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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.

49 **1. Introduction**

50

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

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

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

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99 **2. Materials and methods**

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101 *2.1. Microbiological methods*

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

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

109 The samples were suspended in 1 ml of sterile water and two dilutions were made (10^{-1} and
110 10^{-2}). Ten microliters of each dilution were seeded on two Colombia agars supplemented with
111 5% sheep blood and a blood agar cooked with 10% sheep blood (Oxoid, France). The blood
112 agar plates were incubated at 37 ° C under aerobic and anaerobic using Anaerogen bags
113 (Oxoid, France) conditions, respectively. The chocolate agar plates were incubated at 37 ° C,
114 under 5% CO₂. All agar plates were observed after two and five or seven days depending on
115 their culture conditions. Some colonies were picked out plates for further characterization,
116 and then were stored on beads in cryopreservative solution at -80 ° C (Cryobank®, Lustiner,
117 France).

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

124 software as recommended by the manufacturer. Each identification was done twice. For the
125 bacterial species, identification was considered correct if the score was equal to or greater
126 than 2. Otherwise, identification at the genus level was retained if the score was greater than
127 1.8.

128

129 *2.2. In vitro adhesion model*

130

131 *Bacterial strains and culture conditions*

132

133 Two strains derived from carriers were used to study the adhesion and the internalization of *S.*
134 *aureus* alone on epithelial cells (*S. aureus* II-6 and II-9). Genes coding for virulence factors
135 were determined using the *S. aureus* genotyping kit 2.0 Alere®. For adhesion with the other
136 bacteria of the nasal microbiota (hereinafter referred to as "Community model adhesion"), one
137 *S. aureus* strain (strain II-9) and two strains of the following species: *S. epidermidis*, *C. acnes*
138 and *C. accolens* were selected. The latter six strains came from non-carriers of *S. aureus*. The
139 isolates were grown in Luria-Bertani (LB) medium at 37 ° C with shaking (150 rpm) for *S.*
140 *aureus* and *S. epidermidis*, or in Brain Heart Infusion (BHI) medium at 37 ° C with shaking
141 (150 rpm) under anaerobic conditions for *C. acnes* and *C. accolens*.

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

146

147 *Cell cultures*

148

149 The Ca9-22 cell line (reference JCRB0625, Japanese Cancer Research Resource Bank, Japan)
150 from human gingival carcinoma was used for the adhesion and internalization assays. Cells
151 were cultured in DMEM supplemented with 10% inactivated fetal calf serum (FCS), 100 IU /
152 ml penicillin, 10 mg / ml streptomycin and 5% glutamine (D. Dutscher). Cultures were carried
153 out in cell culture flasks (T75 Falcon®, Corning Incorporated), incubated at 37 ° C, under 5%
154 CO₂ until cells confluence, treated with 0.05% trypsin (D. Dutscher) and then re-suspended in
155 DMEM. For adhesion assay, 5×10^5 cells were placed per well in a 12-well plate (Cellstar®,
156 greiner bio-one) and incubated overnight at 37 ° C, under 5% CO₂.

157

158 *Transmission electron microscopy*

159

160 In order to determine optimal time to avoid internalization, we performed transmission
161 electron microscopy (TEM). Three conditions were retained: pellets of bacteria, Ca9-22 cells
162 alone, and a mixture of Ca9-22 / *S. aureus*. Three contact times were tested: 30, 60 and 90
163 minutes. Succinctly, Ca9-22 cells were incubated at 37°C under 5% CO₂ for 24 hours in wells
164 (Nunclon tm ^Δ Surface, ThermoFisher Scientific) at a rate of 5×10^5 cells / well. Confluent
165 cells were washed twice with PBS prior to contact with *S. aureus* in exponential phase at the
166 MOI 50. After incubation, the samples were prepared as described elsewhere [18]. Briefly,
167 infected and control Ca9-22 cells were washed with 0.15 M cacodylate buffer, then fixed in
168 2.5% glutaraldehyde in cacodylate buffer and stained with 1% osmium in cacodylate buffer.
169 Following dehydration in an alcohol gradient, cells were embedded in resin (mix of Epon and
170 DMP30). Finally, ultrathin sections were observed by TEM. The process was similar for
171 bacteria used alone but the dehydration was realized in an acetone gradient. TEM was also
172 performed with another protocol. After a contact time of 60 minutes, Ca9-22 cells with *S.*
173 *aureus* at the MOI 50 were washed twice with PBS and treated 2h at 37°C with DMEM

174 supplemented with 100 µg.ml⁻¹ gentamicin to kill extracellular bacteria. Samples were
175 prepared as described before.

176

177 *Adhesion assays*

178

179 Adhesion of *S. aureus* alone was achieved in both exponential and stationary phases. Three
180 different Multiplicity of Infection (MOI, e.g. ratio of *S. aureus* cells to epithelial cells) were
181 tested: 10: 1, 50: 1, 100: 1. Confluent Ca9-22 cells were washed twice and then incubated
182 during one hour at 37 ° C, under 5% CO₂ with *S. aureus* suspension in DMEM (not
183 supplemented). After incubation, Ca9-22 cells were washed softly three times with PBS to
184 remove non-adherent bacteria, and then lysed using 200 µl of a 0.05% Triton solution in
185 sterile water. Remaining bacteria, considered to be adherent but not internalized in cells, were
186 counted by spreading on Trypticase Soja agar (TSA) plate and incubation at 37 ° C for 24h.
187 For each MOI tested, inocula were controlled by spreading on TSA and incubated at 37°C
188 during 24h and counting. The ratios were calculated as follows: adhered bacteria / bacteria in
189 contact with cells

190 For the community model, one *S. aureus* isolate in exponential growth was mixed with one
191 isolate of another species (*S. epidermidis*, *C. acnes* or *C. accolens*) in stationary phase. The *S.*
192 *aureus* strain was used at the MOI 10: 1 constantly, but the other bacteria were tested at
193 70:30, 50:50 and 30:70 ratios with *S. aureus* (e.g. suspension corresponding to a 70:30 ratio
194 contained 70% *S. aureus* and 30% of other bacteria).

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

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

200

201 *Internalization assays*

202

203 Internalization of *S. aureus* alone was achieved in exponential phase. Confluent Ca9-22 cells
204 were washed twice and then incubated during one hour at 37 ° C, under 5% CO₂ with *S.*
205 *aureus* suspension in DMEM (not supplemented) at MOI 10:1. After incubation, Ca9-22 cells
206 were washed softly three times with PBS to remove non-adherent bacteria and treated 2h at
207 37°C with DMEM supplemented with 100 µg.ml⁻¹ gentamicin to kill adhered and
208 extracellular bacteria. After incubation, Ca9-22 cells were washed softly three times with PBS
209 and then lysed using 200 µl of a 0.05% Triton solution in sterile water. Remaining bacteria,
210 considered to be internalized in cells, were counted by spreading on Trypticase Soja agar
211 (TSA) plate and incubation at 37 ° C for 24h.

212

213 *2.3. Statistical analysis*

214

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

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

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248 3. Results

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250 3.1. Characteristics of the bacteria constituting the nasal microbiota

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

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

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

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

275

276 *3.2. Differences between S. aureus carriers and non-carriers*

277

278 *S. aureus* was isolated from seven individuals, e.g. 23% (Fig. 1). According to colony counts
279 and species, there was not significant difference either quantitative or qualitative, between the
280 carriers and non-carriers group ($p= 0.31$ and $p= 0.50$). Ten different species were isolated
281 from carriers group including, Firmicutes (50%) and Actinobacteria (50%), but not
282 Proteobacteria. In contrast, 23 species were found in the non-carriers group: Firmicutes
283 (35%), Actinobacteria (44%) and Proteobacteria (21%) (Fig. 2A). Enterobacteriaceae
284 accounted for 17% of bacterial species and constituted the majority of Proteobacteria (80%).
285 There was not significant difference between the two groups ($p= 0.32$) but a trend for
286 decrease of Actinobacteria and Firmicutes in the non-carriers group. In addition, *S. aureus*
287 carriage was negatively associated with two bacterial groups:
288 CoNS and corynebacteria (respectively $p= 0.04$ and $p= 0.003$) (Fig. 2B).

