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1 **Short Communication**

2

3 The extra-intestinal avian pathogenic *Escherichia coli* strain BEN2908 invades avian
4 and human epithelial cells and survives intracellularly

5

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1 **Abstract**

2 Extra-intestinal pathogenic *Escherichia coli* (ExPEC) are responsible for a wide range
3 of diseases in humans and animals. By using *in vitro* invasion assays and transmission
4 electron microscopy, we showed that BEN2908, an ExPEC strain of avian origin (also
5 termed APEC for Avian Pathogenic E. coli), is able to usurp cellular endocytic
6 pathways to invade A549 human type II pneumocytes and LMH avian hepatocytes
7 where it is able to survive over several days. Although type 1 fimbriae are the major
8 adhesin of BEN2908, proportions of adherent fimbriated or afimbriated bacteria that
9 entered cells were comparable. Internalization of BEN2908 into human pneumocytes
10 reinforces previous studies indicating that APEC strains could represent a zoonotic risk.

11

12 ExPEC / APEC/ intracellular/ epithelial cells / type 1 fimbriae/ zoonotic risk

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1 **1. Introduction**

2 Extra-intestinal pathogenic *Escherichia coli* (ExPEC) strains are responsible for a wide
3 range of diseases in humans and animals, including urinary tract infections, neonatal
4 meningitis, septicaemia and pneumonia (Russo and Johnson, 2003). In avian species,
5 ExPEC strains, also termed APEC for avian pathogenic *E. coli*, are responsible for a
6 systemic disease that starts with a respiratory tract infection and evolves into
7 septicaemia and colonization of internal organs (heart, liver, spleen etc.). Phylogenetic
8 studies and virulence-factor pattern analysis have shown close relationships between
9 ExPEC strains of human and avian origin, suggesting that APEC strains could represent
10 a zoonotic risk (Rodriguez-Siek et al., 2005; Moulin-Schouleur et al., 2007).

11 In the present study, to gather new knowledge on the possible zoonotic aspect of APEC,
12 we examined the ability of the APEC strain BEN2908 to enter and survive
13 intracellularly in human type II pneumocytes, an epithelial cell line originating from a
14 target (lung) of early phase of ExPEC infection. As no avian pneumocyte cell lines are
15 available, we then evaluated the ability of BEN2908 to interact with avian hepatocytes,
16 a cell line originating from a target (liver) of late phase of APEC infection.

17

18 **2. Materials and methods**

19 *2.1. Cell lines, bacterial strains and growth conditions*

20 A549 human type II pneumocytes (Giard et al., 1973) and LMH avian hepatocytes
21 (Kawaguchi et al., 1987) were grown with 5% CO₂ at 37 °C in DMEM and at 40 °C in
22 DMEM/Ham's F12 (1:1), respectively. Media were supplemented with 2 mM L-
23 glutamine and 10% heat-inactivated fetal bovine serum. The APEC strain BEN2908,
24 O2:K1:H5 (*fim*⁺, *pap*⁻) is a nalidixic acid-resistant derivative of strain MT78 which was
25 isolated from the trachea of a chicken with respiratory infection (Dho and Lafont,

1 1982). Strain DM34 is an isogenic mutant of BEN2908 lacking the *fim* operon encoding
2 type 1 fimbriae. Electron microscopic examination of DM34 revealed only flagella as
3 cell surface appendages (Marc et al., 1998). Bacteria were routinely grown in Luria-
4 Bertani (LB) broth at 37 °C with shaking. Growth of strains BEN2908 and DM34 in LB
5 and cell culture media was found to be similar, under the conditions as described above.

6 7 2.2. Invasion, association and survival assays

8 For invasion assays, A549 and LMH cells were seeded in 24-well plates to reach
9 approximately 2.5×10^5 and 5×10^5 cells per well after 24 h of culture, respectively. Cell
10 monolayers were infected with mid-log-phase bacteria ($OD_{600} \approx 0.350$) at a multiplicity
11 of infection (MOI) of 10 and incubated for 2 h. Cells were washed and remaining
12 extracellular bacteria were killed by incubating for an additional 1h 30 min in medium
13 containing gentamicin (100 $\mu\text{g/ml}$). Cells were then washed and lysed with sterile water
14 for 30 min at 4 °C. For association assays, the additional incubation with gentamicin
15 was omitted. Inocula, cell-associated (adherent and intracellular) and intracellular
16 bacteria were enumerated by viable counts on LB agar. To provide a true adhesion
17 value, the number of intracellular bacteria was subtracted from the number of cell-
18 associated bacteria. Adhesion and invasion were respectively calculated as the number
19 of adherent or intracellular bacteria, divided by the number of bacteria in the inoculum,
20 and expressed as a percentage. Invasion efficiency was calculated as the number of
21 intracellular bacteria divided by the number of adherent bacteria, and expressed as a
22 percentage.

