion to epithelial cells within a microbiota with few or no *S. epidermidis* and *C. accolens*. However, it is not the number of bacteria but rather the bacterial species that would influence adhesion. Few differences in adhesion are observed by varying number of competitive bacteria. Mechanism(s) responsible for inhibition of *S. aureus* adhesion would not be a simple competition for binding to cellular receptors. It would then be interesting to carry out adhesion tests either according to using exclusion tests (incubation of an inhibitory bacterium before *S. aureus*) and displacement tests (*S. aureus* before the inhibitory bacterium). In addition, *S. aureus* interacts with an already implanted nasal microbiota and inhibitory bacteria can affect the adhesion of *S. aureus* through secreted molecules which are produced and secreted in stationary phase [17, 46]. As we mixed *S. aureus* in exponential growth with potentially inhibiting bacteria at stationary phase, by recovering the culture supernatant from inhibitory bacteria, it might be possible to identify potential molecules that play a role in inhibiting adhesion.

## **Authors statements**

### *Conflicts of interest*

The authors declare that there are no conflicts of interest.

### *Funding information*

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### *Ethical approval*

Ethical approval for this study has been obtained from the ethics committee of the Centre Hospitalier Universitaire (CHU) of Rennes (reference n° 19.146).

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## Figures

Fig. 1. Prevalence of the different species isolated in the 30 volunteers.

Fig. 2. Proportion (%) of the different phyla (A) and distribution of bacterial species/genera/families (B) in *S. aureus* carriers and non-carriers. Significant differences are represented by one ( $p < 0.05$ ) or two ( $p < 0.01$ ) asterisks (\*).

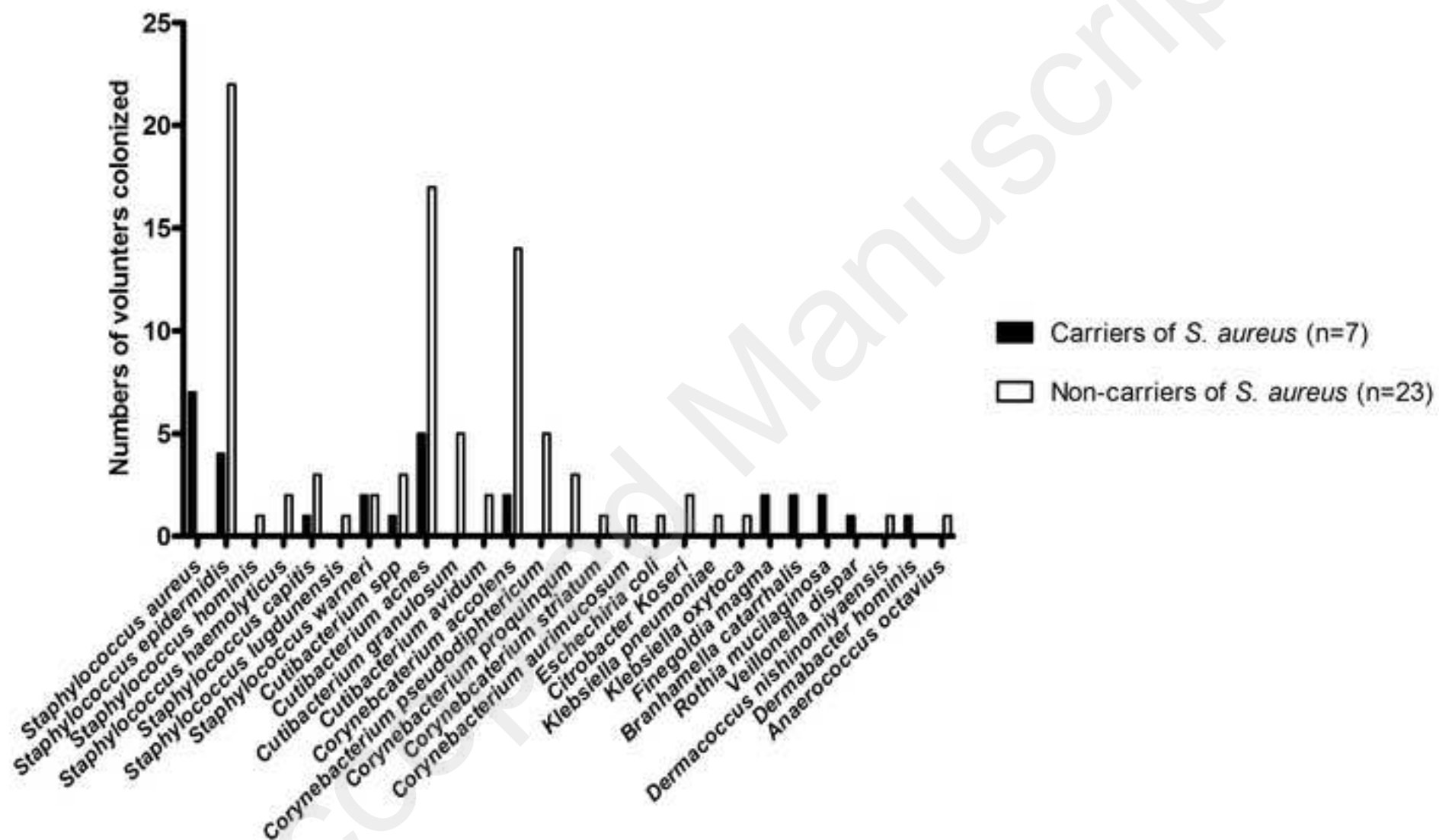
Fig. 3. Transmission Electron Microscopy of *S. aureus* adhesion and internalization to Ca9-22 cells. (A) *S. aureus* alone. (B) Ca9-22 cells without *S. aureus*. (C) *S. aureus* adhesion at the MOI 50:1 after a contact time of 60 minutes. (D) *S. aureus* internalization at the MOI 50:1 after 180 minutes.