289

290 *3.3. Transmission electron microscopy*

291

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

298 *3.4. Adhesion and internalization capacities of S. aureus strain*

299

300 Two strains of *S. aureus* (strains II-9 and II-6) were tested for *in vitro* adhesion and
301 internalization to Ca9-22 cells. Overall, they had the same virulence profile and belonged to
302 the clonal complex 30 lineage. *S. aureus* II-6 differed from II-9 by the presence of *tstI* (toxic
303 shock syndrome toxin 1) and *sea* (enterotoxin A). The main genes involved in adhesion
304 including MSCRAMMs (Microbial surface components recognizing adhesive matrix
305 molecules) were present except *fnbB* (fibronectin-binding protein B) and the profile was also
306 similar (table S1). For both, adhesion was significantly higher in exponential growth phase
307 than stationary growth phase (at *MOI* 10, $p < 0.0001$, *MOI* 50, $p = 0.0009$, *MOI* 100, $p < 0.0001$
308 for strain II-9; and at *MOI* 10, $p = 0.0044$, *MOI* 100, $p = 0.0104$ for strain II-6) (Fig. 4A and
309 4B). When the two strains were compared to each other, no significant difference was found
310 for both exponential and stationary phases.

311 We also found that internalization was significantly lower than adhesion ($p < 0.0001$ for strain
312 II-9 and $p < 0.0001$ for strain II-6) (Fig. 4C). The internalization abilities did not differ
313 significantly between the two strains. Consequently, we considered that their adhesion and
314 internalization capacities were analogous.

315

316 *3.5. Adhesion in community models*

317

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

322 With *S. epidermidis*, *S. aureus* adhesion was lower compared to that observed in the single
323 model (*S. aureus* alone): $p= 0.001$ for the ratio 70/30 and $p< 0.0001$ for the ratio 30/70 with
324 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-
325 6) (Fig. 5A and 5B). The adhesion of *S. aureus* II-9 was similar with the 2 strains of *S.*
326 *epidermidis*. Indeed, no significant difference was showed. Nevertheless, there was a slight
327 difference since the change in ratios with the *S. epidermidis* II-4 strain did not modify the
328 adhesion of *S. aureus*, whereas it was observed with the *S. epidermidis* I-6 strain ($p= 0.0297$).
329 With *C. accolens*, for each ratio tested, adhesion was significantly different in the community
330 model compared to the single-model (respectively $p< 0.0001$ for the three ratios with *C.*
331 *accolens* III-5 and $p= 0.0023$, $p= 0.0065$ and $p= 0.0047$ with *C. accolens* III-7). In the same
332 manner that observed with *S. epidermidis*, there was a variation inhibition of adhesion
333 capacities from *C. accolens* strain to another strain (Fig. 5C and 5D). The inhibition of
334 adhesion was strongly decreased with *C. accolens* III-5 ($p= 0.002$). The use of different ratios
335 did not modify *S. aureus* adhesion for the two *C. accolens* strains.
336 In the presence of *C. acnes* (Fig. 5E and 5F), *S. aureus* adhesion was reduced compared to the
337 single model. There was a significant difference between the single model and the community
338 model for each ratio tested *i.e.* 70/30, 50/50 and 30/70 respectively ($p= 0.0007$, $p<0.0001$ and
339 $p= 0.0002$ with *C. acnes* II-5 and $p= 0.018$, $p= 0.0002$ and $p= 0.0002$ with *C. acnes* III-1).
340 The behaviour of the two *C. acnes* strains on *S. aureus* adhesion was similar. The different
341 ratios tested did not modify the adhesion.

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347 4. Discussion

348

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

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

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

367 Similarly, no *Streptococcus spp* was found. Samples were taken from adult volunteers, and
368 adults with a low prevalence of this bacterial genus are described predominantly for
369 *Streptococcus pneumoniae* [22]. Moreover, species of *Streptococcus spp* colonize more the
370 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

421 exponential phase of growth but is activated in stationary phase, repressing expression of
422 MSCRAMMs [41]. In stationary phase, we observed a significant decrease in adhesion with a
423 low percentage of bacteria adhered. It could be explain through the action of other factors like
424 SasG, another surface protein of *S. aureus* which is not part of the MSCRAMMs group. SasG
425 is expressed in stationary phase and promotes adhesion of *S. aureus* to nasal epithelial cells
426 [42].

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

430 Our objective was to seek an inhibition of the implantation of *S. aureus* in the nasal area by
431 the other key bacteria constituting the nasal microbiota. Our hypothesis assumed a decrease of
432 adhesion in a community model. We confirmed that *S. epidermidis*, *C. acnes* and *C. accolens*
433 significantly reduce *S. aureus* adhesion. There are competition/cooperation phenomena
434 between *S. aureus* and these three species. The literature deals little with inhibition of *S.*
435 *aureus* adhesion. *Corynebacterium spp* may compete at the same sites of *S. aureus* adhesion
436 to epithelial cells [15]. *S. epidermidis* binds to the receptors of keratinocytes and thus reduces
437 *S. aureus* adhesion through a competition phenomenon [43]. *S. epidermidis* is also able to
438 secrete a serine protease, Esp [44], which degrades surface proteins of *S. aureus* such as
439 adhesins [45]. These data may partially explain our data. Results of *S. aureus* adhesion in a
440 community model with *C. acnes* are more surprising: co-operation phenomena are more often
441 described by others, in particular, by promoting the virulence of *S. aureus* [46, 47]. In
442 contrast, some strains of *Propionibacterium freundenreichii sp. shermanii* have the ability to
443 inhibit adhesion of *S. aureus* to intestinal epithelial cells, particularly through competition
444 events [48], perhaps by production of organic acid and H₂O₂ [49].

