

## *Allovahlkampfia spelaea* is a Potential Environmental Host for Pathogenic Bacteria

Mona Embarek Mohamed<sup>1\*</sup>, Enas Abdelhameed Mahmoud Huseein<sup>2</sup>, Haiam Mohammed Mahmoud Farrag<sup>2</sup>, Fatma Abdel Aziz Mostafa<sup>3</sup> and Alaa Thabet Hassan<sup>4</sup>

<sup>1</sup>Department of Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt

<sup>2</sup>Department of Parasitology, Faculty of Medicine, Assiut University, Assiut, Egypt

<sup>3</sup>Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt

<sup>4</sup>Department of Chest Diseases, Assiut University Hospitals, Assiut, Egypt

\*Corresponding author: Mona Embarek Mohamed, Department of Microbiology and Immunology, Faculty of Medicine, Assiut University, 71515 Assiut, Egypt, Tel: +20-882413500/2411899; Fax: +2088233327, E-mail: monasallam2000@yahoo.com

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### Abstract

*Allovahlkampfia spelaea* was identified for the first time in 2009. As a free living amoeba, it has been suggested to be a protective host for some bacterial pathogens against harsh environmental conditions and can transmit them to vulnerable hosts. We aimed in this study to test the interactions between *Allovahlkampfia spelaea* and some waterborne and foodborne bacteria and unravel if the tested bacteria can survive and multiply inside amoeba. We used a keratitis isolate of *Allovahlkampfia spelaea* grown in PYG medium containing proteose peptone, yeast extracts, and glucose. We examined amoeba interactions with Methicillin resistant *Staphylococcus aureus*, *Escherichia coli* 1, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter cloaca*, *Proteus mirabilis*, *Raoultella terrigena*, *Raoultella ornitholytica*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* using the co-culture assays. Amoebal survival rate with different bacterial strains were determined. With the exception of *Proteus mirabilis* that showed decreased survival rates inside amoebal cells, other bacterial isolates could survive and multiply inside *Allovahlkampfia spelaea* that was associated with decreased survival rates of the amoeba. Particularly, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and MRSA exhibited significantly increased multiplication rates inside amoeba. Our study demonstrated that *Allovahlkampfia spelaea* may act as a replicative host for pathogenic bacteria with environmental and clinical implications.

**Keywords:** *Allovahlkampfia spelaea*; Amoeba-bacteria interactions; Co-culture experiments; Gram negative bacteria; Intracellular survival

### Introduction

*Allovahlkampfia spelaea* (*A. spelaea*) belongs to the genus *Allovahlkampfia* in the family *Vahlkampfiidae*, class *Heterolobosea*, and Phylum *Percolozoa* [1] that was identified for the first time in 2009 [2]. *A. spelaea* is a free living amoeba (FLA) and has been found to be associated with keratitis as evident in a research conducted by Tolba et al. FLA is ubiquitous in nature [3] and some of them produce serious human infections [4]. Amoeba in nature may have contact with other microorganisms including bacteria. Amoebae are the dominant bacterial consumers, contributing to recycling of nutrients and maintaining the structure of the microbial community [5]. Most FLA genera are characterized by a biphasic life cycle consisting of a vegetative trophozoite stage and a physiologically static cyst stage [2]. Cysts are highly resistant and remain viable (and infective) for several years which facilitates spreading and colonization of new ecological niches [6]. On the other hand, bacteria have developed several antipredator strategies including cell size reduction, modified cell morphology, modification of cell wall characteristics, high-speed motility, biofilm or microcolony formation, and production of exopolymers or toxins [7]. In this case, amoebae may act as a protective host for some bacterial pathogens against harsh environmental conditions that normally kill. The role of FLA in survival and protection of pathogenic bacteria is increasingly

recognized [8]. The amoebae aid in bacterial transmission to susceptible hosts thus constitutes a problem to the ecosystem health [4]. Additionally, bacteria become more resistant to disinfectants [5,8,9]. Many microorganisms are known to be hosted by FLA including; *Acinetobacter* spp., *Aeromonas* spp., *Enterobacter* spp., *Escherichia coli* (*E. coli*), *Klebsiella pneumonia* (*Kl. pneumonia*), *Pseudomonas aeruginosa* (*Ps. aeruginosa*), *Salmonella* spp., and *Staphylococcus aureus* (*Staph. aureus*) [10]. The hypothesis in our study was that *A. spelaea* may play a role for survival and multiplication of bacterial pathogens.

