Utjecaj metilamina i infektivnih doza na ishod infekcije stanica Dictyostelium discoideum i Acanthamoeba catellanii s Francisella novicida

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Graduate University study

Biotechnology in medicine

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The influence of methylamine and infectious doses on the results of infection of *Dictyostelium discoideum* and *Acanthamoeba castellanii* with *Francisella novicida*

Graduate thesis

Rijeka, 2018.

SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

Biotehnologija u medicini

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Diplomski rad

Rijeka, 2018. godina

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Graduate thesis is approved at the day _____14.09.2018_____

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Thesis have 62 pages, 13 figures, 0 tables and 61 literary allegations.

Acknowledgement

I would first like to thank my thesis advisor prof. dr. sc. Marina Šantić of the Faculty of Medicine at the University of Rijeka who enabled me to work in the Laboratory for Microbiology and Parasitology. The door of Prof. Šantić office was always open whenever I had a question about my research or writing or even ran into a trouble spot. She consistently allowed this paper to be my own work, but steered mi in the right direction whenever she thought I needed it. The research has been done within the framework of the Croatian Science Foundation HRZZ- IP-2016-06-9003 and University Grants 13.06.1.1.11.

I would also like to thank the members of the laboratory: ma. sanit. ing. Valentina Marečić, dipl. san. ing. Mateja Ožanić and dr. vet. med. Mirna Mihelčić, who have always been willing to help and give me valuable advice. It has been an honor and pleasure working with you.

I would like to express my deep love and gratitude to my family members, to dedicate my life for their constant support and understanding throughout my years of study and through process of researching and writing this thesis. This accomplishment would not have been possible without them.

Finally, I must acknowledge to my friends and boyfriend for providing me with unfailing support and continuous encouragement whenever was needed. You made my student life a memorable time.

Thank you!

Summary

Francisella tularensis is a gram-negative facultative intracellular bacterial pathogen that in humans and animals causes the fulminating zoonotic disease tularemia. Free-living amoeba, such as *A. castellanii* and *D. discoideum*, serve as environmental hosts of various intracellular pathogens. Epidemiology of *F. tularensis* is commonly associated with water-borne transmission in some parts of the world, including mosquitoes and amoebae as the host reservoir of bacteria in aquatic environment resources. *In vitro* studies demonstrated intracellular replication of *F. tularensis* within *D. discoideum* and *A. castellanii* cells. It has been established that crucial step in *Francisella* infection is entering the target cells by receptor mediated phagocytosis. This process has been shown to be meaningful in macrophages, arthropod-derived cells and amoeba cells. We used a water-borne amoeba *A. castellanii* and *D. discoideum* as surrogate macrophages as amoeba model systems to study influence of metabolic inhibitor methylamine on results of intracellular and extracellular life of *F. novicida*. For the first time we showed that methylamine has inhibitory effect on *F. novicida* entry and replication in *D. discoideum* and *A. castellanii*. The influence of different levels of infection was also examined and showed a dose-dependent effect on intracellular and extracellular infection. Moreover, methylamine was analyzed to observe whether in concentration of 100 mM induce cytopathogenicity in amoebae mentioned before. We conclude that *F. novicida* cannot entry and replicates in *D. discoideum* and *A. castellanii* after treated with 100 mM methylamine and that this concentration of inhibitor is not toxic to amoeba itself and can be used to inhibit receptor-mediated phagocytosis during *Francisella* infection.

Key words: *Francisella novicida*, *Acanthamoeba castellanii*, *Dictyostelium discoideum*, methylamine

Sažetak

Francisella tularensis je gram-negativni fakultativno unutarstanični bakterijski pathogen koji uzrokuje tularemiju u ljudi i životinja. Amebe kao što su *A. castellanii* i *D. discoideum* služe kao stanice domaćina za različite unutarstanične patogene u prirodi. U određenim dijelovima svijeta epidemiologija *F. tularensis* je usko povezana s prijenosom bolesti putem vode, što uključuje komarce i amebe kao glavni izvor bakterije u vodenom okolišu. *In vitro* studije su pokazale unutarstaničnu replikaciju *F. novicida* u stanicama *D. discoideum* i *A. castellanii*. Utvrđeno je da ključan korak u infekciji je ulazak bakterije u ciljanu stanicu mehanizmom receptorom posredovane fagocizote. Pokazalo se da je taj proces od velike značajnosti u makrofazima, stanicama člankonožaca i amebama. Amebe *A. castellanii* i *D. discoideum*, kao surogat makrofaga, koristili smo kao modele za istraživanje utjecaja metaboličkog inhibitora metilamina na razmnožavanje *F. novicida*. Prvi smo pokazali da metilamin ima inhibitorni učinak na ulazak i replikaciju *F. novicida* u stanice *D. discoideum* i *A. castellanii*. Također, istraživan je utjecaj infektivne doze bakterija na unutarsničnu i izvastaničnu replikaciju *Francisella*, kao i utjecaj metilamina u koncentraciji 100 mM na citotoksičnost u ameba. Naši rezultati pokazuju da *F. novicida* ne ulazi i replicira se u stanicama *D. discoideum* i *A. castellanii* koje su prethodno tretirane sa 100 mM metialminom. Navedena koncentracija nije toksična za same stanice te se može korisiti za sprečavanje receptorom posredovane fagocitoze tijekom *Francisella* infekcije.

Key words: *Francisella novicida*, *Acanthamoeba castellanii*, *Dictyostelium discoideum*, metilamin

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1. Introduction

1.1. History of genus *Francisella*

Since the beginning of the 20th century *Francisella tularensis* has been recognized as a human pathogen. Tularemia was first described in 1911 by McCoy as a plague-like disease of California ground squirrels [1]. Some previous reports of disease date back from 1890s from Norway where cases of disease were called lemming fever [2], and cases of Yato-Byo (hare disease) from Japan as early as 1818 [3]. The first verified clinical case occurred in Ohio in 1914 [4].

F. tularensis is a gram-negative, facultative intracellular bacterium that causes the fulminating zoonotic disease in humans and animals. It is small, asporogenous, pleomorphic and immobile bacteria. After 2 to 4 days of cultivation at 37 °C, *Francisella* colonies appear as smooth, white and moist in 2 mm of diameter on chocolate agar. Optimum pH for growth medium is between 6.8 and 7.3 [5]. This bacterium is auxotrophic for amino acid Cysteine [6] and grows on substrates enriched with animal proteins. Due to its high infectivity it is needed only 10 bacteria to cause a disease [5].