23 For invasion-blocking experiments, inhibitors were added to cell monolayers 30 min
24 prior to inoculation with bacteria and maintained throughout the invasion assay.
25 Chlorpromazine, cytochalasin D, filipin III and nystatine were all purchased from

1 Sigma-Aldrich and stock solutions were prepared in DMSO with the exception of
2 chlorpromazine, which was solubilized in water. The chosen concentration of each
3 inhibitor (0.8 μ M cytochalasin D, 14 μ M chlorpromazine, 25 μ M nystatin and 6 μ M
4 filipin III) was the lowest concentration necessary to obtain the maximal inhibitory
5 effect on bacterial uptake. Invasion efficiency was calculated and expressed in relation
6 to that of the untreated controls. Inhibitors and DMSO, at the final concentrations used,
7 were controlled to have no notable effects on the viability and growth of bacteria (as
8 checked by viable counts) or eukaryotic cells [as checked by trypan blue exclusion
9 assay (Longo-Sorbello et al., 2006)]. DMSO at the used concentrations (0.05% for
10 cytochalasin D and nystatin, 0.1% for filipin III) was proven not to interfere with the
11 adhesion of bacteria with eukaryotic cells. Adhesion, invasion and invasion-blocking
12 experiments were conducted in triplicate and were independently repeated at least three
13 times.

14 For intracellular survival assays, A549 and LMH cells were seeded in 6-well plates to
15 reach 1×10^6 and 2.5×10^5 cells per well after 24 h of culture, respectively. The
16 invasion assay was performed as described above. To delay cell overgrowth after the
17 initial killing of extracellular bacteria with 100 μ g/ml of gentamicin, the infected cells
18 were cultured in medium containing 2.5% of serum only. At that time, the gentamicin
19 concentration was reduced to 10 μ g/ml (a concentration shown to kill BEN2908) to
20 avoid putative accumulation within cells during prolonged incubation. The medium was
21 changed daily. Intracellular and extracellular bacteria were enumerated by viable counts
22 on LB agar every 24 h until 192 h post-inoculation. Intracellular survival assay was
23 conducted in duplicate and was independently repeated at least two times.

24

25

1 2.3. Transmission electron microscopy (TEM)

2 At 2 h post-infection, infected A549 and LMH monolayers were fixed with 2%
3 glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4°C. Infected LMH
4 monolayers were also fixed similarly at 72 h post-infection. They were then postfixed in
5 2% OsO₄ – 1.5% potassium ferrocyanide – 0.1 M cacodylate buffer for 1h 30 min at
6 room temperature. After dehydration using increasing ethanol concentrations, the
7 monolayers were embedded in Epoxy Resin. Ultrathin sections (70 nm) were collected
8 on copper grids, contrasted with uranyl acetate and lead citrate, and observed with a
9 CM10 electron microscope (Philips, Eindhoven, Netherlands). Analysis software (Soft
10 Imaging System, Germany) was used for image acquisition.

11

12 2.4. Statistical analysis

13 Data were analysed using Student's *t*-test with $P < 0.05$ considered as significant.

14

15 3. Results

16 3.1. Interaction of strain BEN2908 with epithelial cells

17 The adhesive and invasive abilities of strain BEN2908 were tested on A549 human type
18 II pneumocytes and LMH avian hepatocytes. Figure 1A (black bars) shows that
19 BEN2908 adhered to LMH and A549 cells to the same extent (366% and 317% of the
20 inoculum, respectively). Bacterial uptake occurred in both cell lines although the
21 internalization of BEN2908 was significantly higher ($P = 0.004$) in LMH cells (23.10%
22 of the inoculum) than in A549 cells (0.756% of the inoculum) (Fig. 1B, black bars). The
23 low internalization level of BEN2908 in A549 cells could not be increased by
24 increasing the MOI from 10 to 100 (data not shown).