### Materials and Methods

Molecular characterization of *A. spelaea* that was conducted at the Department of Medical Genomics, Graduate School of Frontier Sciences, The University of Tokyo, Japan.

### Culture and molecular characterization of *A. spelaea*

*A. spelaea* used in this study was obtained from a patient with keratitis. *A. spelaea* was isolated on 1.5% non-nutrient agar made with Page's saline (PAS) and seeded with *E. coli* kept at 30°C for 7 days. Cultures were examined using inverted microscope for presence of FLA and subcultured every 10 to 14 days by inverting a slice on a new agar plate as described previously [11]. The morphology of the trophozoite and cysts (non-stained and Giemsa's Stained) were identified using light microscope and inverted microscope according

to Smirnov and Goodkov [12]. Molecular characterization of *A. spelaea* by polymerase chain reaction (PCR) of the 18S ribosomal RNA and sequencing was performed using primers described previously [11,13-15].

### Bacterial cultures and antibiotic susceptibility tests

Bacterial strains used in this study were isolated from cases with lower respiratory tract and urinary tract infections. The tested strains were Methicillin-resistant *Staph. aureus* (MRSA), *Enterobacteriaceae* [*E. coli* 1, *Kl. pneumoniae*, *Enterobacter aerogenes* (*E. aerogenes*), *Citrobacter cloaca* (*C. cloacae*), *Proteus mirabilis* (*Pr mirabilis*), *Raoultella terrigena* (*R. terrigena*), *Raoultella ornitholytica* (*R. ornitholytica*)], and other Gram negative bacteria; *Aeromonas hydrophila* (*A. hydrophila*) and *Ps. aeruginosa*. Gram negative bacteria were identified up to the species level by API 20E system (BioMérieux, France) while detection of MRSA based on colonial morphology, Gram staining, and standard biochemical reactions according to the Bergey's Manual of Systematic Bacteriology [16]. Our Gram negative bacteria were all sensitive to imipenem and meropenem (Oxoid, England) while MRSA were sensitive to linezolid. Susceptibility tests were performed using the disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. Bacteria were cultured in brain heart infusion broth overnight at 37°C without shaking prior to experimentation and were used at the stationary growth phase.

### Co-culture experiments

*A. spelaea* was grown without shaking in 15 ml PYG medium (0.75 % w/v, proteose peptone; 0.75 % w/v, yeast extract; 1.5% w/v, glucose) in tissue culture flasks at 30°C, as described previously [18]. The medium being refreshed 17-20h prior to all experimentation. This resulted in more than 95% of the amoebae in the trophozoite form. Supernatants from *A. spelaea* cultures were centrifuged at 2800×g for 30 min. New pellets were resuspended in PYG before being processed to recover cultivable bacteria. Co-culture experiments were performed with a slight modification to a previous method [19]. *A. spelaea* was incubated in a concentration of 1×10<sup>6</sup> amoebae/mL PYG medium/well in 24 well plates until confluent. The cells were washed once with PAS. Next, Different bacterial strains were added in a concentration of 1×10<sup>7</sup> colony forming units (c.f.u)/well/mL PYG giving a multiplicity