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

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470 **Authors statements**

471

472 *Conflicts of interest*

473

474 The authors declare that there are no conflicts of interest.

475

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480 *Ethical approval*

481

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484

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486

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646

647 **Figures**

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

649

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

653

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

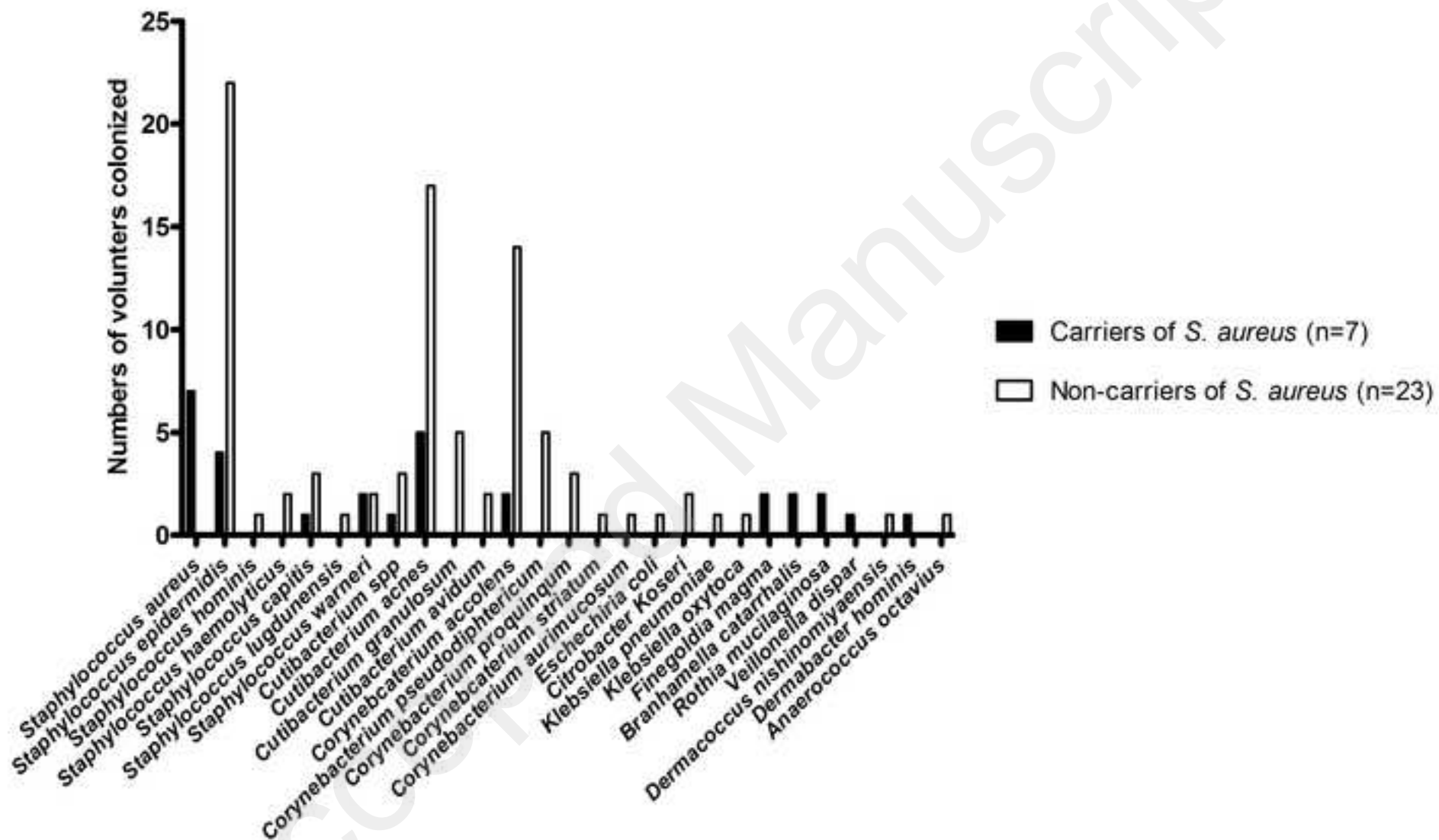
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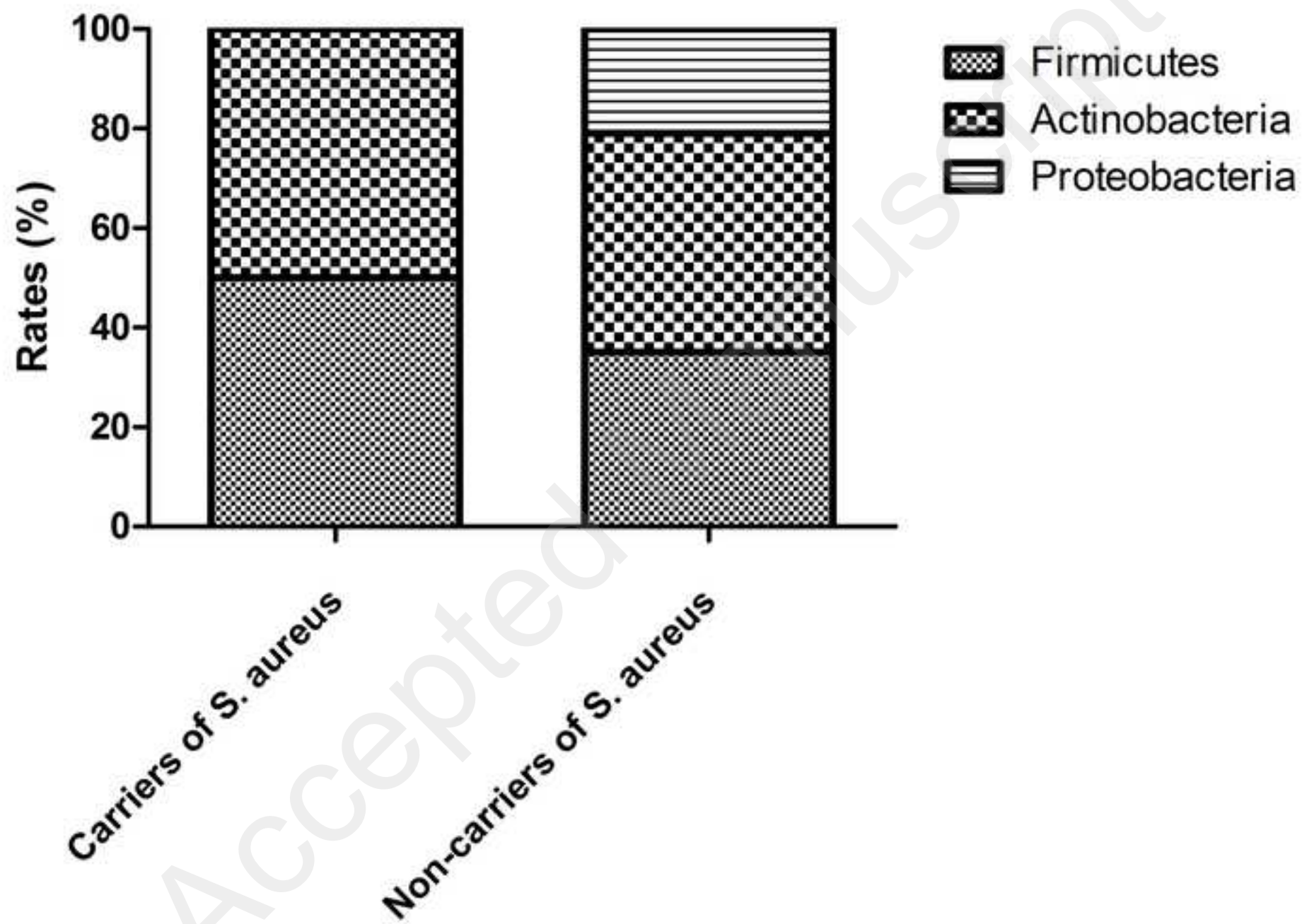
659 Fig. 4. Adhesion of *S. aureus* II-9 and II-6 and internalization on Ca9-22 cells. (A) Adhesion
660 of *S. aureus* II-9. (B) Adhesion of *S. aureus* II-6. Results are expressed as a percentage.
661 Adhesion tests were performed both in exponential and stationary phases and for each
662 condition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