1 The ability of BEN2908 to invade LMH and A549 cells was also examined by
2 transmission electron microscopy. Figure 2 illustrates evidence of invasion of both cell
3 lines by BEN2908. Intracellular bacteria were observed both singly and in groups and
4 were clearly enclosed in membrane-bound vacuoles (Figs. 2A to 2D). *E. coli* were
5 observed bound to the cell surface of LMH cells and engulfed by lamellipodia
6 suggesting a zipper-like mechanism of internalization (Fig. 2B, arrowhead). Moreover,
7 some images of synchronized dividing intracellular bacteria suggest that intravacuolar
8 multiplication may occur during infection of LMH cells (Fig. 2C, arrowheads). In
9 infected A549 monolayers, *E. coli* were observed bound to the cell surface and
10 surrounded by microvilli (Figs. 2D and 2F, arrowheads). In some cases, bacteria were
11 associated with endocytic pits at the cell membrane (Fig. 2E, arrowheads), suggesting
12 that lipid rafts/caveolae- or clathrin- mediated endocytic pathways could be involved in
13 BEN2908 internalization.

14

15 3.2. Role of type I fimbriae in the interaction of the APEC strain BEN2908 with avian 16 hepatocytes and human type II pneumocytes

17 As type I fimbriae have been shown to be involved in the interaction of BEN2908 with
18 avian primary pharyngeal or tracheal cells (Marc et al., 1998), we quantified their
19 impact on the interactions of BEN2908 with LMH and A549 cells. To that end, we
20 monitored the adhesive and invasive abilities of strain DM34, a *fim* operon deletion
21 mutant of BEN2908 that does not express type I fimbriae (Marc et al., 1998). As shown
22 in Figure 1A, the adhesion of DM34 with LMH and A549 cells was drastically reduced
23 compared to that of BEN2908 (11.55% versus 366% of the inoculum for LMH cells and
24 1.805% versus 317% for A549 cells). Similarly, the internalization of DM34 in LMH
25 and A549 cells was significantly lower than that of BEN2908 (0.923% versus 23.10%

1 of the inoculum and 0.003% versus 0.756%, respectively) (Fig. 1B). Although bacterial
2 uptake occurred in both cell lines, the adhesive and invasive abilities of DM34 towards
3 LMH cells were significantly higher than towards A549 cells ($P = 0.031$ for the capacity
4 of adhesion, $P = 0.022$ for the capacity of invasion). Electron microscopy examination
5 of LMH cells infected with DM34 confirmed the presence of cell-surface adherent and
6 intravacuolar bacteria (data not shown). Such investigations were not performed on
7 A549 cells regarding the very low level of DM34 internalization in this cell type.

8

9 *3.3. Influence of endocytosis inhibitors on the internalization of BEN2908 into*

10 *eukaryotic cells*

11 As bacteria were frequently observed near endocytic pits at the cell membrane, we
12 investigated the involvement of the lipid rafts/caveolae and of the clathrin endocytic
13 pathways in BEN2908 uptake. To that end, LMH and A549 cells were treated with
14 inhibitors known to affect these endocytic processes. Nystatin and filipin III, two
15 inhibitors of the lipid rafts/caveolae endocytic pathway, have no effect on bacterial
16 uptake by LMH and A549 cells (Fig. 3A and 3B, black bars). However, cytochalasin D,
17 an inhibitor of actin polymerization, and chlorpromazine, an inhibitor of clathrin-coated
18 pit formation, significantly reduced entry of strain BEN2908 into LMH (96% and 95%
19 inhibition, respectively) and A549 cells (82% and 97% inhibition, respectively) in a
20 dose-dependent manner, giving good indication of the involvement of clathrin in the
21 internalization process (Fig. 3A and 3B, black bars; data not shown). As BEN2908 is
22 able to invade cells independently of type 1 fimbriae, we tested the role of actin and
23 clathrin in this process and we found that cytochalasin D and chlorpromazine also
24 significantly reduced (50% and 76% inhibition, respectively) the entry of strain DM34

1 into LMH cells (Fig. 3A, white bars). Such tests could not be done on A549 cells due to
2 the very low invasion rate of these cells by DM34.

3

4 *3.4. Fate of internalized BEN2908 bacteria*

5 We investigated the ability of strain BEN2908 to survive inside LMH and A549 cells
6 (Fig. 4). In LMH cells, the number of intracellular bacteria decreased slowly during the
7 first 48 h post-infection and then remained stable until 192 h post-infection. Electron
8 microscopy examination of LMH cells infected since 72 h confirmed the presence of
9 intracellular bacteria (Fig. 2G). In contrast, in A549 cells the number of intracellular
10 bacteria decreased steadily, reaching 0.6% of the initial number of intracellular bacteria
11 at 120 h post-infection. These data demonstrate that BEN2908 is able to survive inside
12 epithelial cells to a greater or lesser extent depending on the cell lines or host species.