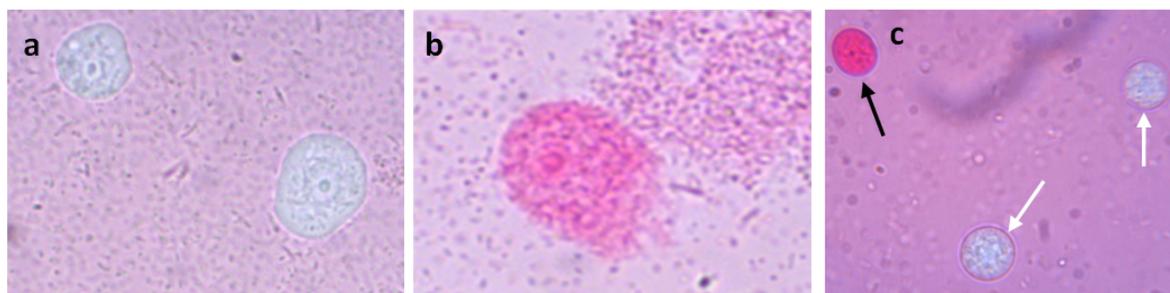
of infection (MOI) of 10. PH was adjusted to 7.2 and the plates were incubated for 1 h at 30°C to permit bacterial uptake. To kill residual extracellular bacteria, medium was replaced with PYG supplemented with 16 mg/L imipenem, a concentration greater than the highest MIC observed for all Gram-negative strains and linezolid in concentration of 1.5 µg/ml was used for MRSA. Plates were incubated for 1 h at 30°C. Antibiotics were removed by washing three-times with PAS. At the final wash, the discarded supernatants were also plated onto nutrient agar plates to determine bacterial presence and 100 µl fresh PAS were added to wells. The microtiter plates were incubated again at 30°C (designated time 0 h). The wells were processed at 0 h, 8 h, 24 h, 48 h, and 72 h. To count the extracellular bacteria, PAS was carefully aspirated and sampled. To determine the number of intracellular bacteria, 100 µl of fresh PAS were added to the wells and the surface of each well bottom was scraped to remove adherent cells. Finally, amoebae were lysed by adding sodium dodecyl sulphate (SDS) in 0.5% final concentration to each well for 20 min, and the number of bacteria was enumerated by plating on nutrient agar plates [20]. *A. spelaea* viability was monitored using the eosin dye exclusion assay using light and inverted microscope according to Wang and Ahearn [21].

### Statistical analysis

The SPSS program version 20.0 was used for the statistical analysis of data. Data were presented as number and percentage, or mean ± SD as appropriate. ANOVA test was used before data were transformed (Log<sub>10</sub>), p value <0.05 was considered statistically significant.

### Results and Discussion

As newly discovered in 2009 in the karst caves of Slovenia, data on *A. spelaea* (Figure 1) and their interactions with bacteria are lacking. For identification and characterization of *A. spelaea*, we depended upon 18S rRNA gene sequencing that revealed our strain to be *A. spelaea* strain SK1. Being a member of FLA, the role of *A. spelaea* in survival and multiplication of pathogenic bacteria should be considered. So, we aimed in this study to investigate survival and/or multiplication of the tested bacteria inside amoeba cells. Our bacterial strains were isolated from cases of urinary and respiratory tract infections and the majority of them are known to be natural contaminants of the water and food systems.



**Figure 1:** Representative Micrograph of *Allovalhikampia spelaea* trophozoite (a) living, (b) dead stained with eosin stain, and (c) cyst with perinuclear ring (white arrow: living; black arrow: dead stained with eosin stain) (oil immersion x100 objective lens).

## Co-culture of *A. spelaea* and bacteria

In our co-culture system, we followed the survival of bacteria inside *A. spelaea* at 30°C. Bacteria alone were incubated with various concentrations of SDS, and it was found that 0.5% SDS had no effect on bacterial viability.