For many years, *F. tularensis* has been considered as a potential biological weapon, and in 1930s and 1940s, Japanese germ-warfare units examined the possibility of intentionally causing tularemia in humans. There were also offensive programs in both the United States and former Soviet Union to develop weapons containing *F. tularensis*. Ken Alibek, a former Soviet Union scientist involved in the bioweapons program, claimed that such offensive programs resulted in production of *Francisella* strains engineered to be resistant to vaccines and antibiotics [7].

The high virulence and high morbidity justify the inclusion of *F. tularensis* as an agent as the most likely biological treat agents. According to Centers for Disease Control and Prevention (CDC), it is designated as a category A Select Agent [8].

1.2. The taxonomy and classification of *Francisella tularensis*

In year 1919, Edward Francis first suggested original name of *F. tularensis*, *Bacterium tularensis* named after Tulare County, California [9]. During 1920s, bacterium was designated as *Pasteurella tularensis* until 1966 when it was determined that the genus was not intently related to *Pasteurella* [10]. Later, 16S rDNA sequence analysis disclosed that *Francisella* belonged to γ-subclass of *Proteobacteria* [11]. Current taxonomy affirm that *Francisella* still is the only recognized genus within family *Francisellaceae* [12].

1.2.1. Species of genus *Francisella tularensis*

Five species of genus *Francisella* has been recognized: *F. tularensis*, *F. hispaniensis*, *F. noatunensis*, *F. novicida* and *F. philomiragia* [13]. The *F. tularensis* species causes an incapacitating disease in humans, although *F. hispaniensis*, *F. novicida* and *F. philomiragia* are less pathogenic and predominantly pernicious for humans with a compromised immune system [12,13]. Three closely related subspecies of *F. tularensis* have been identified: *tularensis* (Type A), *holarctica* (Type B) and *mediasiatica*.

F. tularensis subsp. *tularensis* (Type A) endemic in North America is a cause of approximately all fatalities due to tularemia, which mortality rates are up to 30%.

F. tularensis subsp. *holarctica* (Type B) found throughout the Northern hemisphere causes a milder disease with rarely occurred fatalities.

F. tularensis subsp. *mediasiatica* found in central Asia display level of virulence related to strains of subspecies *holarctica* with rarely cause of human infections [13].

1.2.1.1. *F. novicida*

In 1951, a bacterium related to *F. tularensis* was isolated from a water sample collected from Ogden Bay, Utah. Although it was originally classified in genus Pasteurella [14], in 1959 it was transferred to genus *Francisella* and originally designates as *F. novicida*. Based on biochemical characteristics and DNA relatedness, in 1989 *F. novicida* was suggested to constitute a subspecies of *F. tularensis* [15]. Today, despite genetic identity, genomic, ecological, virulence, pathogenic, and clinical differences between *F. tularensis* and *F. novicida* clearly affirm maintaining *F. tularensis* and *F. novicida* as separate species [16,17].

F. novicida type strain U112 is not virulent in immunocompetent humans but is highly virulent in laboratory mice. Only several bacteria cause tularemia and death, similar to subsp. *tularensis* and is likely an attractive model to study pathogenesis of *F. tularensis*. Only 12 cases of *F. novicida*-like human infection has been documented in immunocompromised patients [13,17]. Association with water-borne transmission indicates mosquitos and amebae as the potential aquatic reservoir of the bacteria [18] and demonstrate that *F. novicida* resides in environmental niche by propagating via mechanism that does not involve arthropod or mammalian hosts [17].

1.3. Epidemiology of tularemia

Tularemia is transmitted to humans by direct contact with infected animals, by arthropod bites (mosquitoes, ticks, flies), by contact with infected tissues and animal fluids, by ingestion of contaminated water and food and by inhalation of contaminated aerosols. There is no documented evidence for human-to-human transmission of tularemia. *F. tularensis* has been isolated from a number of different wildlife species which may act as vectors for transmission to humans [19]. *F. tularensis* subsp. *tularensis* is most commonly find in rabbits, rodents, hares and ticks, whereas *F. tularensis* subsp. *holarctica* is often find in ponds, streams, lakes and rivers. According to demonstration of *F. tularensis* in amoebal cysts it has also

been proposed that protozoa play important role as hosts in aquatic life cycles [19,20].

1.3.1. Geographical distribution

Tularemia has been reported in many countries since its first original description in the United States. Endemic areas have existed for a long time, especially in countries of the northern hemisphere [19] such as Nordic countries and North America, as well as in Russian Federation and Eastern Europe. A large outbreaks have also occurred in Germany [21], Czech Republic, Turkey, Finland, Sweden and France. In recent decades the rise in a number of the cases were remarkable in the Balkan countries, particularly in the period 2001-2010 with the highest annual incidence in Kosovo because of the negative impact of the war. Croatia followed with rates of 0.15 per 100,000 people. There is no documented report of tularemia in the Antarctica, South America, Africa, Iceland and United Kingdom [22]. Only few tularemia related cases and isolate of *F. novicida* has been found in Australia [19,22]. Climate change, natural disasters, wars, human travels and animal movements have been increasing causes of tularemia among the world. One study estimated that in coming decades global warming would cause an increase in tularemia cases [22].

1.3.2. Vectors

1.3.2.1. Arthropods

Ticks are recognized as the most important vectors in the USA, where at least 13 species from genera: *Ixodes*, *Haemaphysalis*, *Dermacentor* and *Amblyomma* have been infected with *F. tularensis*. Flies (*Chrysops* spp.) are prevalent vectors in California, Utah and Nevada [23]. In the former Soviet Union, bacteria are mostly transmitted by mosquitoes (*Anopheles* spp., *Aedes* spp., *Culex* spp.) and ticks (*Ixodes* spp.). In central European countries, such as Austria and the Czech Republic ticks *Ixodes ricinus* and *Dermacentor reiculatus* are considered as important vectors for the disease. Mosquitoes of the species *A. cinereus* are discussed to have a role as mechanical vectors for transmission of *F. tularensis* to humans [19].