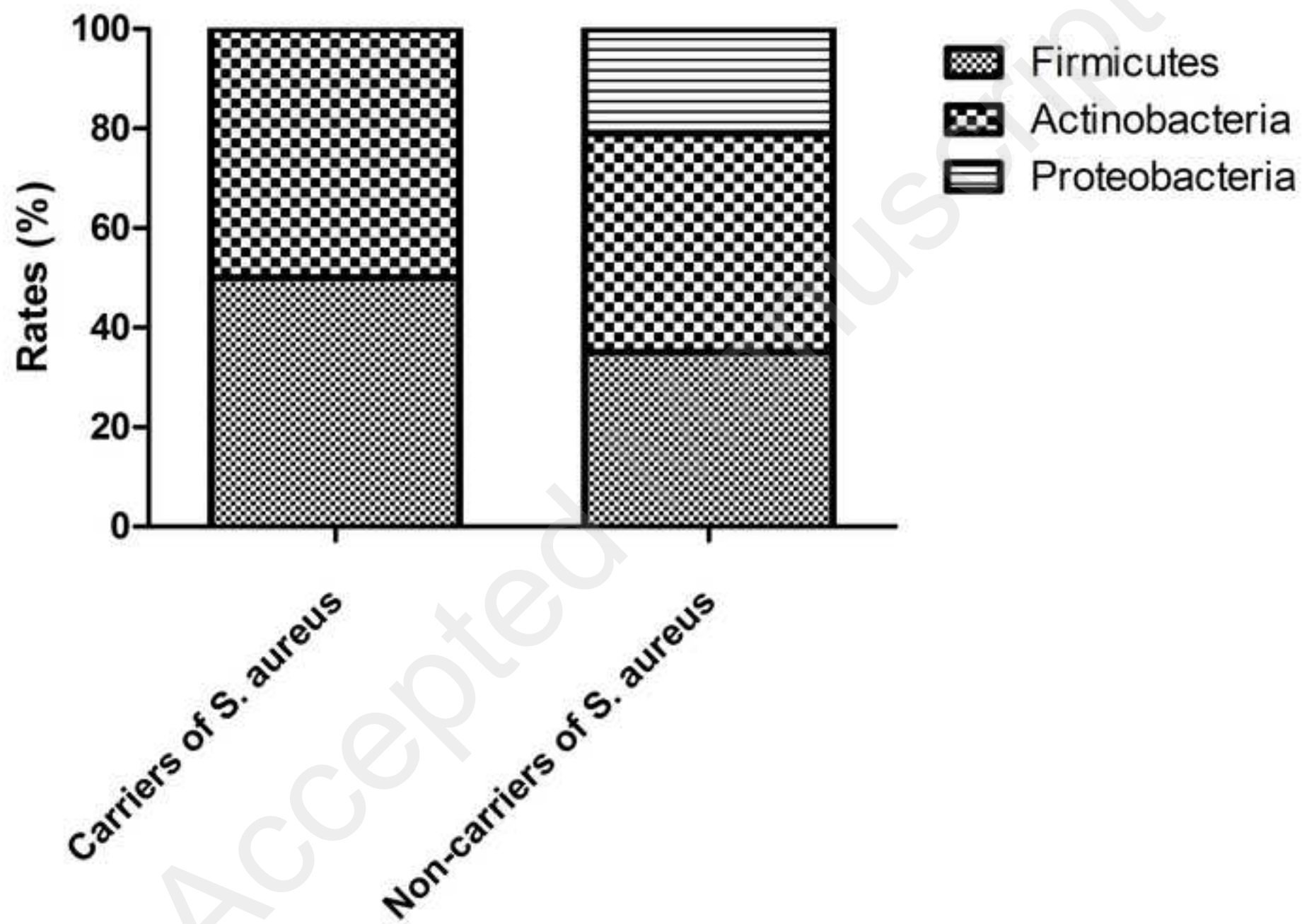
Fig. 4. Adhesion of *S. aureus* II-9 and II-6 and internalization on Ca9-22 cells. (A) Adhesion of *S. aureus* II-9. (B) Adhesion of *S. aureus* II-6. Results are expressed as a percentage. Adhesion tests were performed both in exponential and stationary phases and for each condition 3 MOI were tested (10:1, 50:1 and 100:1). (C) Comparison of adhesion and internalization. Results are expressed as a percentage. Adhesion and Internalization tests were performed in exponential phase and one MOI was tested (10:1). Each experiment was carried out in triplicate. Significant differences are represented by one ( $p < 0.05$ ), two ( $p < 0.01$ ) or three ( $p < 0.001$ ) asterisks (\*). STAU: *S. aureus*.

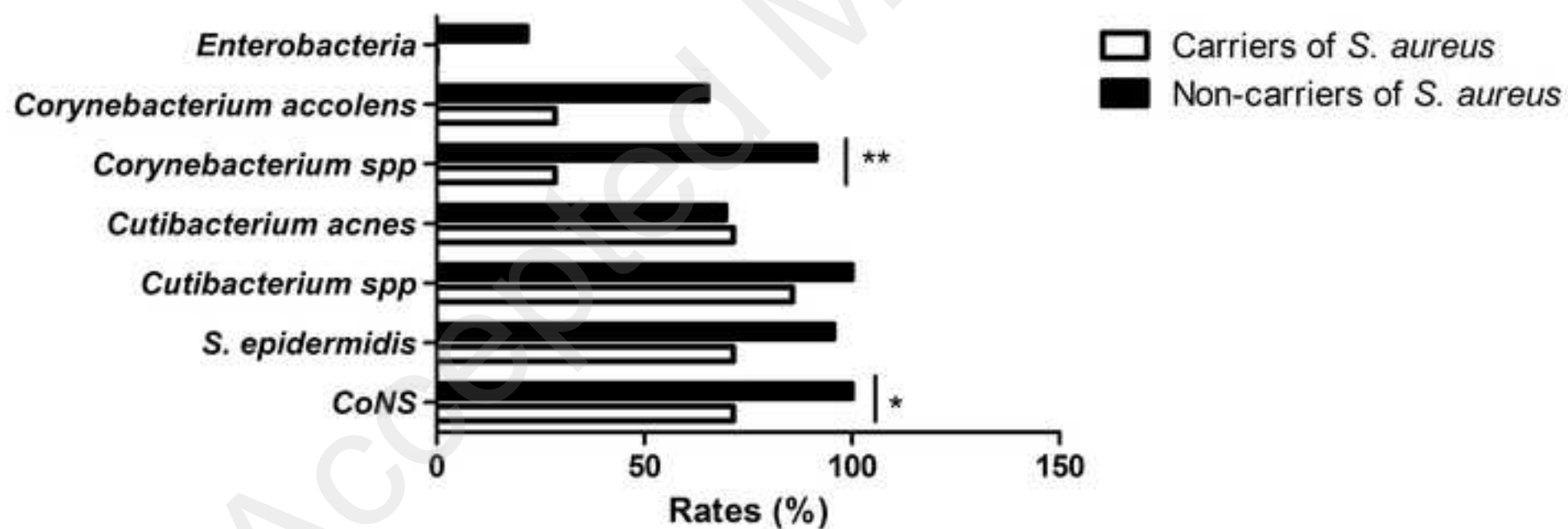
Fig. 5. Adhesion of *S. aureus* II-9 on Ca9-22 cells in community model respectively with *S. epidermidis* (A, B), *C. accolens* (C, D) and *C. acnes* (E, F). *S. aureus* was in exponential phase at the constant MOI of 10:1 and it serves as a reference (adhesion of *S. aureus* II-9 alone). Other species were in stationary phase. Several ratios were used: 70/30 (70% *S. aureus*