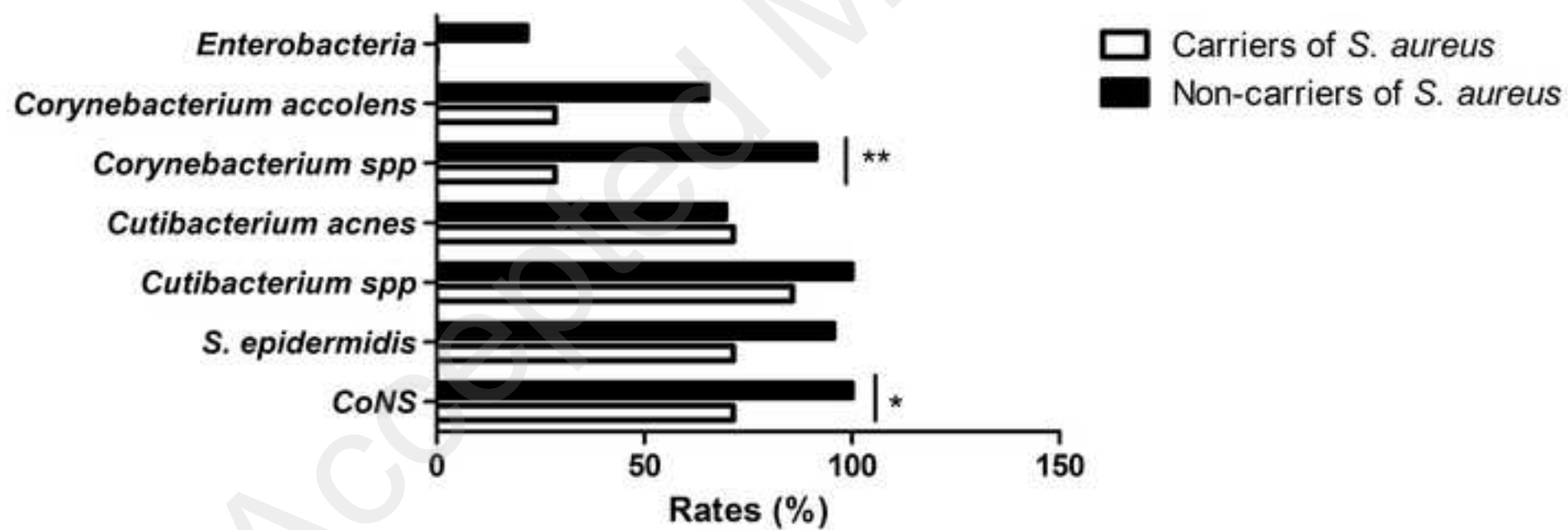
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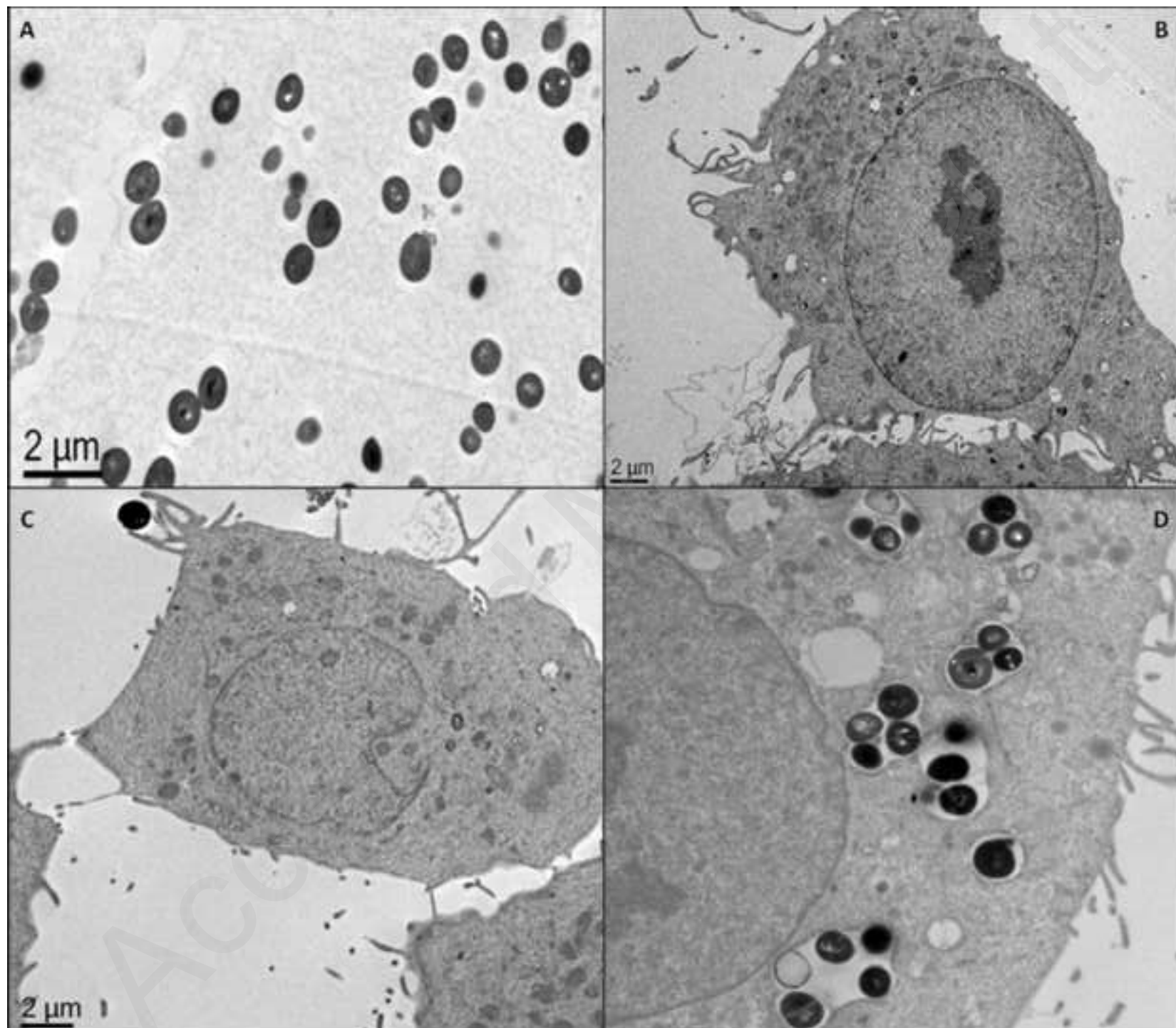
668 Fig. 5. Adhesion of *S. aureus* II-9 on Ca9-22 cells in community model respectively with *S.*
669 *epidermidis* (A, B), *C. accolens* (C, D) and *C. acnes* (E, F). *S. aureus* was in exponential
670 phase at the constant MOI of 10:1 and it serves as a reference (adhesion of *S. aureus* II-9
671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

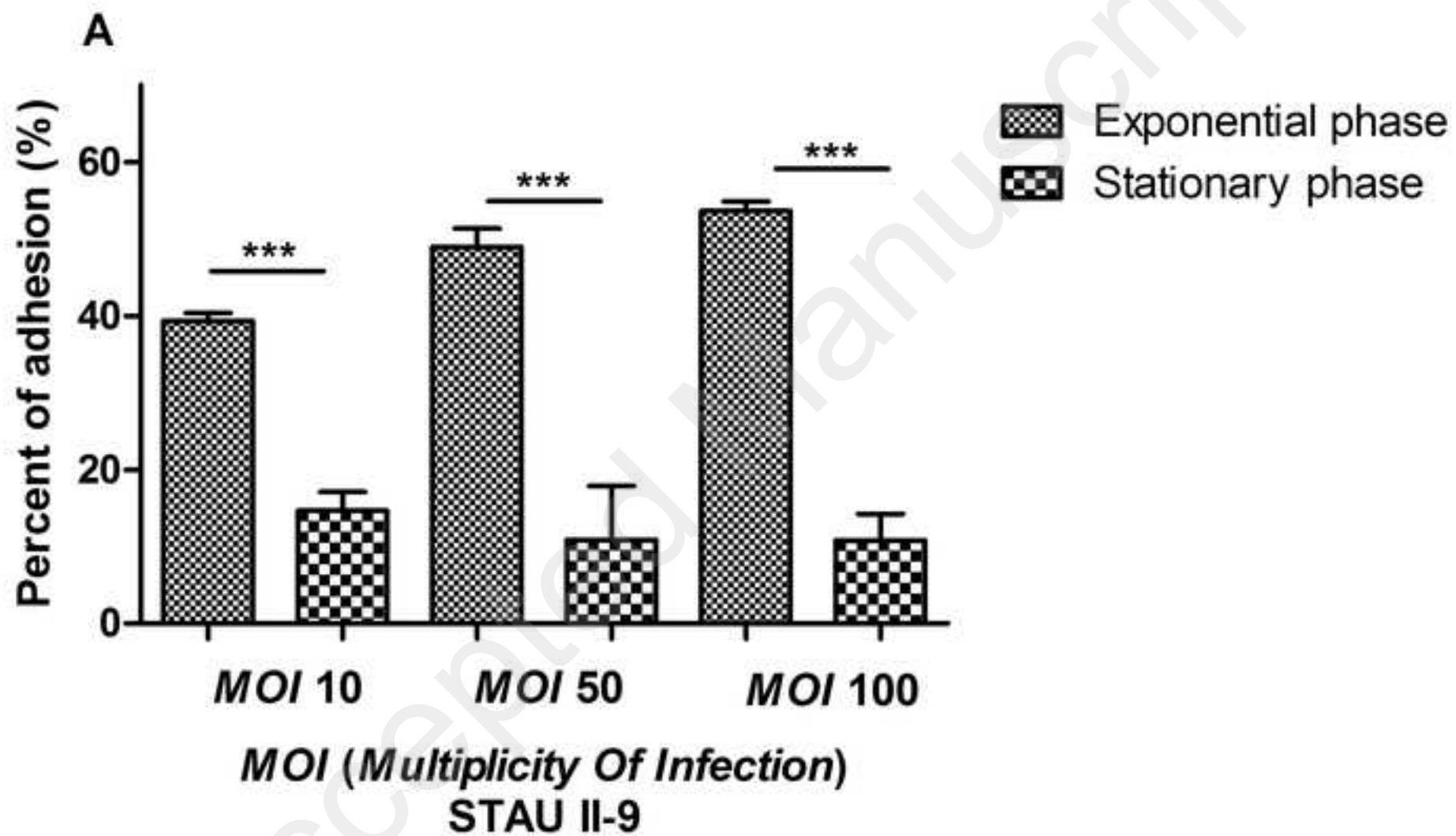
672 and 30% of another species), 50/50 and finally 30/70. Results are expressed as a percentage.
673 Each experiment was carried out in triplicate. Significant differences are represented by one
674 ($p < 0.05$), or two ($p < 0.01$) or three ($p < 0.001$) asterisks (*). STAU: *S. aureus*, STEP: *S.*
675 *epidermidis*, COAC: *C. accolens*, CUAC: *C. acnes*.
676