Flies of the family *Tabanidae* which includes true deer flies (*Chrysops* spp.) and horse flies (*Tabanus* spp. and *Chrysozoma* spp.) may serve as vector for disease in former Soviet Union and the USA [23].

1.3.2.2. Mammals

First tularemia outbreaks have been characterized in hares, prairie dogs and mink. Numerous terrestrial and aquatic mammals such as muskrats, beavers, ground squirrels, rabbits, hares and, rodents such as water voles and meadow voles are thought to spread *F. tularensis* in the environment. The most common vectors for the spread of disease to humans are the common vole (*Microtus arvalis*), the water vole (*Arvicola terestris*), the red-backed vole (*Clethrionomys* spp.) and the domestic mouse (*Mus musculus*) [19]. In central Europe, a non-rodent species such as hares and rabbits are suggested to be primary reservoir of tularemia and may spread tularemia by infecting hunters or trappers [20] .

1.3.2.3. Environment

F. tularensis is considered as highly resistant to external environmental conditions. Bacteria can survive months and years at low temperatures in water, soil, swamps, mud, barns, frozen meats and animal wastes [5,22]. If stored at -70 °C or lyophilized, virulence can be retained for years. *F. tularensis* can also survive heating at the temperatures of 42-65 °C, in food and drinking water. Some studies have shown that *F. novicida* exponentially propagates in cow, goat, soy and chocolate milk, which is corresponding to results that bacteria best replicates on the nutrient substrates that contains amino acid cysteine [5].

Previous studies have shown that *F. tularensis* can survive and proliferate in amoebae living in water, *Acanthamoeba castellanii*, as new a source of infection for animals and people [5,22].

1.3.3. Tularemia

Tularemia is more prevalent in certain groups of people. Groups with a higher risk of developing disease include laboratory workers,

veterinarians, foresters, hikers, hunters, people from rural areas and people in contact with animals and meat [22].

1.3.3.1. Clinical manifestations

Preliminary symptoms (flu-like symptoms) such as fever, chills, malaise, sore throat and headache are quite nonspecific and occur after an incubation period of 3-5 days [24]. The clinical manifestations of tularemia differ because of different route of infection. Humans can get infected by *F. tularensis* through the skin, mucous membrane, respiratory and gastrointestinal tract [20].

There are several clinical forms of disease including ulceroglandular, glandular, oculoglandular, oropharyngeal, respiratory and typhoidal [20,24]. In ulceroglandular form, skin or mucous membranes are often considered the route of infection. Normally, it results from arthropod bites, ticks and mosquito bites. A primary ulcer develops at the site of arthropod bite, followed by painful swelling of the lymph nodes. The term glandular tularemia pertains to a similar clinical presentation, but without primary skin lesion. In the most parts of the world, around 90% of cases are represented by glandular and ulceroglandular forms [5,20,24]. The oculoglandular is transmitted by patient's fingers followed by conjunctivitis and lymph node enlargement [5,24].

The oropharyngeal form of tularemia appears after the ingestion of contaminated water or food and presents with stomatitis, pharyngitis and with tonsillar involvement. The respiratory form of tularemia is often related to inhalation of aerosolized *F. tularensis*, or inhalation of dust containing remains from infected animals, which occurs during farming activities. Symptoms are often presented as a systemic illness with high fever. Although respiratory tularemia is the most severe form of disease, mortality rate in Europe is highly unusual [19,20, 24]. Typhoidal tularemia represents a clinical manifestation with severe systemic manifestations but without specific regional symptoms such as mucosal or cutaneous lesions or swollen lymph nodes [20,24].

1.4. *Francisella* virulence factors

During the past, there has been a dramatically increase in studies on the virulence of the highly infectious intracellular pathogen *F. tularensis*. The organism produces a capsule, an insert LPS, escapes the phagosome (*Francisella* pathogenicity island, FPI mediate phagosomal escape) to grow in cytosol of variety of host cell types [25].

1.4.1. *Francisella* pathogenicity island (FPI)

Francisella pathogenicity island was first reported in 2004 when it was described a large cluster of genes on an island of the *F. novicida* chromosome [26]. This 30-kb island encodes 18 genes, 14 of which are essential for growth in macrophages. All, except *pdpE* and *anmK* genes are required to produce virulence phenotype in *F. tularensis*. This island is also recognizable by regions of DNA with a relatively low GC content that differs from that of the rest of the chromosome. Due to some proteins encoded on the FPI that show homology to the core components of Type VI secretion system (T6SSs) of other Gram-negative bacteria, it was recommended that the FPI encoded a T6SS [27].

The FPI is present in single copy in *F. novicida* and duplicated in *F. tularensis* and *F. tularensis* subsp. *holarctica*. Because of single copy of the FPI and low virulence for humans, *F. novicida* is more tractable species for study and investigations of the FPI [27].

1.4.2. Capsule

Polysaccharide capsule are produced by bacteria to maintain the cells shape [28] and typically protect them from phagocytosis, complement-mediated lysis and immune recognition. Biochemical analyses showed that capsule was distinct from cell wall material, and composed of lipids (51%), amino acids (35%) and carbohydrates (21%). Majority of studies have indicated that *F. tularensis* produces a polysaccharide capsule identical to the O-antigen. It is well-known that pathogens like *F. tularensis* manipulate host cell entry and mechanisms involved in intracellular trafficking to limit

inflammation and promote intracellular survival. Capsule can also draw out protective immunity, as proved by successful commercial capsule- based vaccines against *S. pneumoniae*. In addition, capsule-like substances may vary among different *Francisella* strains and can depend on culture conditions and environmental stimuli [29].

1.4.3. Lipopolysaccharide (LPS)

For most Gram-negative bacteria, lipopolysaccharide (LPS) makes up the outer leaflet of the outer membrane. LPS is made up of three main components: lipid A, O-antigen and the core oligosaccharide. Of these, lipid A (endotoxin) has high immunostimulatory and virulence characteristics. Differences in macrophage activation and virulence appeared to be due to lipid A alternations. The lipid A component is conserved across all *Francisella* species and all have typically loci flanked by transposases with lower G-C content than the rest of *Francisella* genome. Taken together, specific LPS modifications provide virulence strategy of *F. tularensis*, resulting in high infectivity and serious disease. In future, vaccine development efforts may need to take into account incorporating anti-LPS responses [29].