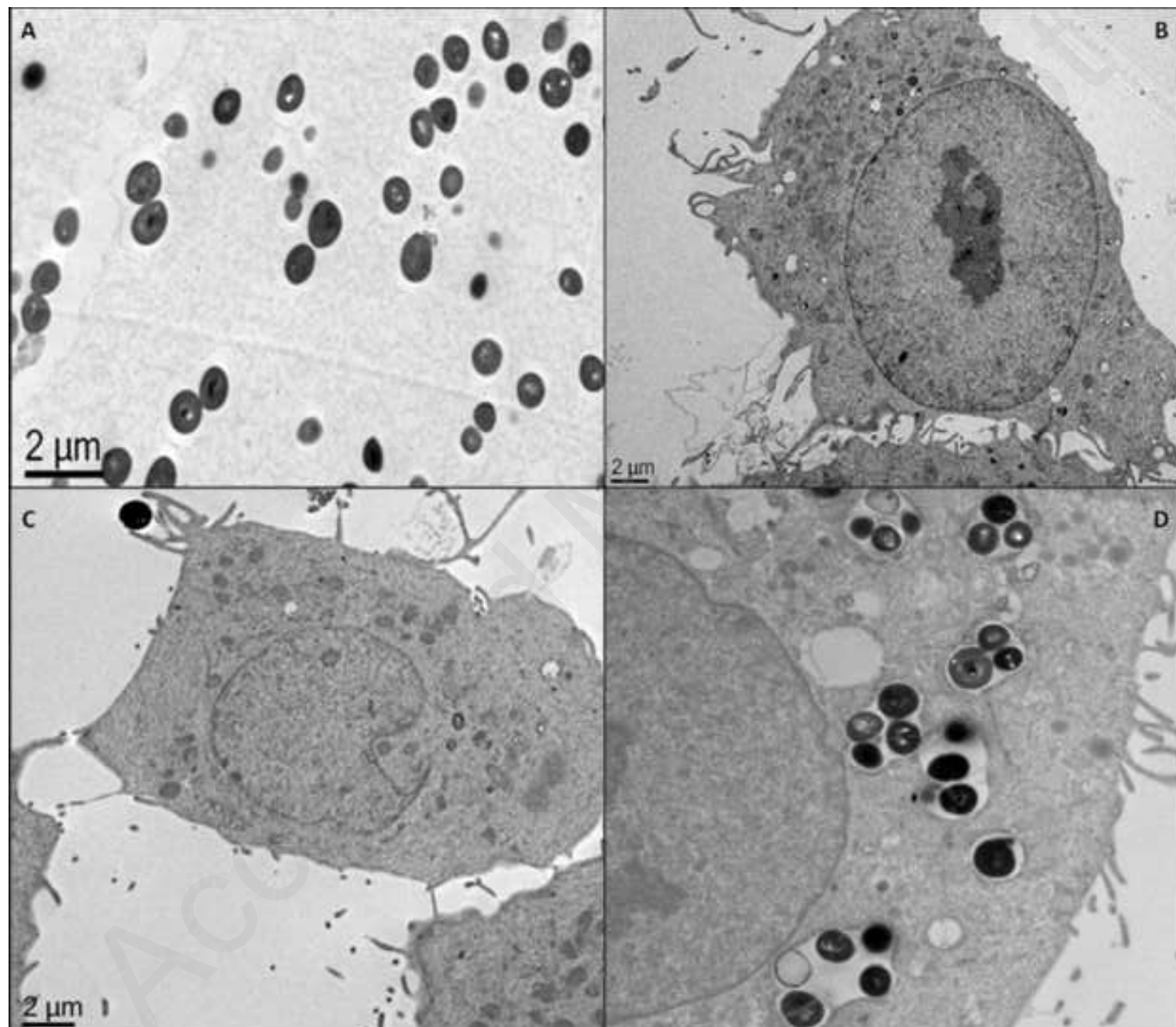
672 and 30% of another species), 50/50 and finally 30/70. Results are expressed as a percentage.  
673 Each experiment was carried out in triplicate. Significant differences are represented by one  
674 ( $p < 0.05$ ), or two ( $p < 0.01$ ) or three ( $p < 0.001$ ) asterisks (\*). STAU: *S. aureus*, STEP: *S.*  
675 *epidermidis*, COAC: *C. accolens*, CUAC: *C. acnes*.