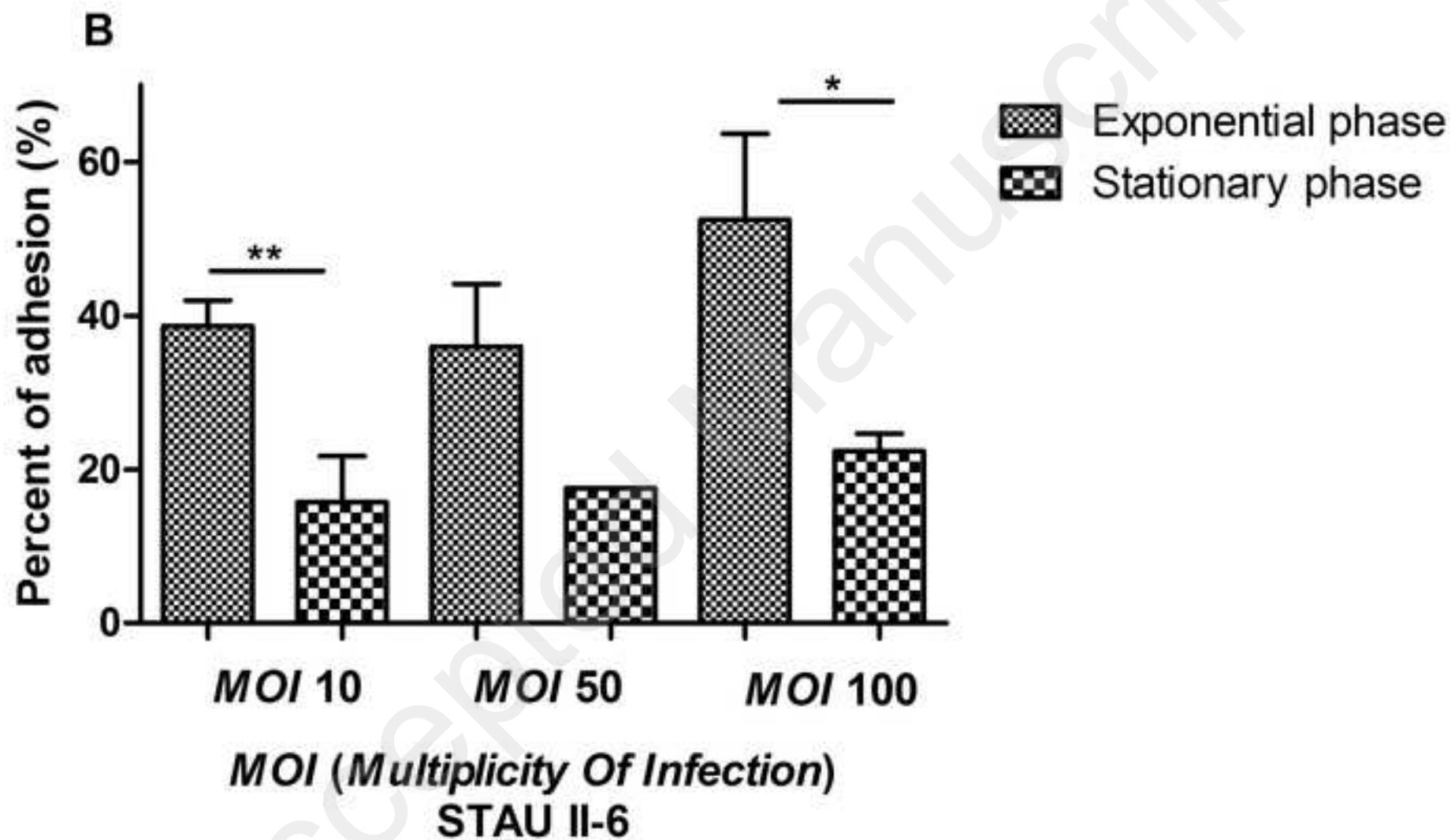


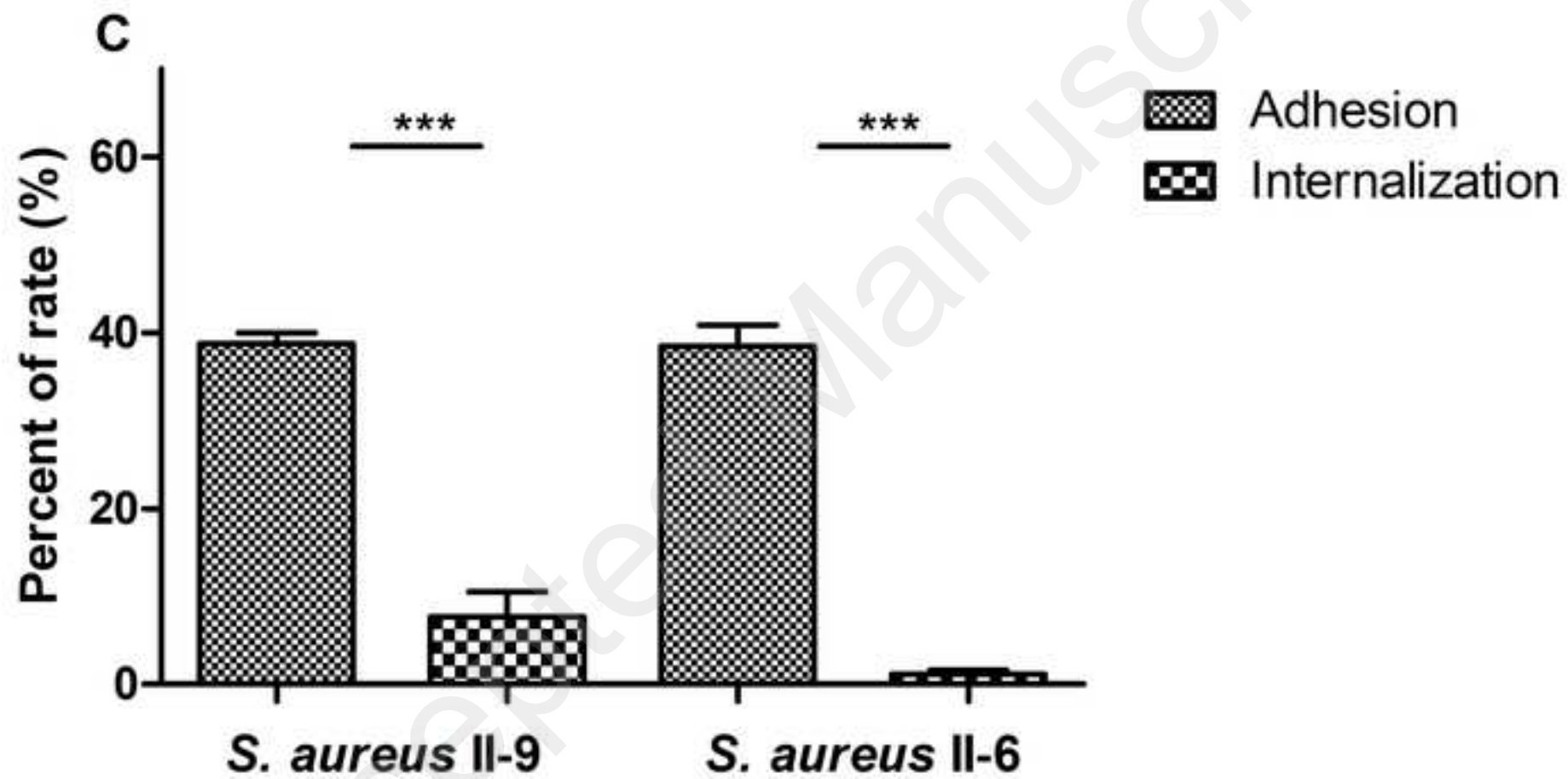


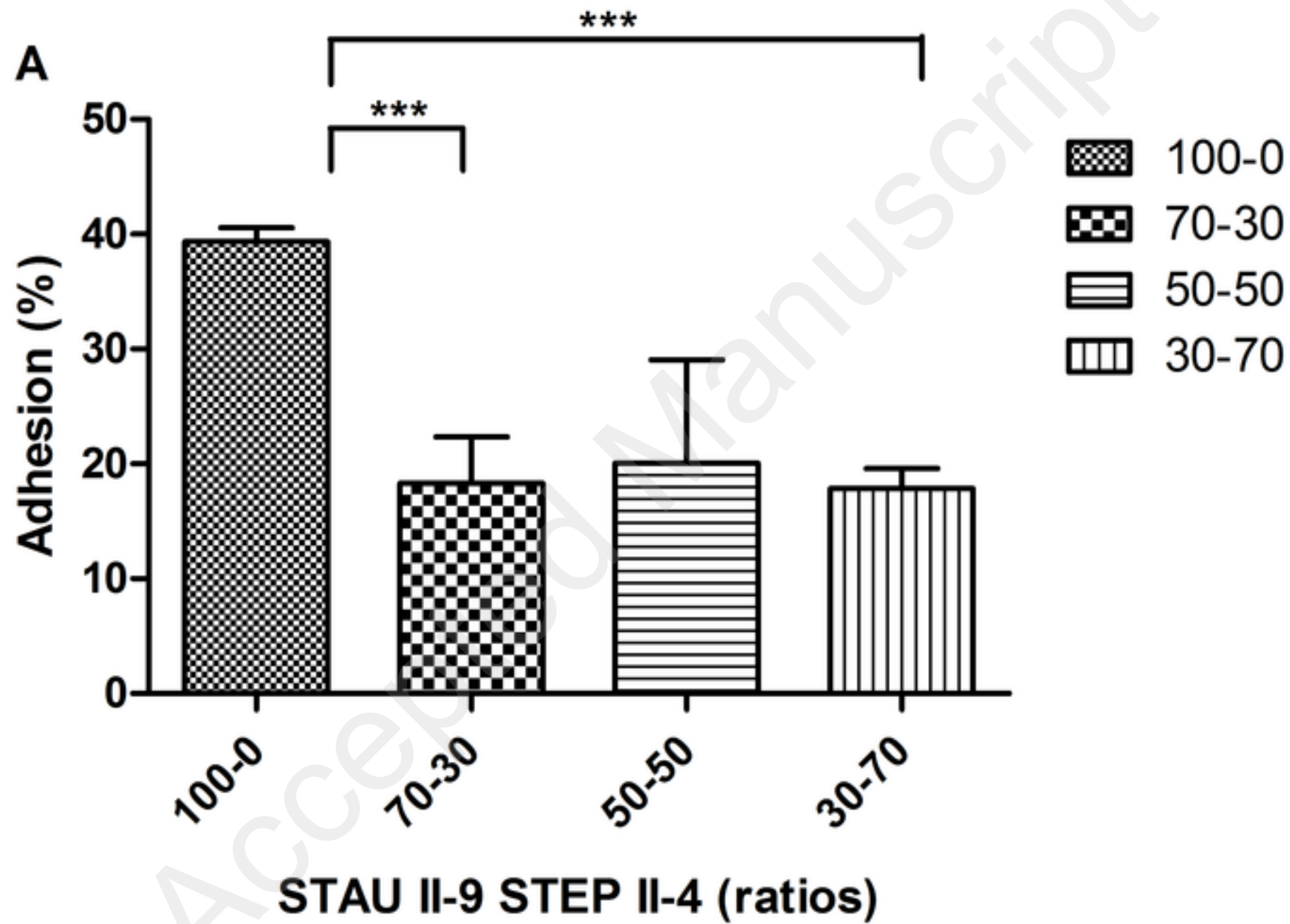