1.4.4. Type IV pili

Type IV pili are important virulence factors for Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Neisseria meningitides* with important role in bacterial adhesion, aggregation, DNA uptake and twitching motility. The *Francisella* gene cluster found in those bacteria appear to contain 14 genes, including inner and outer membrane, ATPase and pilin subunit proteins. The inner or outer membrane protein deletions resulted in attenuation refer to requirement of many type IV pili assembly for *Francisella* virulence. In *F. novicida*, components of the type IV pili homologous genes have been described to be involved in type II-like protein secretion system [29].

1.5. Intracellular lifestyle of *F. tularensis* in mammalian, arthropod- derived and amoeba cells

1.5.1. Role of the T6SS in phagosomal escape and intracellular replication

As previously illustrated FPI genes encode T6SS which is required for phagosomal escape and intracytoplasmic replication of *F. tularensis* in macrophages and necessary for virulence in animals [27,30]. The existence of the T6SS secretion system is affirmed by finding that VgrG and IglI proteins are secreted by *F. novicida* into the cytosol of macrophages. It has been recently shown that lipoprotein IglE encoded by FPI, interacts with other FPI proteins and localizes to the outer membrane resulting in a channel formation. The T6SS assembles a phage tail-like injectosome to translocate effector proteins crosswise the host cell membrane and two bacterial membrane into the cytosol of the host cell. The most researched FPI protein, global regulator MglA and IglC are crucial for the capacity of *Francisella* to escape *Francisella*-containing phagosome (FCP) into the cytoplasm [30].

1.5.2. Intracellular life of *F. tularensis* in macrophages

F. tularensis invade and multiply within various cells, including neutrophils, alveolar epithelial cells, and hepatocytes, but macrophages are considered its primary target *in vivo* [27].

Francisella enters into macrophages by binding to surface receptors and looping phagocytosis through "lipid rafts" (cholesterol-rich membrane domains) with caveolin-1 [30]. Lipid-rafts associated components and cholesterol, as a key regulatory and structural element for the integrity of lipid-rafts, are incorporated into FCP membrane during entry and initial phase of intracellular infection of host cells [31]. Following uptake, *Francisella* reside within the FCP which matures to an early endosome state characterized by Rab-5 and early endosome antigen 1 (EEA1). The FCP matures into late endosome and therefore acquires late endosomal markers

Rab7, CD_{63}, mannose-6-phospathe, and lysosomal associated proteins (LAMP-1 and LAMP-2). Ultimately, the late endosome become acidified through the acquisition of the vATPase proton pump that imports hydrogen inside the vacuole. To avoid lysosome-mediated killing, bacteria escapes from the FCP to the cytosol (Figure 1A) [30,31].

Despite the fact that brief time spent in the FCP is dynamic step in the infection for *Francisella* by evading host antimicrobial defense (antimicrobial peptides and reactive oxygen species), recent study has brought controversy about *Francisella* vacuolar escape kinetics that vary from 15 min to 8 h after infection [30,31]. It appears that *Francisella* escape from phagosome is requisite to intracellular proliferation and is crucial in its intracellular life cycle [31]. Eventually, the bacterial escape from the host cell is followed by cell-to-cell transmission, or by inducing host cell death (pryoptosis, apoptosis) [32].

1.5.3. Intracellular life style of *F. tularensis* in arthropod-derived cells

Although little is known about the molecular interaction of *Francisella* with arthropod vectors, transmission of tularemia borne by vector has a very important role in pathogenesis. Recent studies have shown that *F. tularensis* induce infection and kills adult *Drosophila* files. The intracellular lifestyle of bacteria within human macrophages is similar to arthropod-derived cells. *F. tularensis* temporarily reside within acidified phagosome that consequently matures to a late endosomal stage, accompanied by bacterial escape into the cytosol [30,33].

1.5.4. Intravacuolar proliferation of *F. tularensis* within amoeba cells

Previous studies have shown that *Francisella* enters and multiply within *Acanthamoeba castellanii* [34], *Hartmannella vermioformis* [18] and *Dictyostelium discoideum* cells [32,34]. After being engulfed by trophozoites [34], *Francisella* resides and replicates within amoeba cells in non-acidifies, membrane-bound vacuoles known as *Francisella*-containing vacuoles (FCVs) [31,36]. In contrast to mammalian and arthropod-derived cells, bacteria do not escape from FCV for replication in water-borne amoebae (*A. castellanii* and *H. vermioformis*), which is the main difference between these host cells (Figure 1B) [30,32,36]. In addition, one study has shown that FCVs did not acquire the dye that concentrates in acidified vesicles during the *F. novicida* infection of amoebae [37]. It has also been indicated that lysosomal fusion within *A. castellanii* is blocked by *F. novicida*. Interestingly, the IglC (FPI-encoded protein) does not serve for phagosomal escape in the water-borne amoebae but is important growth factor inside bacteria's vacuoles [18,32].

Studies have also illustrated replication of *F. novicida* within *H. vermioformis* in non-acidified vacuoles [18]. The fact that bacteria was observed in cysts of *A. castellanii* suggests the importance of the protists in sustaining the *Francisella* life-cycle and its long-term survival in aquatic environment [32]. Even though, amoebae serve as a training ground for other bacterial pathogens, it has been shown that *F. novicida* infected amoebae does not enhance bacterial virulence in mice, which may result from difference in virulence strategies and life cycles in macrophages and amoebae [38].

On the other hand, there is a model to study *Francisella* infection using the amoeba *D. discoideum* as a macrophage surrogate. This study points out similarities between infection of *D. discoideum* with *F. noatunensis* and *Francisella* infection of mammalian macrophages, such as phagocytosis, phagosomal maturation with acidification of this

compartment, and consequently bacterial escape and replication into the cytosol, and autophagy, thereby presenting *Dictyostelium* as a useful model to examine the interactions between *Francisella* and its phagocytic host cells [32,35].