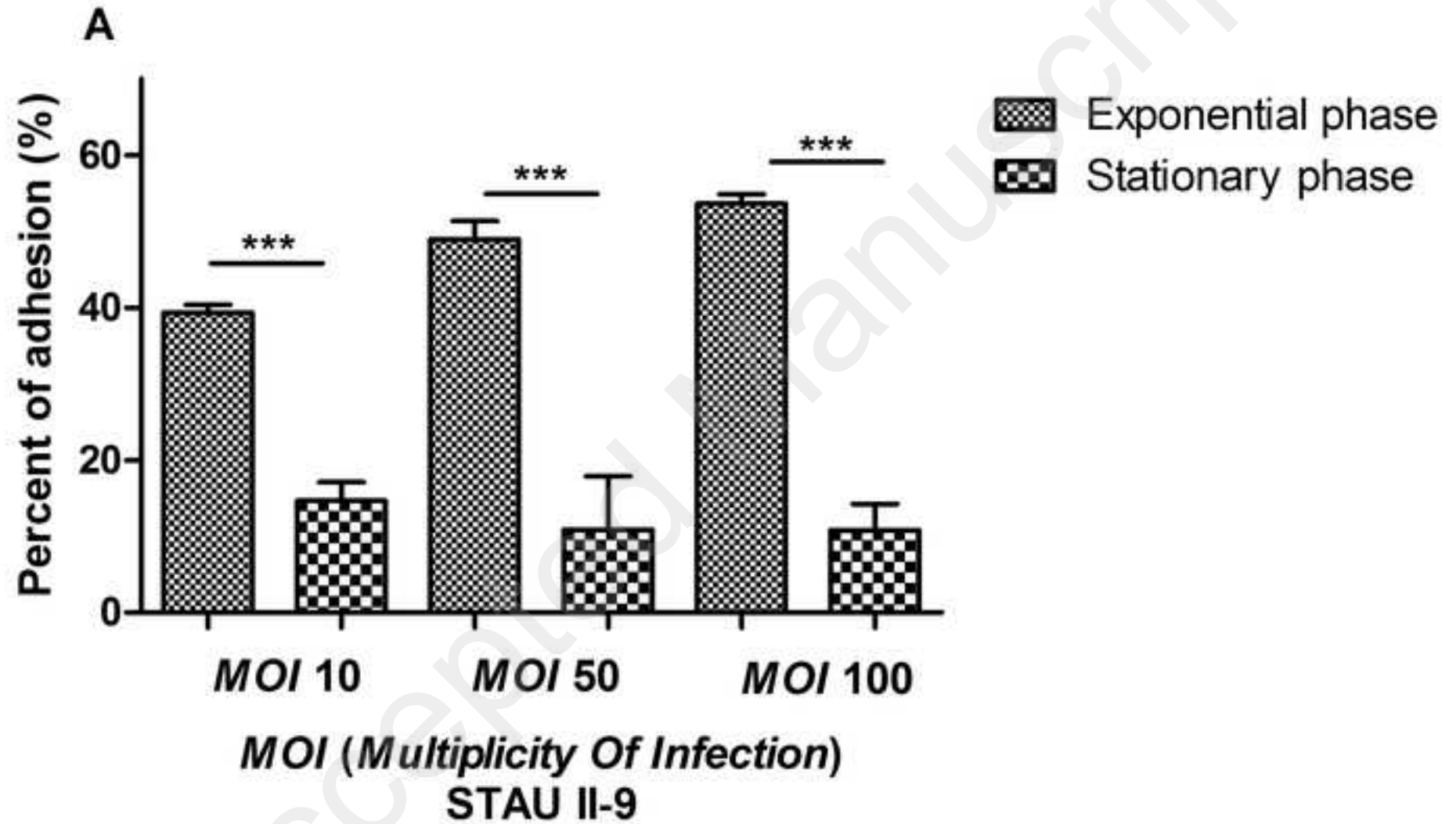
676

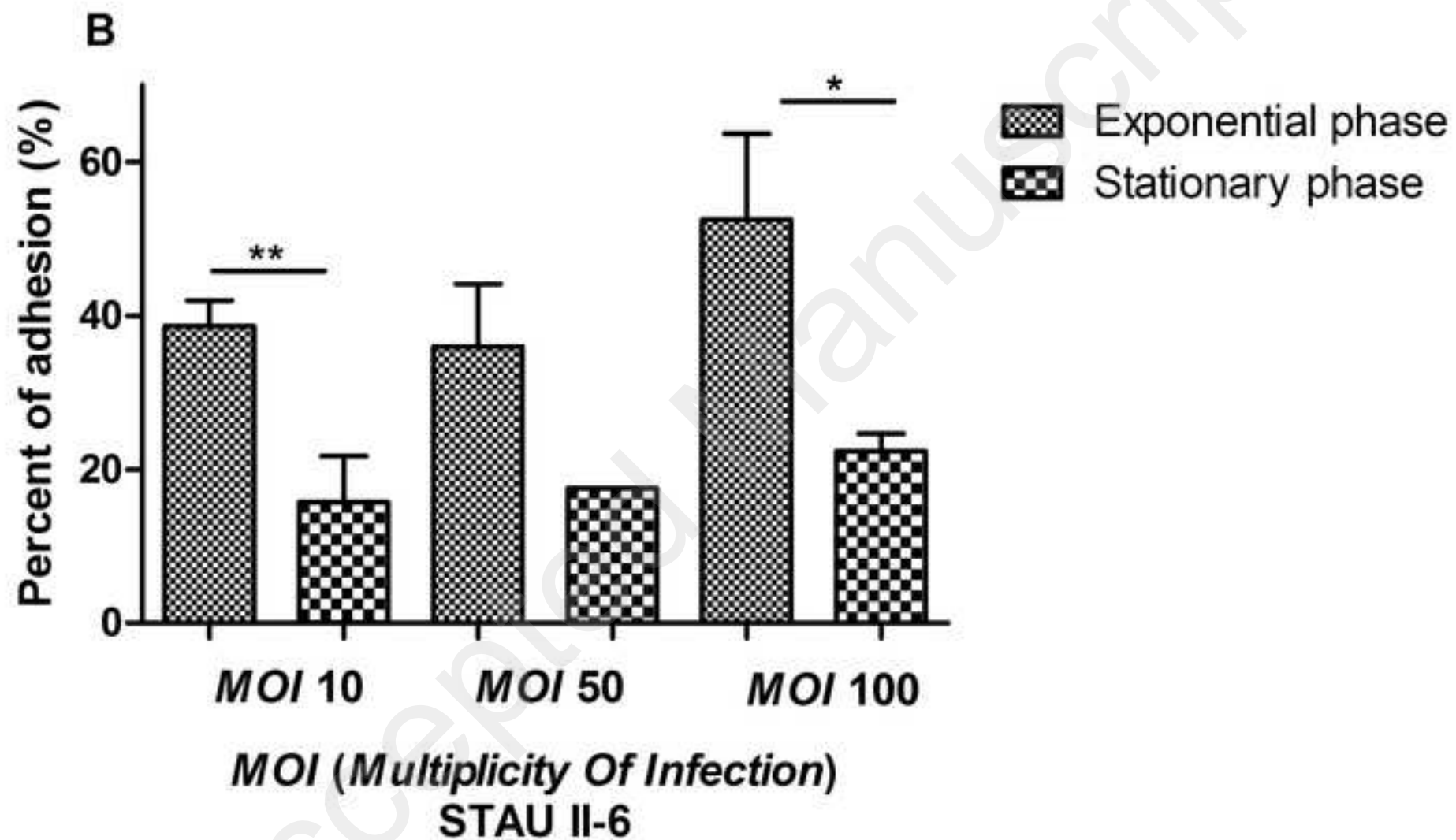