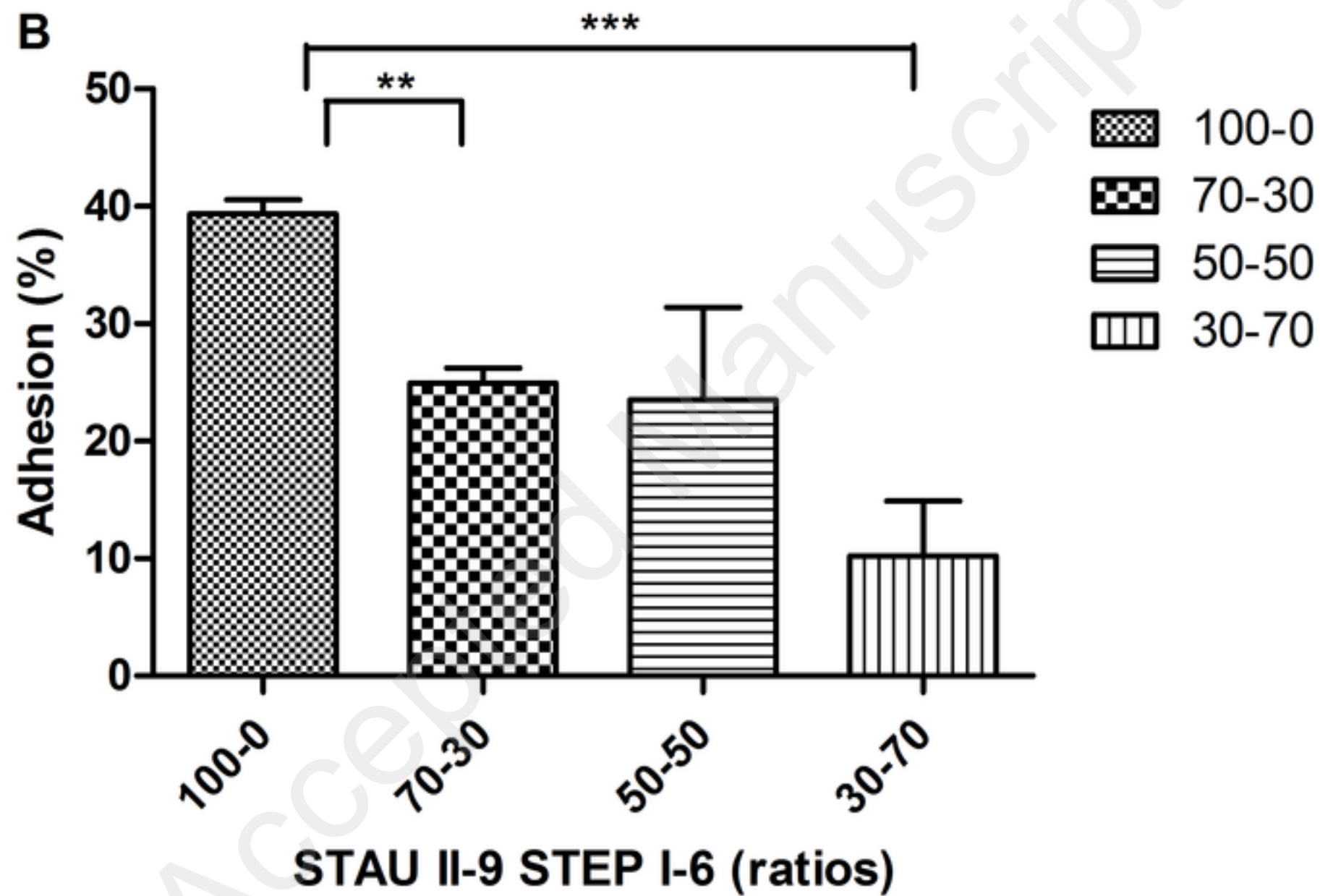


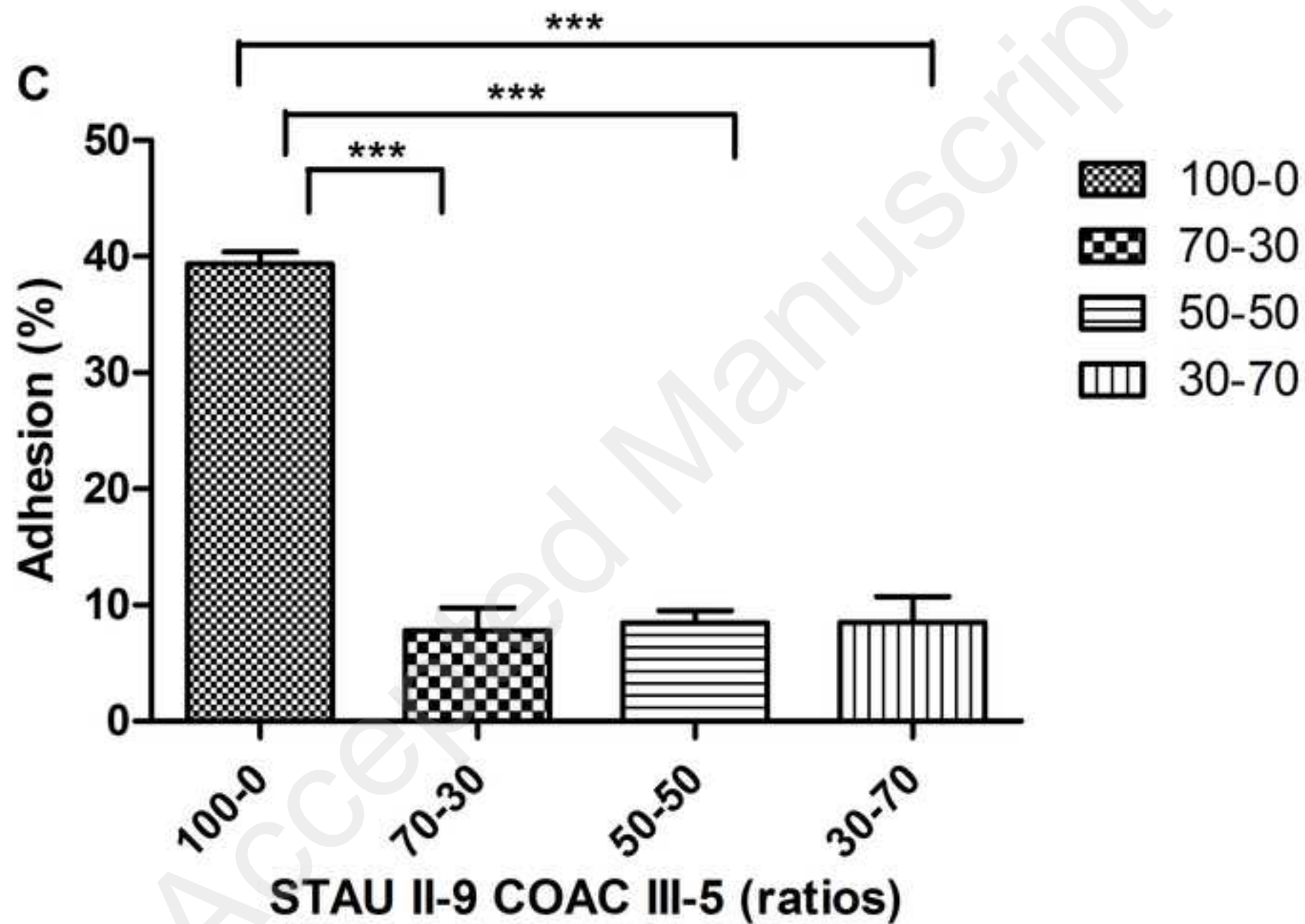


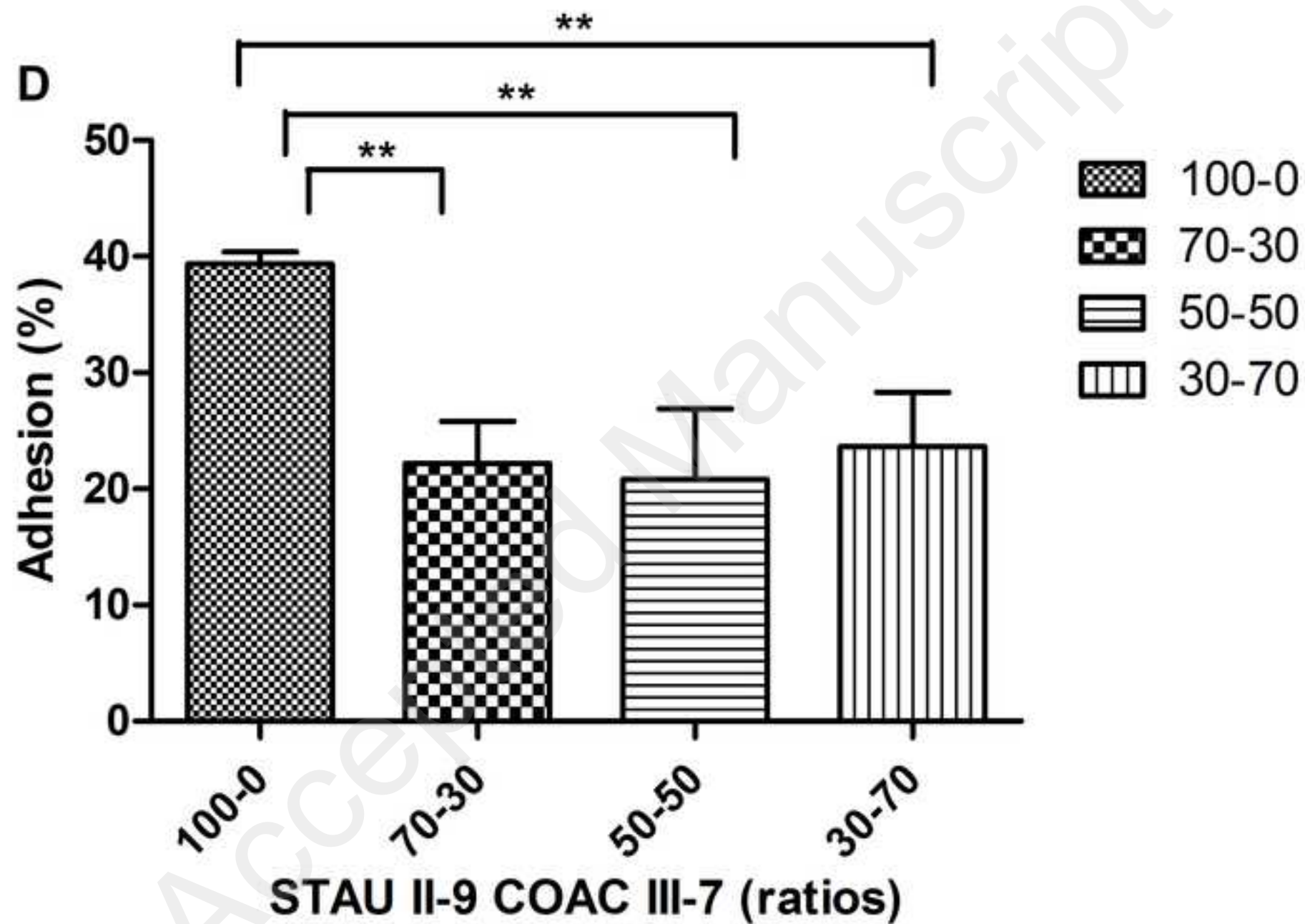