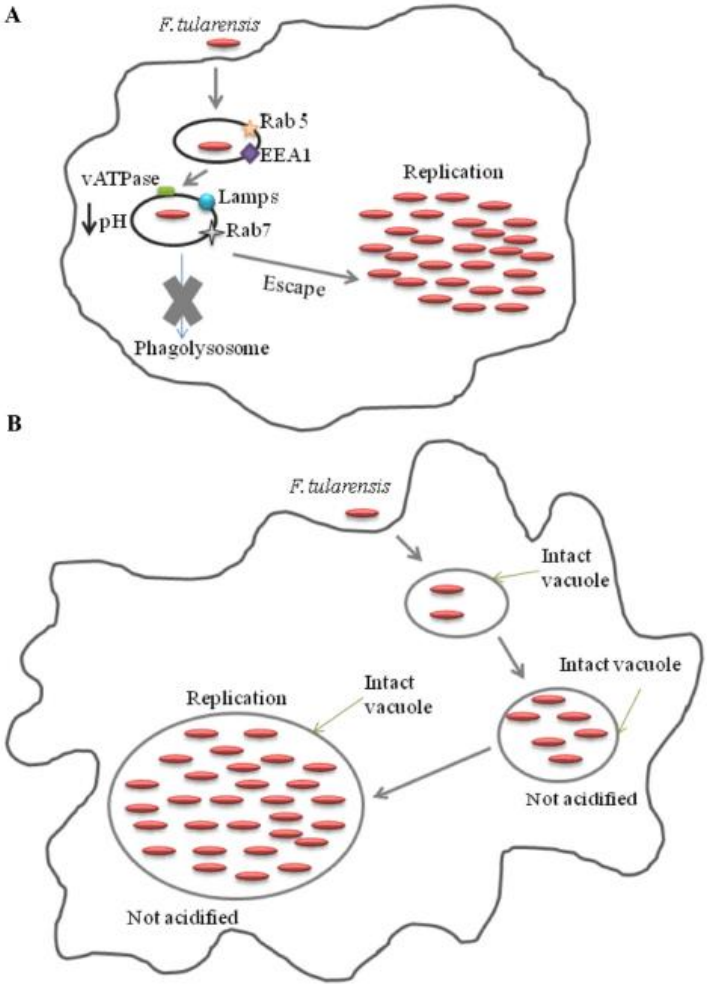

Figure 1. Intracellular proliferation of *Francisella* within macrophages (A) and amoebae cells (B). (**A**) After phagocytosis by macrophages, Francisella reside in the FCP which matures in early (EEA1 and Rab 5) and late (Lamos and Rab7) endosomal stage. The FCP become acidified by the vATPase proton pump with the result of bacterial escape into the cytosol and replication. (**B**) After phagocytosis by amoebae, bacteria reside and replicate in intact vacuole. "Retrieved from: M. Ozanic et al, "The Divergent Intracellular Lifestyle of Francisella tularensis in Evolutionarily Distinct Host Cells," PLoS Pathog., vol. 11, no. 12, pp. 1–8, 2015."

1.6. Model to study tularemia

1.6.1. *Acanthamoeba castellanii*

A. castellanii belongs to genus *Acanthamoeba* that was first established in 1930 by Volkonsky [39]. The genome of 45 Mb makes *A. castellanii* suitable for the wide range of studies. Free-living *Acanthamoeba* spp. are commonly found in natural sources such as soil, lakes, seawater and fresh water. They are also isolated from chlorinated water from swimming pool, jacuzzi tubs, hydrotherapy pools, tap water, contact lens, dental treatment units and heating and air conditioning units [36,39,40]. Several *Acanthamoeba* species have ability to cause a human disease, including *A. castellanii*. Free-living amoeba feed on fungi, bacteria and algae by phagocytosis. *Acanthamoeba* species can serve as environmental hosts for large number of intracellular pathogens, including *F. tularensis*, *Legionella pneumophila*, *Listeria monocytogenes*, *Chlamydia*, *Coxiella burnetii*, many *Mycobacterium* spp., and *Escherichia coli* [34,36,39].

1.6.1.1. Life cycle of *A. castellanii*

A. castellanii adopts a bi-phasic life cycle, an active vegetative trophozoite stage and dormant cyst stage [34,39,41], both with a diameter of 13-23 μm [42]. During trophozoite stage amoeba replicates by binary fission under optimal culturing conditions such as food supply, neutral pH and ~30 °C [42], has a doubling time ranged from 8 to 18 h (Figure 2). The amoeba are highly motile producing short, spine-like projections known as acanthopodia, and have ability to use chemotaxis to "hunt" [41]. When exposed to a harsh conditions, amoebae differentiate into a double-walled cyst form (Figure 2) [42].