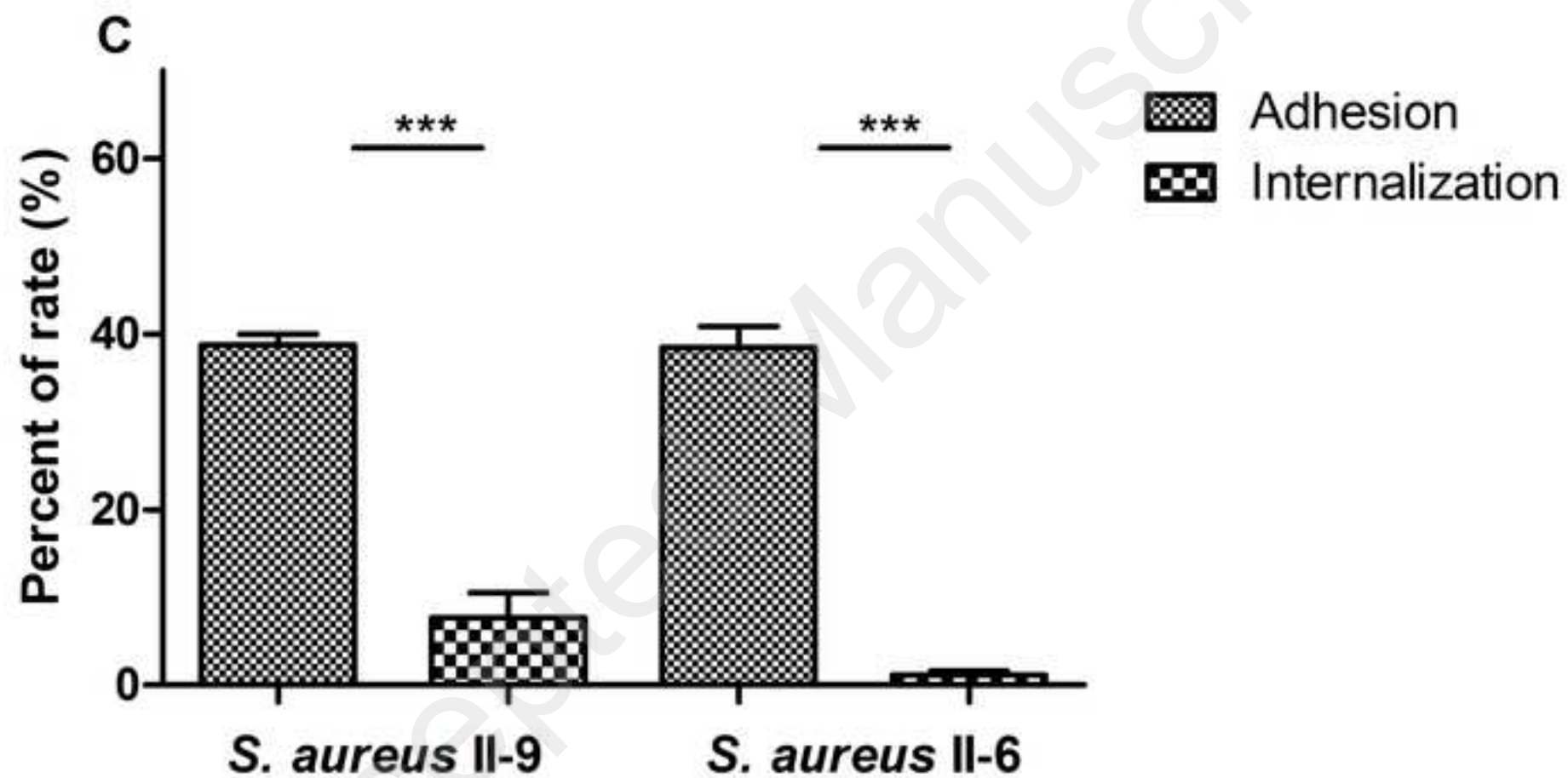


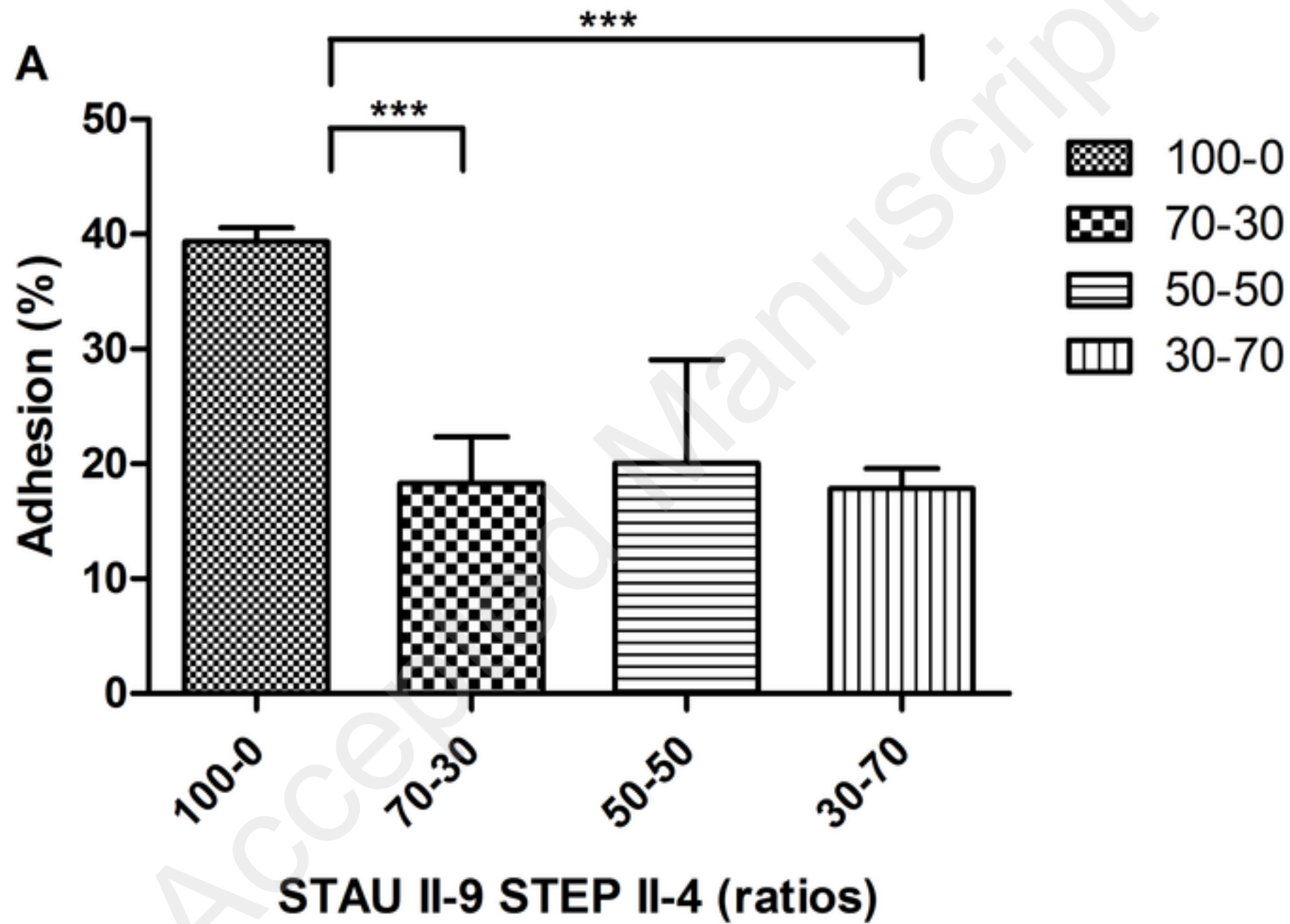


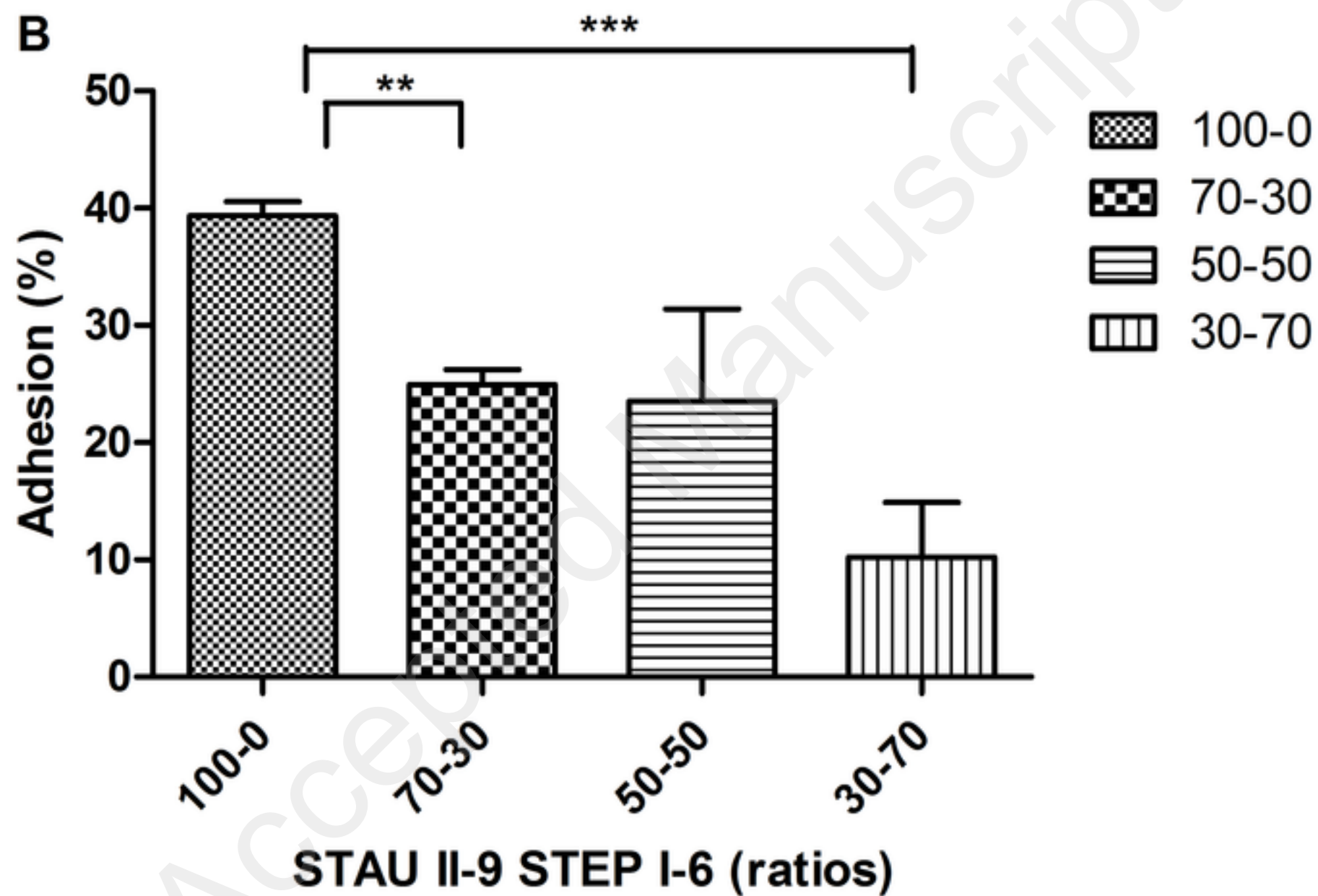