13

14 **4. Discussion**

15 In this study, we found that BEN2908, an ExPEC strain of avian origin is able to adhere
16 to and invade human pneumocytes and avian hepatocytes. Intracellular bacteria were
17 observed enclosed in membrane-bound vacuoles (Fig. 2). Internalization of this ExPEC
18 strain of avian origin into human pneumocytes indicates that APEC do not have an
19 absolute host specificity for at least one of their entry site. This reinforces previous
20 studies showing the close phylogenetic proximity of ExPEC strains of avian and human
21 origin and indicating that APEC could represent a zoonotic risk (Rodriguez-Siek et al.,
22 2005; Moulin-Schouleur et al., 2007). BEN2908 invades LMH hepatocytes with greater
23 efficiency than A549 pneumocytes, although it adheres similarly to both cell lines (Fig.
24 1). This difference in invasiveness could be due to cellular type per se and/or species
25 origin of the cells.

1 Previous *in vivo* studies have shown that BEN2908 is still present in the lungs and liver
2 of chicken 144 h post-inoculation (Dozois et al., 1994). This could be explained in part
3 by the ability of strain BEN2908 to survive intracellularly as shown by intracellular
4 persistence of BEN2908 in the cell lines used in this study (Fig. 4). Although
5 synchronized dividing intravacuolar bacteria were observed in the first time of invasion
6 of LMH cells (Fig. 2C), the number of intracellular bacteria did not increase post-
7 infection (Fig. 4), suggesting that BEN2908 does not replicate within cells. This
8 apparently stable number of intracellular bacteria observed from 48 h to 192 h in LMH
9 cells could have resulted from a balance between intracellular replication and
10 intracellular degradation. However, as the antibiotic was maintained in extracellular
11 media throughout the survival assay, we cannot exclude the possibility of some
12 extracellular release of bacteria which were subsequently killed by the antibiotic.

13 Our data (Fig. 1) confirm that type 1 fimbriae are the major adhesin of strain BEN2908
14 but suggest that other minor components are also involved in adhesion and invasion.
15 These putative BEN2908 components have not been identified to date. Unpublished
16 PCR experiments from our laboratory indicated that BEN2908 is devoided of the *pap*
17 genes coding for P fimbriae but factors such as curli, Tsh and Yqi were described to be
18 involved in adhesion and/or invasion of other APEC strains (Provence and Curtiss,
19 1994; Gophna et al., 2001; Antao et al., 2009). Although type 1 fimbriae are the major
20 adhesin of BEN2908 no significant difference was nevertheless observed between the
21 invasive efficiency of fimbriated and afimbriated strains. Indeed, 7.83% of adherent
22 DM34 bacteria were internalized in LMH cells versus 6.32% of BEN2908 bacteria. In
23 A549 cells, these percentages were 0.232% and 0.207% for DM34 and BEN2908,
24 respectively. This suggests either that the host cell receptors for type 1 fimbriae and for

1 the other minor adhesin(s) of BEN2908 allow similar rates of bacterial internalization,
2 or that type 1 fimbriae are not directly involved in the BEN2908 internalization process.
3 Bacterial pathogens have developed various strategies to usurp host endocytic pathways
4 and enter epithelial cells (Pizarro-Cerda and Cossart, 2006). Studies of ExPEC entry
5 into epithelial cells have mainly focused on strains responsible for human urinary tract
6 infections. Interaction between type 1 fimbriae of uropathogenic ExPEC and host cells
7 has been shown to trigger localized actin rearrangements (Martinez et al., 2000; Eto et
8 al., 2008) leading to bacterial internalization via endocytosis pathway. Type 1 fimbriae
9 were first described to mediate endocytosis via the caveola pathway (Baorto et al.,
10 1997; Shin et al., 2000; Duncan et al., 2004), but internalization mediated by the
11 clathrin endocytic pathway has recently been reported (Eto et al., 2008). By using
12 inhibitors affecting each of these pathways, we found that only inhibitors affecting actin
13 polymerization and clathrin-coated pits formation reduced BEN2908 entry, providing
14 good indication that its internalization in avian hepatocytes and in human pneumocytes
15 depends on the clathrin endocytic pathway.

16 In conclusion, our work indicates that the APEC strain BEN2908 is able to usurp
17 cellular endocytic pathways to invade human and avian epithelial cells where it is able
18 to survive over several days.