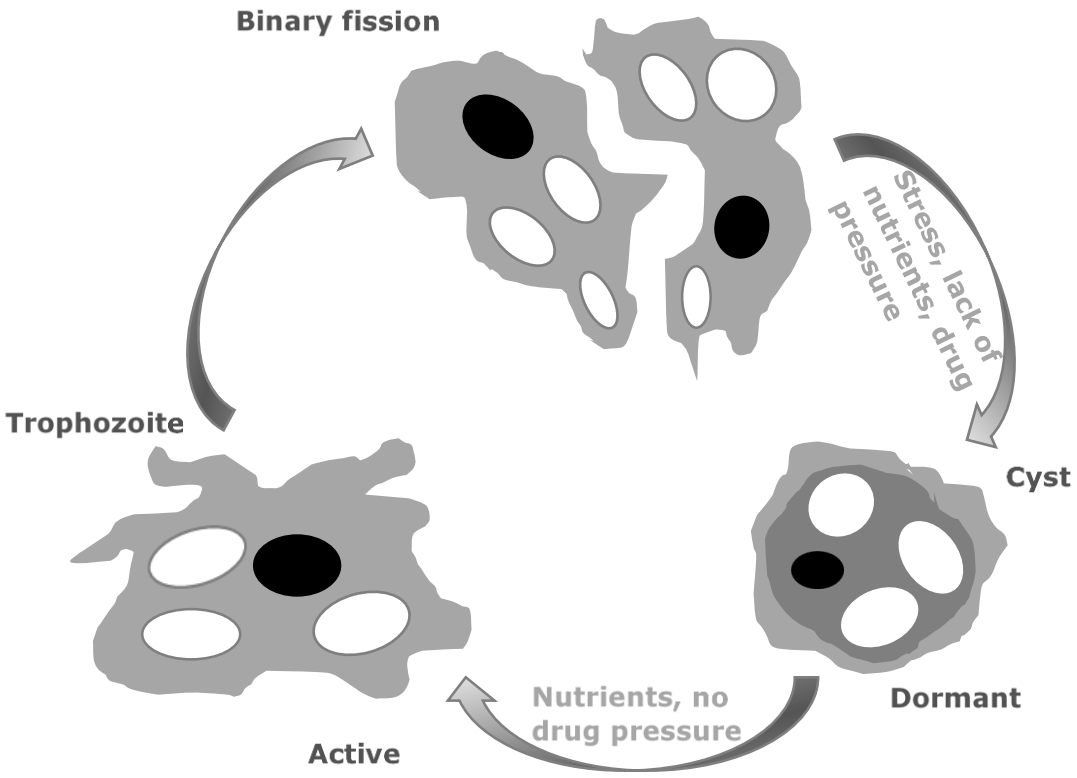

Figure 2. The life cycle of *A. castellanii*. Amoeba life cycle stages: active vegetative trophozoite and dormant cyst stage. In conditions with nutrients and without drug pressure, amoeba replicates during trophozoite stage by binary fission. After exposure to a stress conditions, drug pressure and the lack of nutrients, amoeba differentiate into double-walled cyst form.

1.6.1.2. Experimental approaches of *A. castellanii*

A. castellanii imply as a good training model eukaryote due to its rapid growth, as short as 8 h at 30 °C, in complex media, and its effective encystment on starvation. Amoebic motility and phagocytosis provide new insights in examining cellular structure and function aspects. Indication that bacterial pathogens such as *F. tularensis* can survive in *A. castellanii* cysts for at least 3 weeks after infection [43], gives special impact on future *Francisella* studies on protozoa. Moreover, amoebae are characterized by contractile vacuoles, wherein *F. tularensis* seems to replicate [34,41]. The bacteria are able to survive inside *A. castellanii* as endosymbionts and use of amoeba as vectors for their dissemination [34]. Despite polyploid genome complexity and no genetic manipulation tools, a water-borne *A. castellanii* remains possible model for environmental reservoir [32,41].

1.6.2. *Dictyostelium discoideum*

The social amoeba, *D. discoideum* is a member of a group *Dictyostelia* which belong to a supergroup Amoebozoa, one of the major divisions of eukaryotes [44,45]. The genome of 34 Mb, about 100 times smaller than human, makes *D. discoideum* as increasingly used model for the study of genes that are defective in human diseases [41]. There are ~120 species of *Dictyostelia* subdivided into four major groups, whereas *D. discoideum* belongs to group 4, species that have ability to secrete cyclic adenosine monophosphate (cAMP) to serve as a chemoattractant for aggregation [41,45]. Social amoebae are commonly found in a wide range of natural habitats such as from arctic to tropical soil, and from rainforest to desert with most prevalence in the leaf litter , which is the supplied source of their bacterial food [45]. Considering the fact that amoebae can be infected by *Francisella*, *Legionella*, *Mycobacteria*, *Pseudomonas* or *Klebsiella*, makes *Dictyostelium* as a suitable cellular model for intracellular replication of human pathogenic bacteria [32].

1.6.2.1. Life cycle of *D. discoideum*

The *D. discoideum* life cycle is fast at just 24 hours [45] with the narrow temperature tolerance of 20-25 °C [41]. The amoeba replicates by mitotic division of only one amoebae and is feeding on bacteria taken up by phagocytosis [41]. When food runs out *D. discoideum* utilize one of three survival strategies. The amoebae come together to assemble a fruiting structure, in which a part of cells are sacrificed to form a stalk and remainder differentiate into solid dormant spores. After single cell growth, amoebae cells stop proliferating and start chemotactic aggregation in response to cAMP signals (Figure 3A,B), thereby forming a multicellular mounds (Figure 3C). Movement of the cells underneath cause a toppling over of an emerging column of cells to form a slug (Figure 3D). After migration to the top layer of the soil, slug build the fruiting structure (Figure 1E,F). At the final stage of morphogenesis a formed fruiting body structure

composed of a tapered column of stalk cells with a spherical mass of spores carried aloft (Figure 1G) [45].