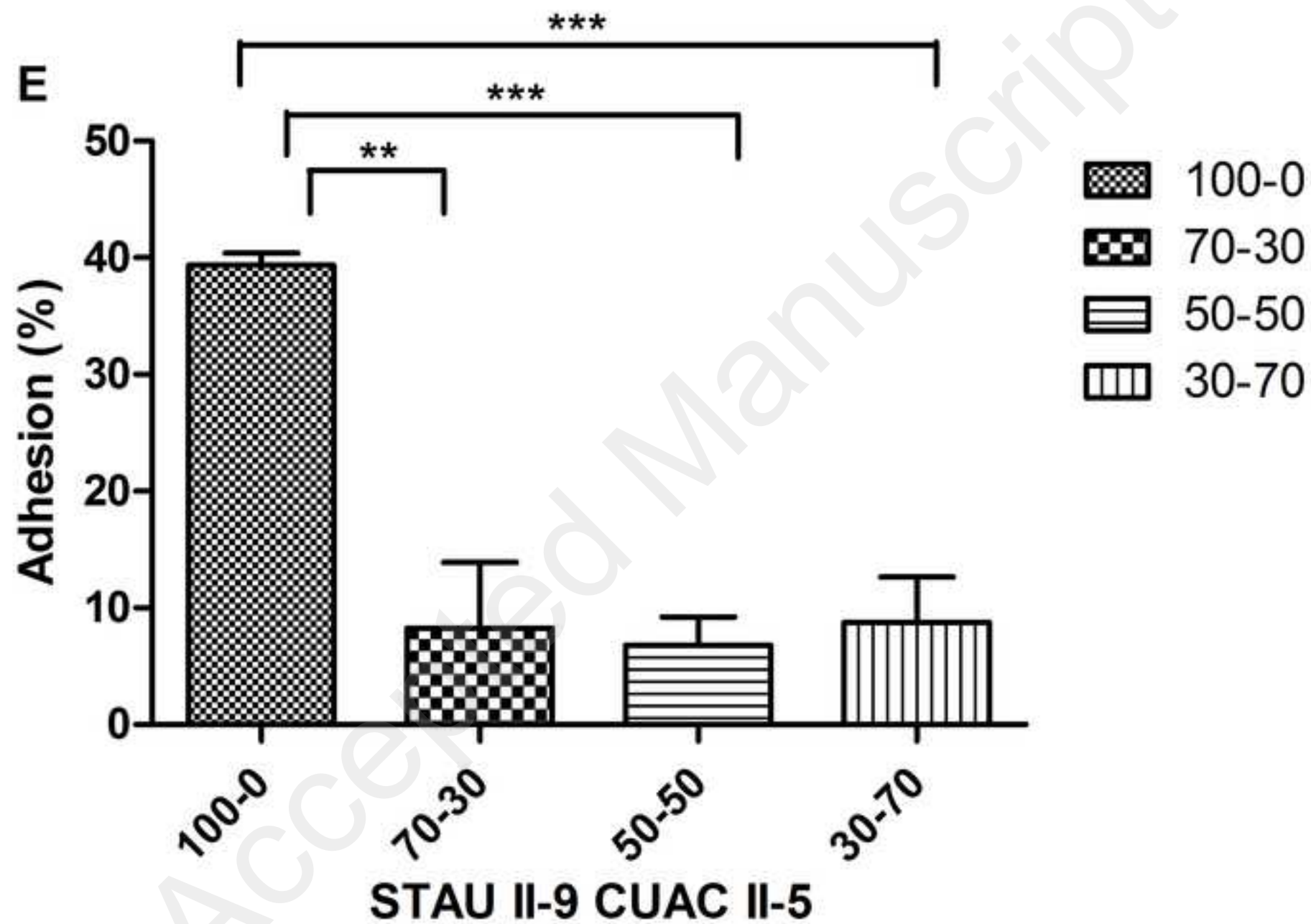


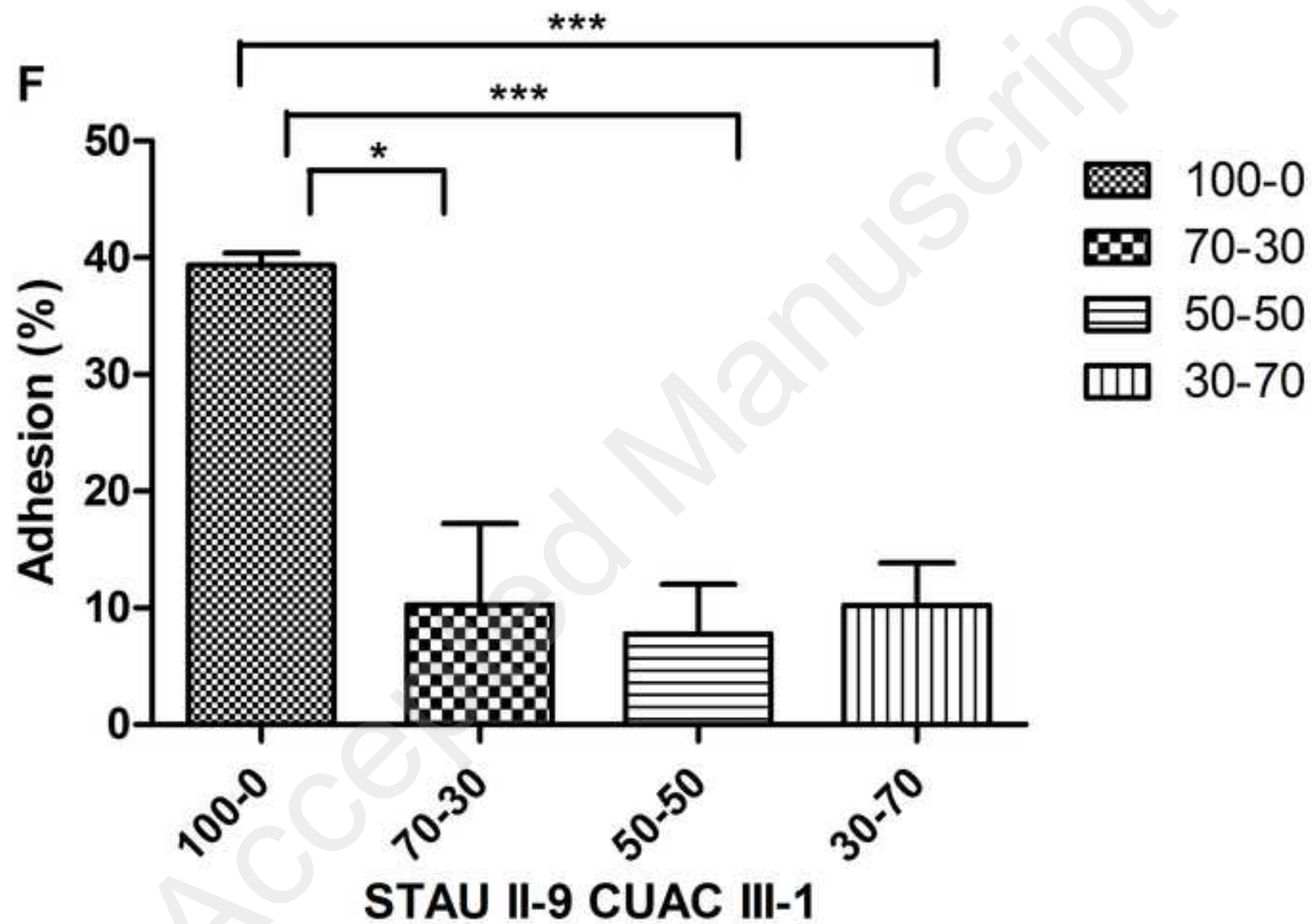












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Supplementary Material - Excel file
Table S1.xlsx

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