19

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11

1 **Figure legends**

2 **Figure 1.** Interaction of *E. coli* strains BEN2908 and DM34 with LMH and A549 cells.
3 Adhesive and invasion abilities of strains BEN2908 (■) and DM34 (□) are expressed,
4 on a logarithmic scale, as the percentage of adherent (A) or intracellular (B) bacteria in
5 relation to the number of bacteria in the inoculum. Data are means \pm SEM of at least
6 three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.005$ by
7 Student's *t*-test.

8

9 **Figure 2.** Transmission electron micrographs of epithelial cells infected with *E. coli*
10 strain BEN2908. Ultrathin sections of LMH (A to C and G) and A549 (D to F)
11 monolayers fixed at 2 h (A to F) or 72 h (G) post-infection were observed. Panel E
12 shows a high magnification of cell membrane-associated bacteria of panel D.
13 Arrowheads indicate lamellipodia (B), synchronized dividing intracellular bacteria (C),
14 endocytic pits (E) and microvilli (F).

15

16 **Figure 3.** Effect of different inhibitors on internalization of *E. coli* strains BEN2908 and
17 DM34 by epithelial cells. Invasion efficiency of LMH (A) and A549 (B) cells by strains
18 BEN2908 (■) or DM34 (□) in the presence of inhibitors was calculated and expressed in
19 relation to that of the untreated controls. The inhibitors are cytochalasin D (Cyt D) at
20 0.8 μ M, chlorpromazine (Chlor) at 14 μ M, nystatine (Nys) at 25 μ M and filipin III (Fil)
21 at 6 μ M. Data are means \pm SEM of at least three independent experiments performed in
22 triplicate. * $P < 0.05$ ** $P < 0.0005$ versus respective control by Student's *t*-test.
23 Internalization of DM34 was not tested in the presence of nystatine and filipin in LMH
24 cells or of any inhibitors in A549 cells.

25

1 **Figure 4.** Intracellular survival of *E. coli* BEN2908 in LMH and A549 cells.
2 Intracellular and extracellular bacteria were enumerated at different times post-
3 infection. Data of intracellular bacteria are means of at least two independent
4 experiments performed in duplicate. SEM not comprised within symbols are indicated
5 by bars. Extracellular bacteria accounted for less than 2% of the total number of bacteria
6 throughout the assay. Viability and growth of infected and non-infected cells were the
7 same during the entire incubation period.
8