## Bacterial survival and multiplication inside *A. spelaea*

Figure 2 shows the intracellular bacterial counts in the presence of *A. spelaea*. With the exception of *Pr. mirabilis* that showed low counts throughout the experiment, our results showed that pathogenic bacteria survived and multiplied within the amoeba host. In particular, *Ps. aeruginosa* and *A. hydrophila* where the cell counts exceeded 5 log cycles at time 0h with highly significant differences versus other bacterial strains ( $P < 0.001$  for both) and increased significantly in number to reach  $>8$  and  $>7$  log cycle at time 24h of co-incubation, respectively in comparison to other isolates ( $p < 0.001$  for both). The high multiplication rate of *A. hydrophila* inside FLA in our study has been demonstrated before [22,23]. *Ps. aeruginosa* is an environmental Gram-negative bacillus that colonizes hospital water systems and causes nosocomial infections [24]. Additionally, *Ps. aeruginosa*-amoeba co-infections have been described in keratitis patients [25]. Our *A. spelaea* was isolated from a patient with keratitis, so the interactions between *A. spelaea* and *Ps. aeruginosa* are of special concern in those patients. The isolation of FLA naturally infected with *Ps. aeruginosa* [5,26-29] demonstrated the role of amoebae and their cysts as vectors for these intracellular bacteria [30]. Our results showed the intracellular multiplication of *Ps. aeruginosa* as supported previously [20,31,32]. In contrast to our findings, another report [33] supported the extracellular multiplication mode of *Ps. aeruginosa* with better growth outside than inside eukaryotic cells. In our work, MRSA intracellular counts were  $>4$  log cycle at time 0h that significantly differed from other bacteria ( $p < 0.001$ ) and increased by  $>1$  log cycle at time 24 h of co-incubation that differed from *Ps. aeruginosa* and *A. hydrophila* ( $p < 0.001$ ) but showed no significant differences ( $p > 0.05$ ) against the *Enterobacteriaceae* group. Huws et al. [34] demonstrated the proliferation of epidemic strains of MRSA inside FLA. Our *Enterobacteriaceae* group started with  $\sim 1$  log cycle growth at time 0 h that increased up to 4 log cycle for *C. cloacae* and *E. coli* 1, and  $>2$  log cycle for *Kl. pneumoniae*, *E. aerogenes*, *R. terrigena*, and *R. ornitholytica*. Previous data demonstrated that *Enterobacteriaceae* can survive and multiply within amoeba host [23, 35-39] which is consistent with our findings. At 48h co-incubation, the intracellular counts for all bacteria in our study decreased onwards. The decrease after 2-3 days of incubation, has been reported previously [19,40] which may be attributed to the limited intracellular life of bacteria, or the presence of viable not cultivable cells [41]. In our work, the intracellular viable count of *Pr. mirabilis* showed significantly the highest levels at time 0h ( $p=0.045$ , 0.021, 0.01, and 0.005 versus time 8h, 24h, 48h, and 72h, respectively). As the comparative counts of *Pr. mirabilis* in the presence of amoebae were lower, then, this was evidence of predation by *A. spelaea*.