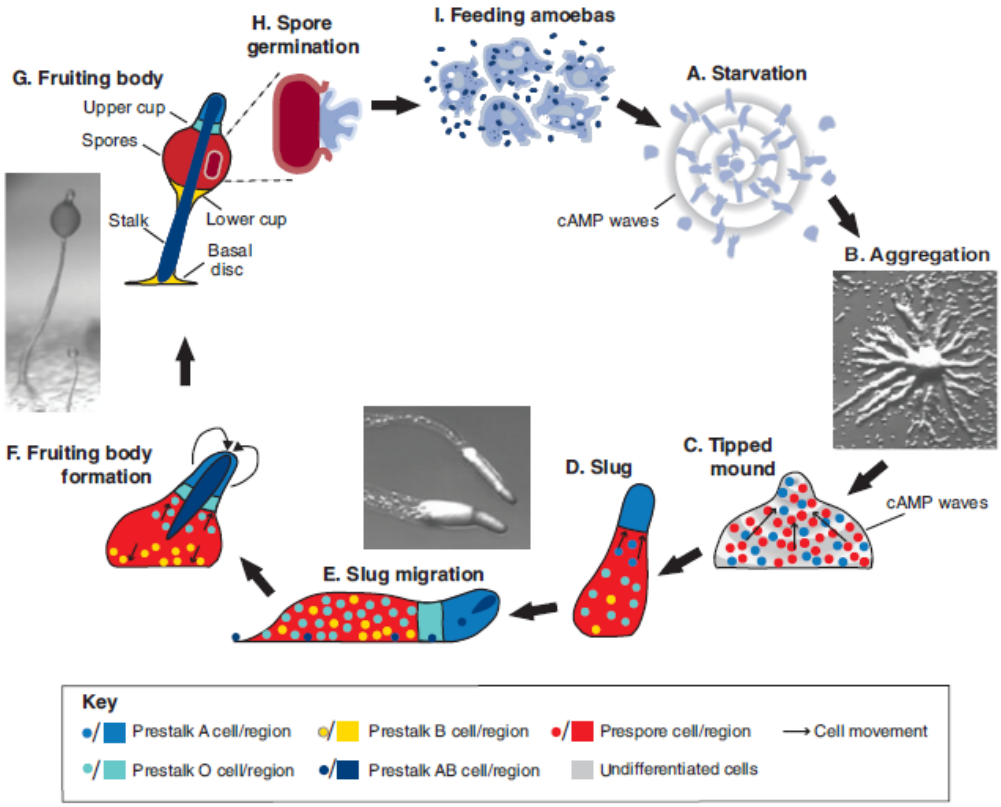

Figure 3. The life cycle of *D. discoideum.* (**A**) Starving amoebae secrete pulses of cAMP, that (**B**) cause the chemotactic aggregation of cells forming multicellular mounds. (**C**) Cell movement underneath the mound cause a formation of a tipped mound. (**D**) A mound then develops into a slug. (**E**) After a slug migration (**F**) it undergoes fruiting body formation and (**G**) differentiation of cells into spores, stalk cells and structures for their support. (**H**) After dispersion to nutrient-rich habitats, formed spores germinate and (**I**) continue proliferation as individual amoebae. "Retrieved from: P. Schaap, "Evolutionary crossroads in developmental biology: *Dictyostelium discoideum*.," Development, vol. 138, no. 3, pp. 387–96, Feb. 2011.")

1.6.2.2. Experimental approaches of *D. discoideum*

The *D. discoideum* can be easily and cheaply cultured in the laboratory on the bacterial lawns on agar [45], whereby some strains e.g. Ax2 can thrive in liquid medium containing peptone, glucose or in a defined mixture of vitamins and amino acids through micropinocytosis [41,45]. This facilitates the purification and isolation of cellular products for proteomics and biochemical analysis. Regarding amoeba life cycle its multicellular structures are transparent, allowing the visualization of gene expression and cell movement [45].

D. discoideum cells are easy to manipulate from biochemical and genetic level using integrating and extrachromosomal expression plasmids. Due to their haploid genome, knockout cell lines or alteration of a gene of interest can be easily generated by homologous recombination [32,41,45]. The complete genome sequence of *D. discoideum*, intrinsic biological features and molecular genetic tools of amoebae allow to research many fundamental cellular processes [41].

Owing to these experimental approaches with high similarities with human phagocytes, *D. discoideum* has become primary organism in which to study essential processes in cell biology, such as phagocytosis, phagosomal maturation, autophagy, actin cytoskeleton [32] chemotaxis, cytokinesis, vesicle trafficking and cell motility [45]. Furthermore, *Dictyostelium-Francisella* system is a great addition to present models of *Francisella* infection and facilitates detailed dissection of host-pathogen interactions [35].

1.7. Approaches for treatment of tularemia

1.7.1. Antibiotic treatment

The antibiotic classes recommended for tularemia treatment are the aminoglycosides, the tetracyclines and the fluoroquinolones. New data indicate that among fluoroquinolones, ciprofloxacin and levofloxacin are rapidly and strongly bactericidal in cell models and are extremely effective in curing *F. tularensis* infection in mice. In contrast, tetracycline doxycycline is considered less effective due to its bactericidal result only in cell models [46,47]. According to WHO guidelines [19], the aminoglycosides, especially streptomycin and gentamicin, are still considered the most trustworthy treatment of severe tularemia cases, including the typhoidal and pneumonic forms, due to its lower relapse rates and better outcomes of disease [46,47]. Moreover, the macrolides, such as azithromycin, could be an alternative treatment in patients infected with *F. tularensis* type A and B, which are erythromycin-susceptible strains, including pregnant woman and children [46].

1.7.2. Vaccines

In 1900s, several vaccines against tularemia were developed, including *F. tularensis* Live Vaccine Strain (LVS) [48]. Despite that the LVS vaccine retains toxicity in animals and humans, it is efficient in humans, thereby serving as gold standard for future vaccine efficacy studies [49].

Since 2001, several promising LVS vaccine candidates have been developed including LVS mutants, *F. tularensis* type B mutant, LVS Δ*capB* with overexpressed *F. tularensis* T6SS proteins, a LVS Δ*capB*-rLm/IglC heterologous prime-boost vaccine, and a SCHU S4 mutant with single deletion (Δ*purMCD*). These new vaccines have been better and greatest when administered by the intranasal. Nevertheless, application through another respiratory route has displayed higher significance, but raised additional safety issues. Regardless these outcomes, vaccines that are at least as efficacious as LVS, and safer, have been developed, and they are promising candidates for future advanced non-human primate model studies and human safety trials [49].

1.7.3. Cytoskeletal and metabolic inhibitors

As mentioned, crucial step for *Francisella* infection and replication in mammalian and amoeba cells is phagocytosis by macrophages and amoebae. Due to revealed mechanism and receptors involved in phagocytosis, some potential inhibitors have demonstrated significance impact on entering bacteria into cells.

1.7.3.1. Cytochalasin D

A small organic fungus-derived compound, cytochalasin D (CytoD), is widely used for studying the role of actin dynamics in numerous cellular process, including phagocytosis, migration, division and morphological changes. CytoD inhibits, not only the depolymerization and polymerization of actin sub-units by binding with high affinity to the ends of actin filaments, but also inhibits interaction between G-actin and cofilin, including

disassembly of cofilin-mediated actin filament [50]. It has been demonstrated that treating U-937 and macrophages CytoD effectively inhibits the entry of *L. pneumophila* and consequently preventing replication of this intracellular bacteria. In the protozoa *A. castellanii* and *H. vermioformis*, CytoD has almost no effect on entry and replication of *L. pneumophila* [51,52].

1.7.3.2. Methylamine

Methylamine is a small organic compound with a molecular formula of CH_3NH_2 that belongs to the group C_1 alkylamine [53], which are easily synthesized by interaction of methanol (MeOH) and ammonia (NH_3) over a catalyst [54]. Methylamines are used as intermediates for a wide range of agricultural chemicals (e.g. herbicides, fungicides, insecticides, biocides and miticides), pharmaceuticals (e.g. ephedrine), animal nutrients, explosives, surfactants and water treatment [53].