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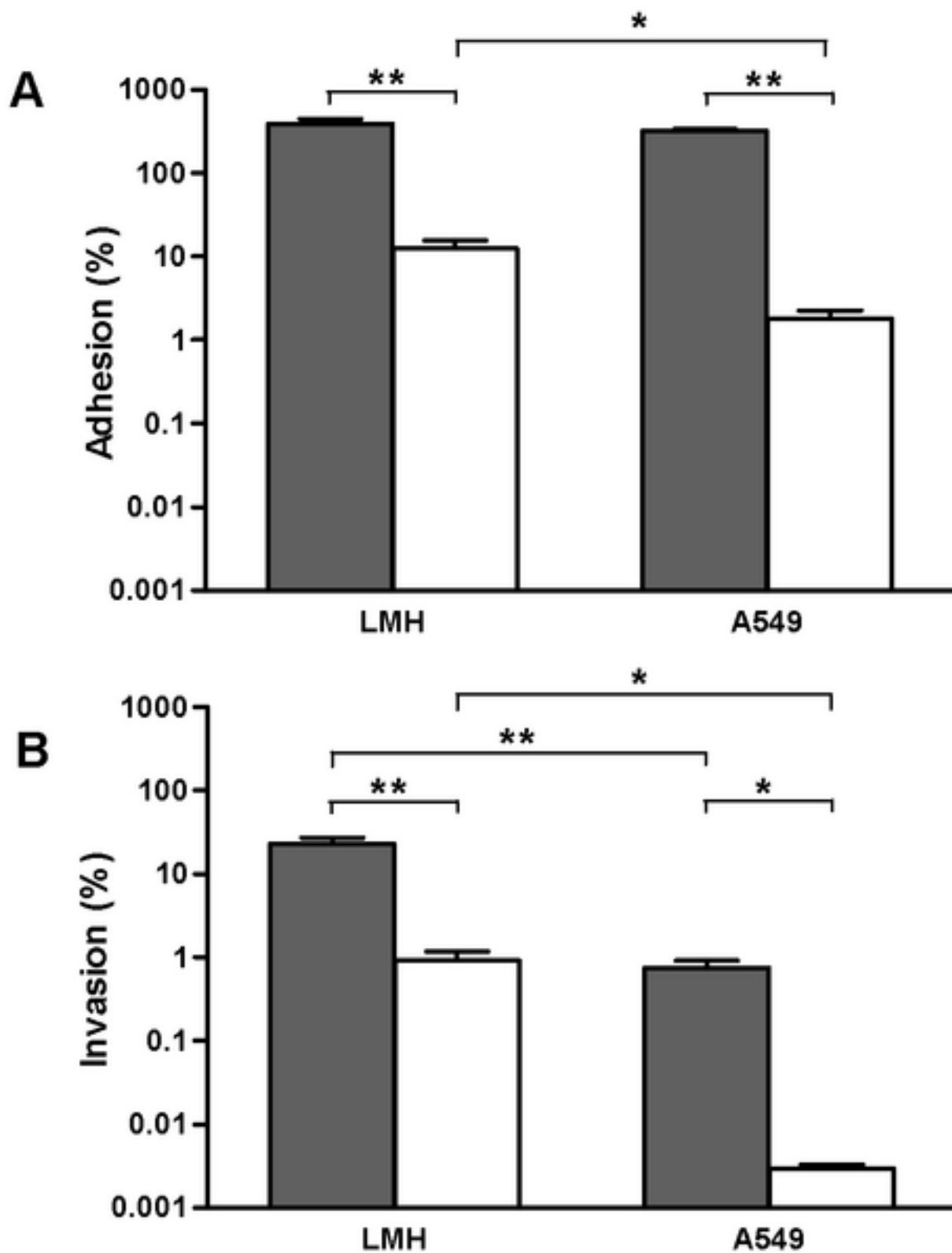
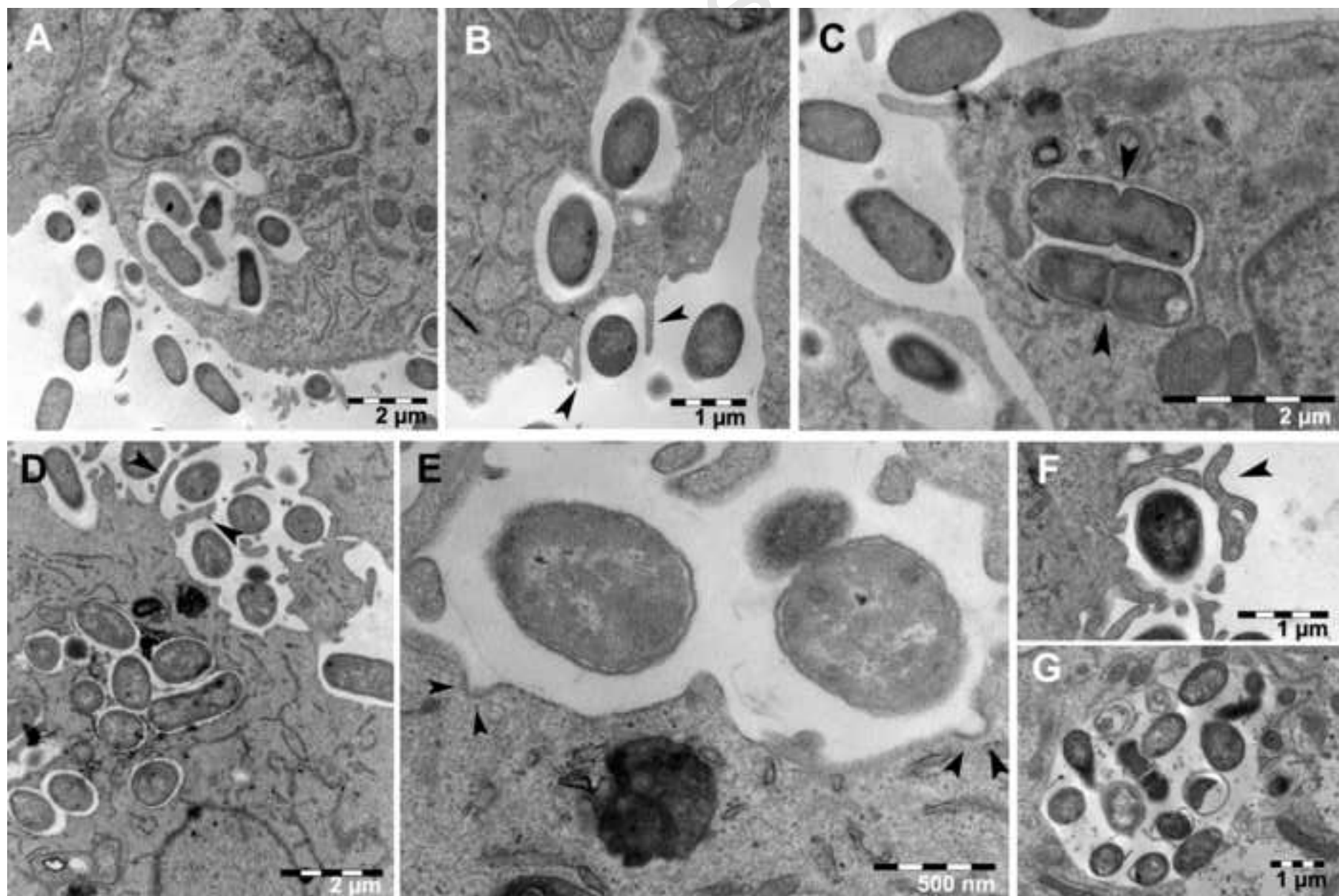