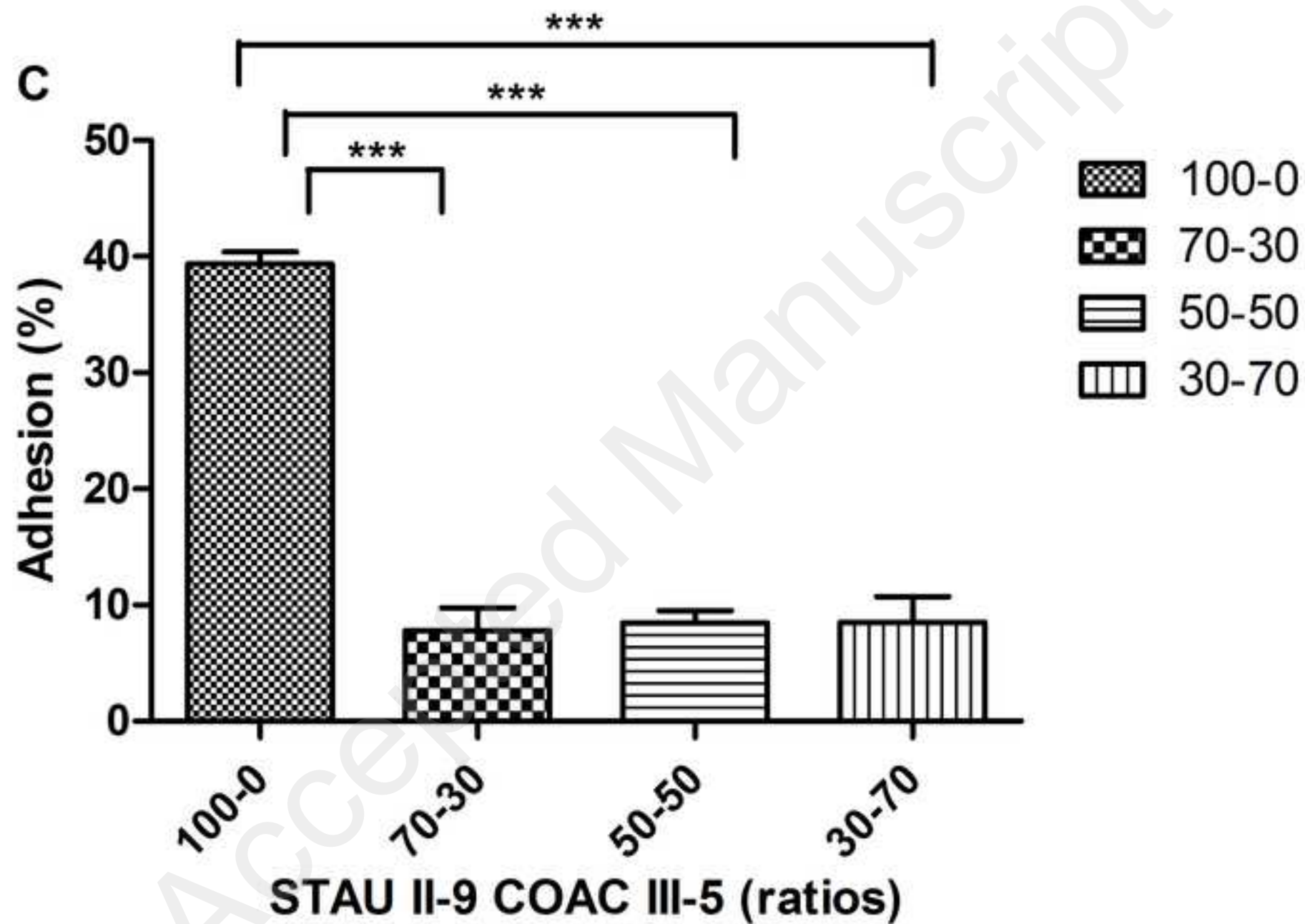


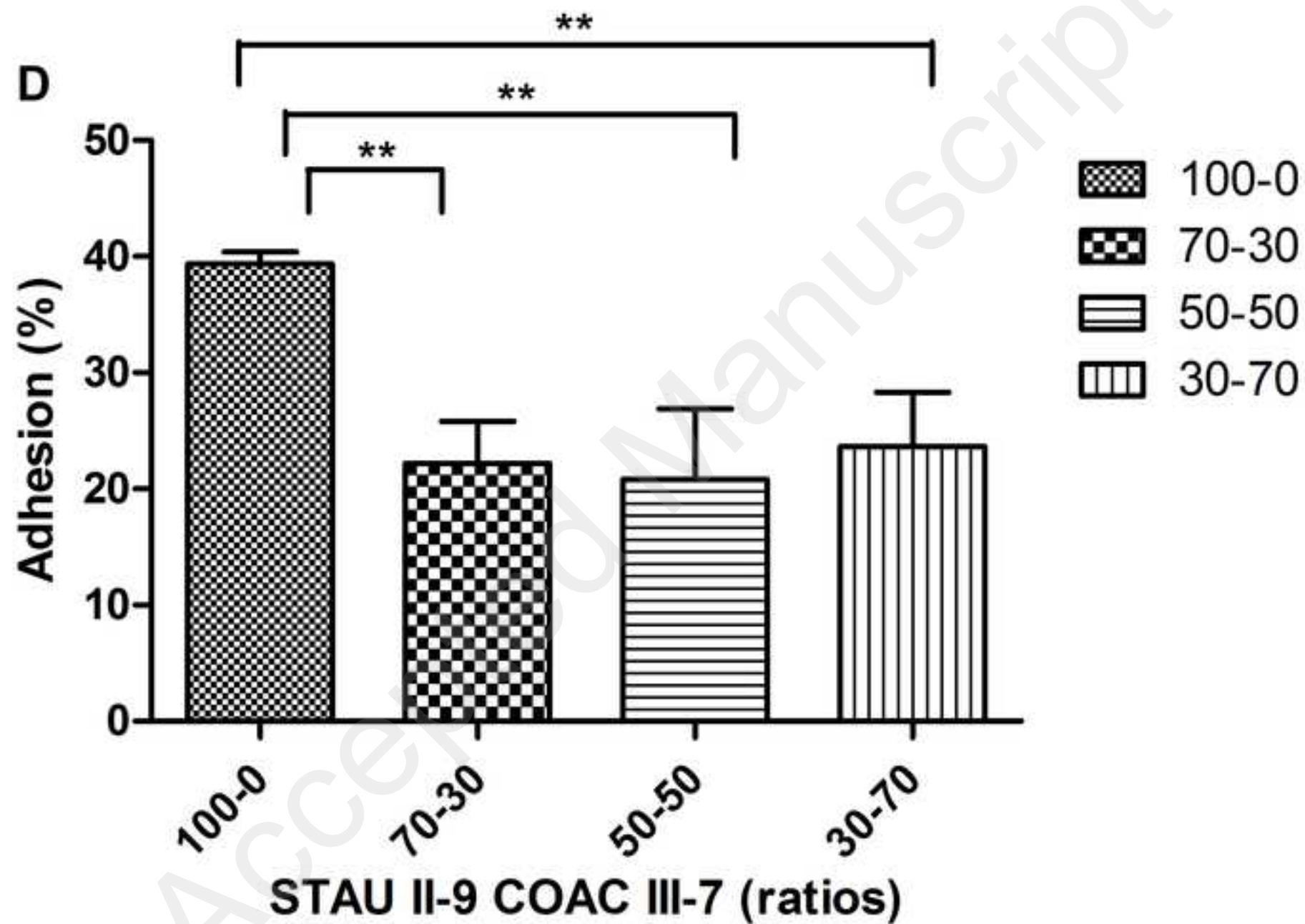