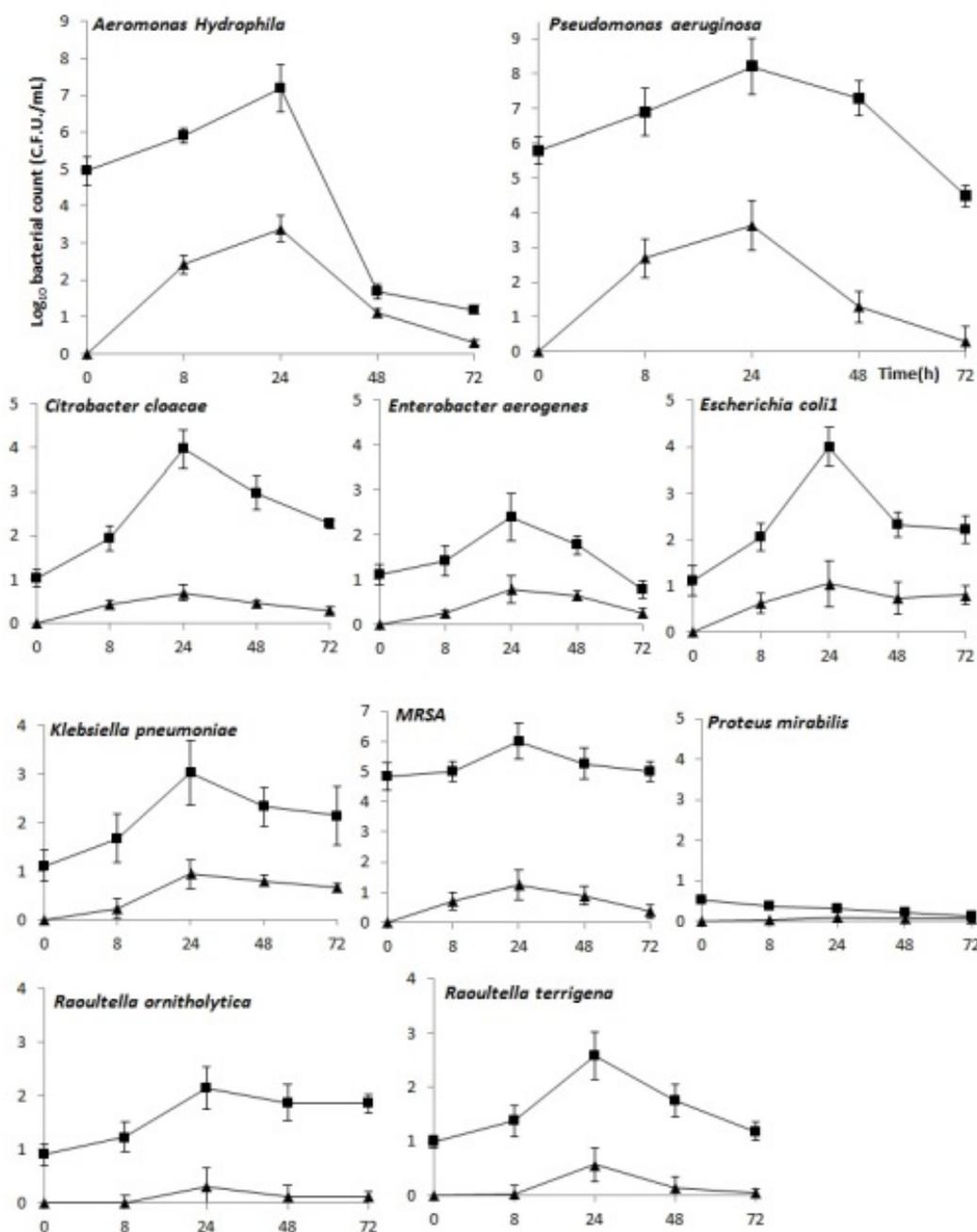
## Extracellular bacterial counts

The extracellular bacterial counts in our work, as shown in figure 2, occurred as a result of intracellular multiplication and subsequent release of vesicles containing live bacteria as reported [8,42]. They were characterized by gradual increase from time 0h to 8h co-incubation ( $p \leq 0.001$ ) until significantly maximum extracellular counts at time 24h co-incubation ( $p < 0.001$ ). Then bacterial counts decreased

significantly to very low or undetected levels at 72h co-incubation ( $p > 0.05$  versus time 0h). Significant maximum extracellular viable counts at 24h co-incubation were detected for bacteria that showed the highest growth intracellularly; *Ps. aeruginosa* and *A. hydrophila* ( $p < 0.001$  for both versus other bacteria tested). *Pr. mirabilis* extracellular counts reached very low levels with insignificant differences between time 8h co-incubation until the end of experiment ( $p > 0.05$ ). There are varying accounts in the literature on the types of interaction between bacteria and FLA with different types of endocytosis and intracellular behaviors including intracellular lysis of bacteria, followed by its digestion by amoebae or intracellular survival and multiplication of bacteria leading to amoebal lysis which may be dependent on virulence of bacteria [43]. As shown in our work, the non-invasive bacteria are taken up by amoeba as a food source (in our case *Pr. mirabilis*), while the invasive bacteria are able to reside and multiply inside amoebae without being killed (in our case the other bacteria) [35], where they use amoebae as a transmission vehicle and develop resistance against other phagocytic cells in host tissues [4,44]. Nevertheless, the precise mechanisms of intracellular survival of our tested bacteria remain unclear and have to be determined. Although a previous report has demonstrated the ability of bacteria to inhibit the fusion of lysosomes with phagosomes as a critical step in the intracellular survival inside *Acanthamoeba castellanii* (*A. castellanii*) [45], so, our bacterial strains may use similar mechanisms to evade the amoeba-cell defenses. Other reports [34,40,46] that demonstrated the multiplication of *Staph. aureus* within FLA, suggested that *Staph. aureus* possess no specific mechanism for evading digestion but have post-ingestion defenses such as a thicker cell wall, or an antioxidant yellow carotenoid. Pickup et al. [46] stated that *Kl. pneumoniae* has the ability to resist phagocytosis and digestion as a result of polysaccharide capsule. The ability of many bacterial pathogens to survive intracellularly in *A. spelaea* may be a key step in the evolution of those bacteria to produce human and animal infections.