Figure 1

Figure 2
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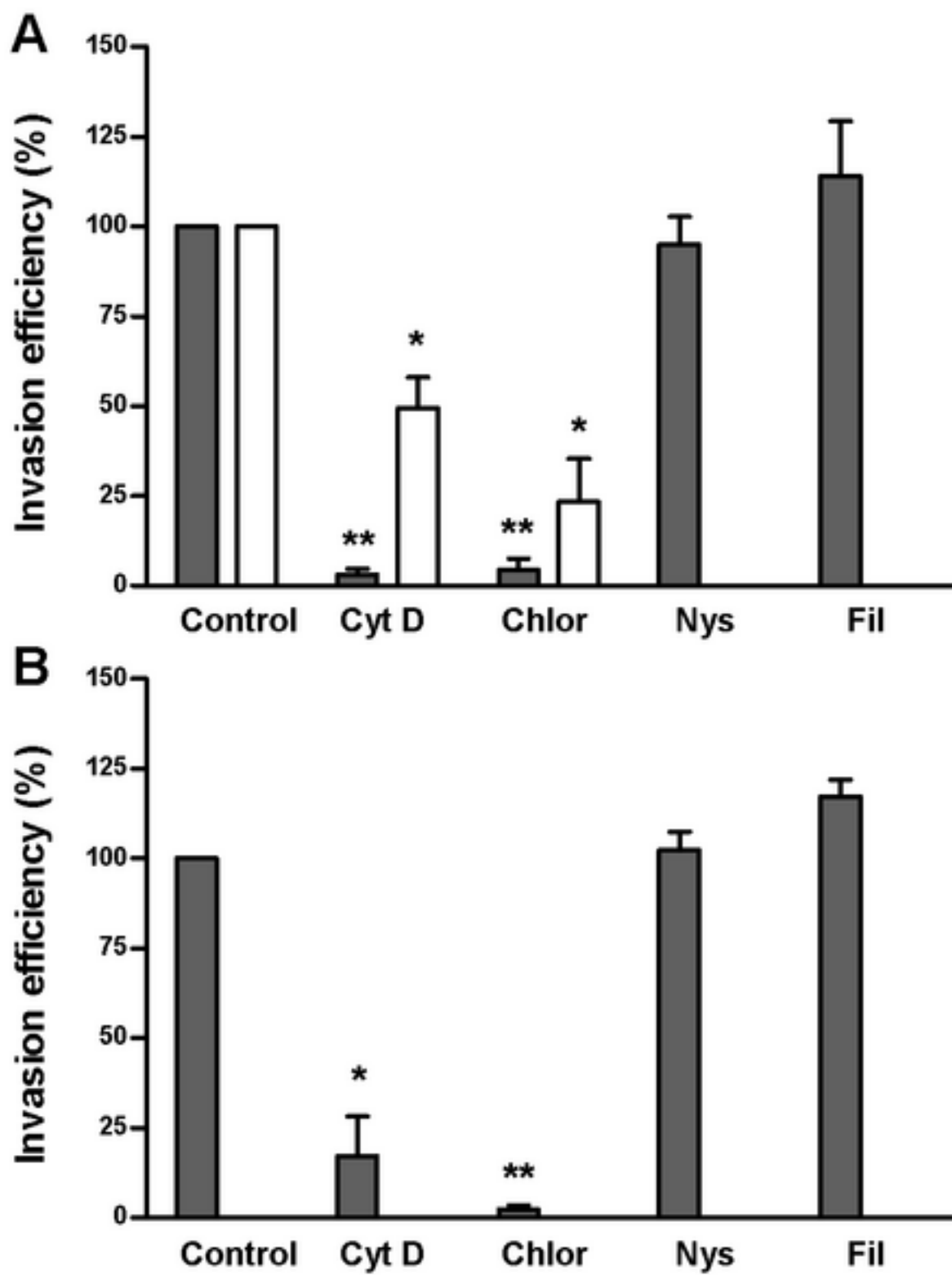