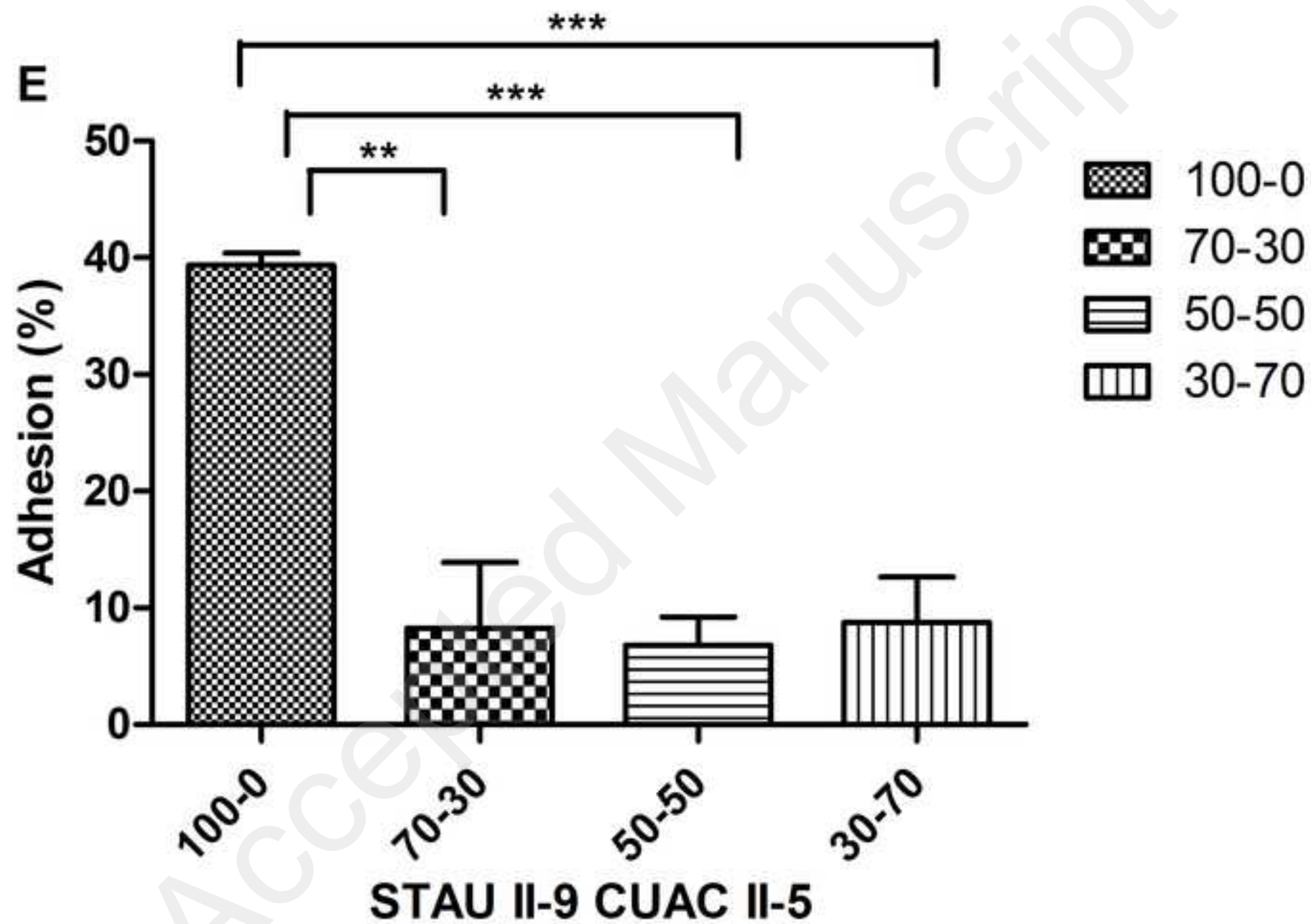


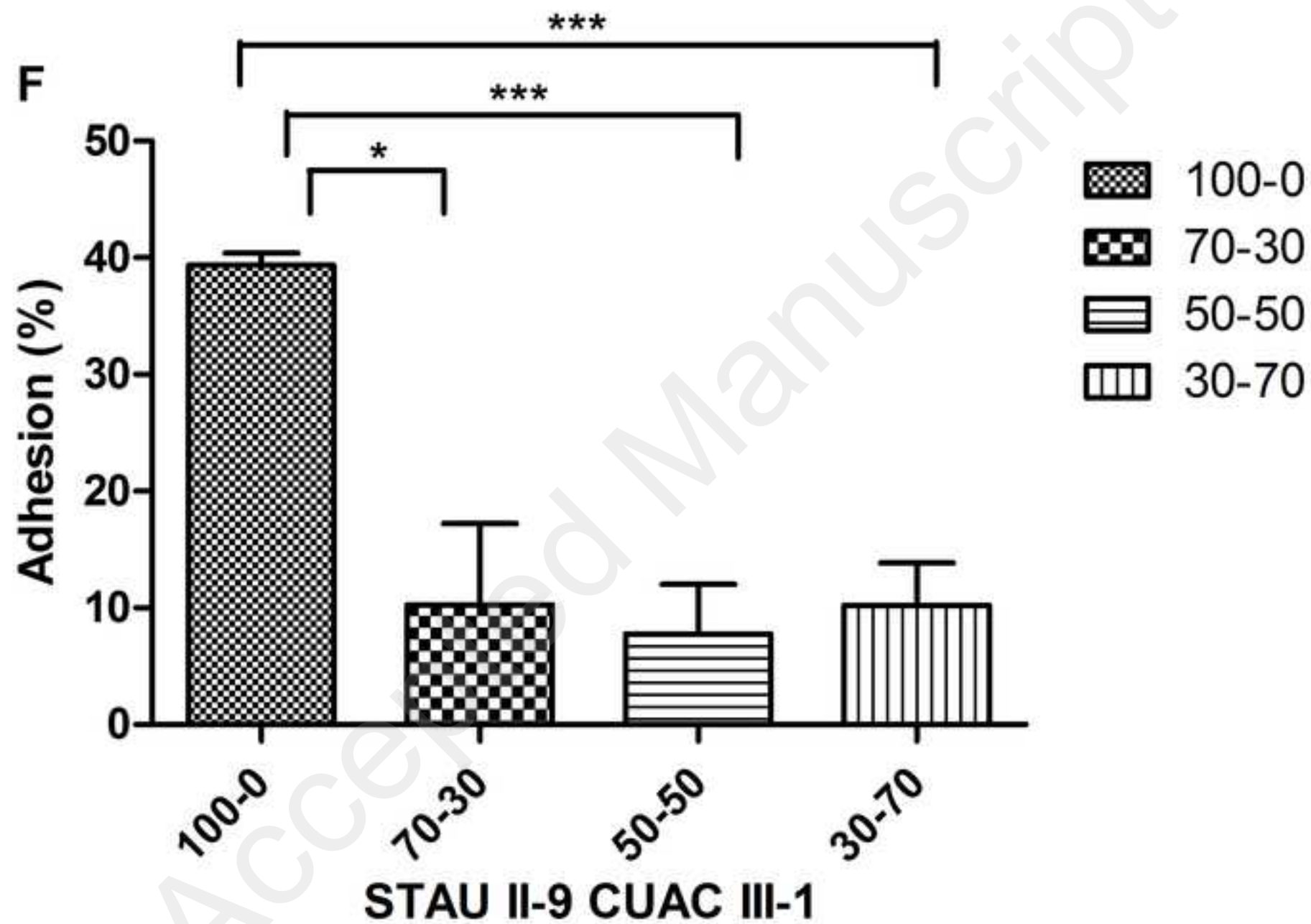












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**Supplementary Material - Excel file**  
**Table S1.xlsx**