## The survival rate of *A. spelaea* in the presence of bacteria

Number of viable *A. spelaea* in absence of bacteria increased from  $1 \times 10^6$  cell/mL (100%) on time 0h to  $\sim 0.96 \times 10^7$  cells/mL (110%) and  $1.88 \times 10^7$  (119%) cells/mL on 24h and 48h, respectively, and then survived to  $\sim 0.8 \times 10^6$  cell/mL (82%) at 72h (Figure 3). Growth of cocultivated *A. spelaea*, except with *Pr. mirabilis*, was inhibited with varying degrees. The statistical analysis showed highly significant differences in survival rates of alone-cultivated compared to the cocultivated *A. spelaea* ( $p < 0.001$  for 24h and 48h, and  $p < 0.002$  for 72h except for *R. ornitholytica* and *E. aerogenes* where  $p=0.097$  for both). *A. spelaea* growth at 24h co-incubation was highly significantly affected by *Ps. aeruginosa* and *A. hydrophila* where  $p$  were  $< 0.008$  and  $< 0.01$  versus other bacterial strains, respectively. In this context, it is well known that the invasive property of *Ps. aeruginosa* target the amoeba with their toxins that cause cell lysis [33,47]. *A. spelaea* growth was significantly enhanced by *Pr. mirabilis* ( $p < 0.002$ ,  $< 0.003$ , and  $< 0.003$  for 24h, 48h, and 72h co-incubation versus other bacterial strains, respectively). The decrease in survival rates of *A. spelaea* when co-incubated with our bacterial strains suggests that amoebal lysis occurred as a result of bacterial multiplication. Nevertheless, bacterial growth did not result in total killing of the amoebal cells that survived until end of the experiment at 72 h, which suggests the adaptation of our bacteria to the intracellular environment without causing total protozoal lysis as reported previously [19]. Our results are in accordance with previously published data [5,38,48-50] that detected decreased amoebal survival in presence of pathogenic bacteria.