Figure 3

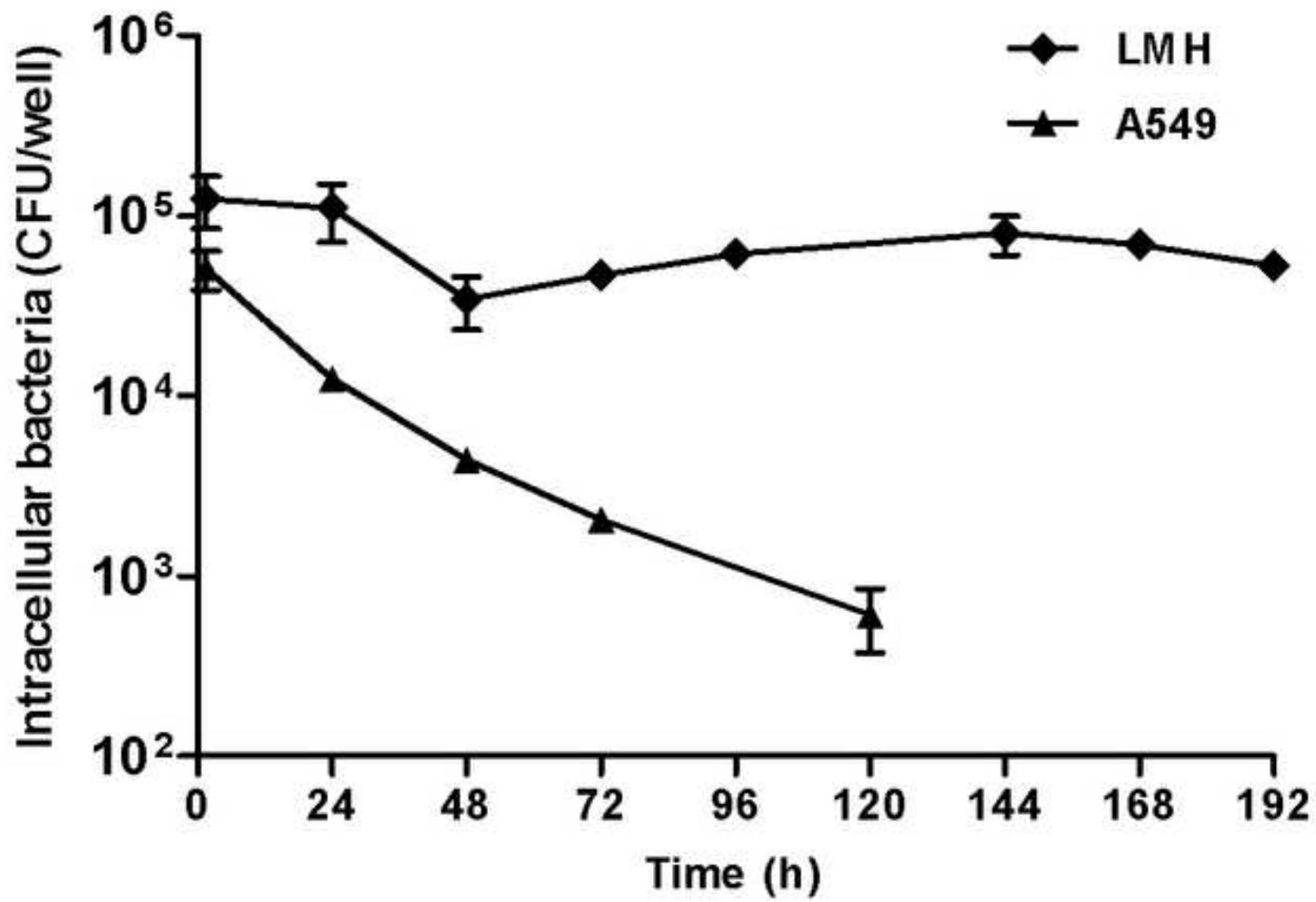


Figure 4