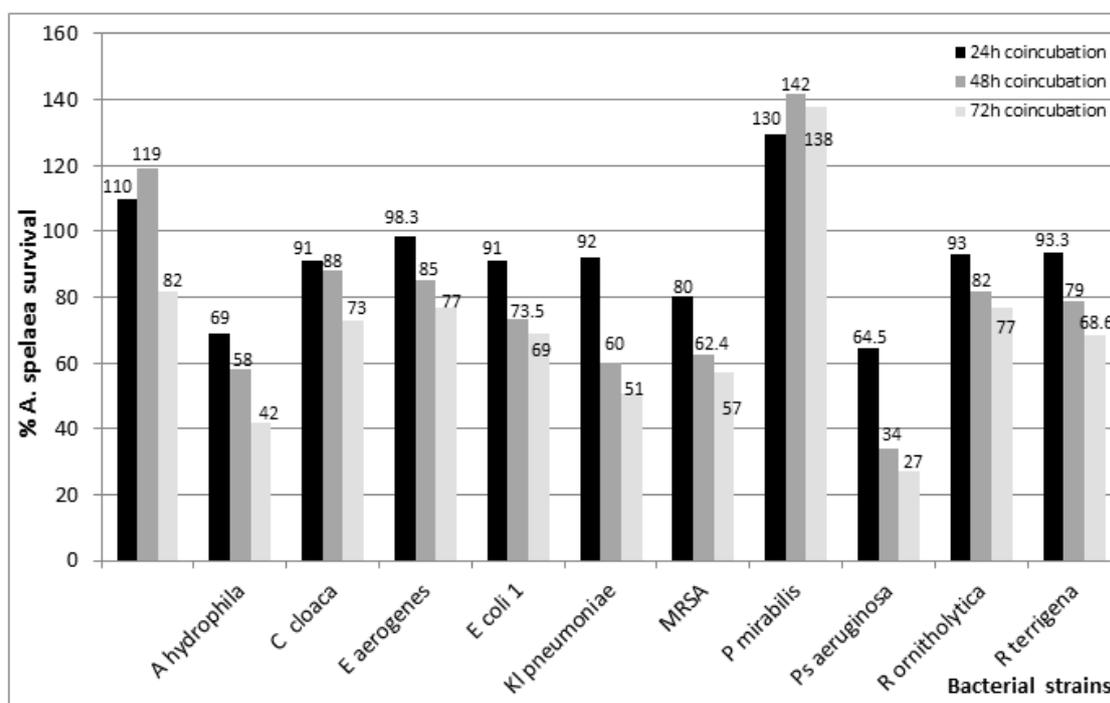
**Figure 2:** Extracellular (▲) and intracellular (■) bacterial counts following co-incubation for 72h with *Allovalhkampfia spelaea* (*A. spelaea*). Data are SE (bars) of the mean for three replicate experiments. The values of some error bars were too small to be presented.

The predation/survival/intracellular replication data for our bacteria differed in some instances from some work on bacteria and FLA interactions already published [6,51] that revealed a dose-dependent proliferative response of FLA when co-incubated with bacteria like *E. coli*, *Staph. aureus*, *C. cloacae*, and *Ps. aeruginosa*. We attribute this discrepancy between our results and previous data to the use of *A. spelaea* isolate that has not been investigated before, different bacterial

strains used, different MOI, or different co-culture conditions as yet reported [19]. The predatory activity of FLA is known to be influenced by several factors including the type and amount of surrounding bacteria [52,53]. As obvious in our findings, *A. spelaea* may act as a bacterial predator, or as a reservoir for bacteria, with environmental and clinical implications. Our results although may not reflect all possible modality of interactions as a single amoebal host have been

employed, demonstrated that many pathogenic bacteria are able to interact with *A. spelaea*, even species which were not expected to have an intracellular life cycle. *A. spelaea* effect on health ecosystem has two problems. First, *A. spelaea* serve as reservoirs for pathogenic bacteria. Second, *A. spelaea* species can themselves cause disease in humans or animals. In the environment, the interactions of bacteria and *A.*

*spelaea* are expected to be much more complex than reported here, as different bacterial prey are present in different niches that can be colonized by competing bacterial and protozoan predators. Deciphering the mechanisms of bacteria-protzoa interaction will assist in a better understanding of *A. spelaea* and bacterial lifestyle.



**Figure 3:** Viability of *A. spelaea* (106 cells/ml) following 72h incubation with different bacterial strains (107 CFU/ml). Results illustrate percentage survival relative to control (absence of bacteria) amoebal counts. Abbreviations; *A. hydrophila*: *Aeromonas hydrophila*, *Ps. aeruginosa*: *Pseudomonas aeruginosa*, *C. cloaca*: *Citrobacter cloacae*, *E. aerogenes*: *Enterobacter aerogenes*, *E. coli 1*: *Escherichia coli 1*, *Kl. pneumoniae*: *Klebsiella pneumoniae*, MRSA: Methicillin-resistant *Staph. aureus*, *P. mirabilis*: *Proteus mirabilis*, *R. ornitholytica*: *Raoultella ornitholytica*, *R. terrigena*: *Raoultella terrigena*. Mean value of three replicate experiments are shown. The values of error bars were too small to be presented.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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