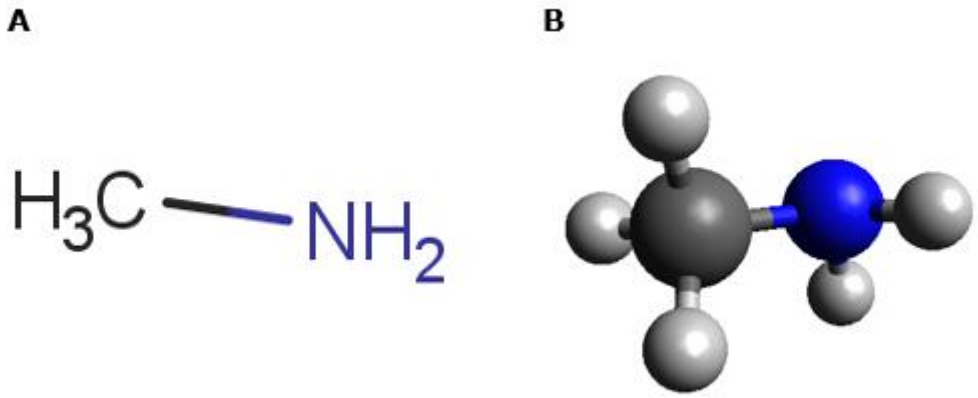

Figure 4. Molecular structure of methylamine. (A)2D molecular structure of methylamine. **(B)**3D molecular structure, ball and stick model of methylamine. Drawings of molecular structures are made with MarvinSketch command line program.

In biological aspects, methylamine inhibits transglutaminase, a plasma membrane enzyme included in the aggregation of ligand-receptor complexes, thereby inhibiting receptor-dependent endocytosis [52,55]. This inhibitor is also characterized as adsorptive inhibitor of receptor-mediated pinocytosis in macrophages, fibroblasts [56] and amoeba cells

[51,52]. Methylamine has been used to inhibit intracellular development and internalization of *Chlamydia trachomatis* into McCoy cells [57], and for inhibition of adsorptive endocytosis of Semliki Forest virus in BHK-21 cells [58]. Previous studies have shown that the ability of *L. pneumophila* to multiplicate in *H. vermioformis* and U-937 cells was inhibited by a dose-dependent effect of methylamine, which means that concentration of 100 mM completely inhibit multiplication of bacteria in amoebae. They have also established that concentrations of methylamine higher than 250 mM cause encystation of amoebae within 24 h, whereas lower concentrations are not toxic to either the U-937 monocytes and amoebae [52]. Some experiments have also indicated that when treated with 10 µM, 1mM and 10 mM 1 hour before and 3 hours after infection, methylamine incompletely inhibit entry of *F. novicida* into *D. discoideum* cells (unpublished data).

Moreover, other study has indicated that methylamine in concentration of 100 mM greatly reduce entry and replication of *L. pneumophila* into *A. castellanii* into early stages of infection when added 1 h prior to inoculation with bacteria [51]. Considering *L. pneumophila* and *F. tularensis* similarities of life cycle in protozoa cells [36], methylamine could be a potential cytoskeletal and metabolic inhibitor of entry and multiplication of *Francisella* within various amoeba cells, such as *A. castellanii* and *D. discoideum*.

2. Objective

Examine the influence of methylamine in concentration 100 mM on extracellular and intracellular growth of *F. novicida* in *D. discoideum* and *A. castellanii* cells.

Examine whether different doses of infection (MOI) have influence on extracellular and intracellular growth of *F. novicida* in *D. discoideum* and *A. castellanii* cells after treatment with 100 mM methylamine.

Examine the influence of methylamine in concentration 100 mM on LDH release from *D. discoideum* and *A. castellanii* cells infected with different MOI of *F. novicida*.

3. Materials and methods

3.1. Materials

3.1.1. Bacteria and amoeba cultures

The wt strain of *F. novicida* was obtained by the courtesy of prof. Andreas Sjostedta (Umei University, Umea, Sweden). *D. discoideum* strain AX2 was obtained by the courtesy of prof. Michaela Steiner (Technische Universitat Braunschweig, Germany). *A. castellanii* was obtained from American Type Culture Collection, 30234.

3.1.2. Nutrient substrates and other materials

The HL5 medium for growth of *Dictyostelium* was prepared by dissolving 7.15 g of Yeast extract (Oxoid), 14.3 g of Bacterial Protease Peptone (Biolife), 1.28 g Na_2HPO_4 in 900 mL of sterile distilled H_2O. The pH was adjusted to 7.5 with sterile solutions of 10 N KOH. After autoclaving and cooling, 15.4 g of glucose monohydrate was dissolved in 100 mL of sterile distilled H_2O and sterilized by filtration through a 0.22 μm filter. Concentrated sorens buffer (200x, TCS Biosciences Ltd.) was diluted with sterile distilled H_2O 1:200. The pH buffer was checked before use.

The ATCC 30234 medium for growth of *Acanthamoeba* was prepared by dissolving 1 g of Yeast extract, 20 g of Bacterial Protease Peptone in sterile distilled H_2O. Inorganic stock solutions were prepared separately in order and amount of 8 mL 0.05 M $CaCl_2$, 10 mL 0.4 M $MgSO_4$ x $7H_2O$, 10 mL 0.25 M Na_2HPO_4 x $7H_2O$, 10 mL 0.25 M KH_2PO_4 and 10 mL 0.005 M $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$. After autoclaving, cooling and mixing, 18 g of glucose monohydrate and 1 g of $Na_3C_6H_5O_7$ x $2H_2O$ was dissolved in 50 mL of sterile distilled H_2O and sterilized by filtration. After mixing, the pH of the complete medium was adjusted to 6.5 with sterile solutions of 1N HCL or 1N Na OH. Final volume was brought up to 1L by the addition of sterile distilled water.

Triton X-100 (0.9 %), a non-ionic surfactant which causes permeabilization of the living eukaryotic cell membrane was used to release intracellular bacteria from amoeba.

To assay the influence of methylamine on *F. novicida* growth kinetics in amoeba, it was used in concentration of 100 mM.

3.2. Methods

3.2.1. Cultivation of *F. novicida*

F. novicida was grown on buffered charcoal-yeast extract (BCYE) agar at 37 °C with 5 % CO_2 atmosphere.

3.2.1.1. Gramm staining for identification of bacteria

Pure culture for stained with Gramm.

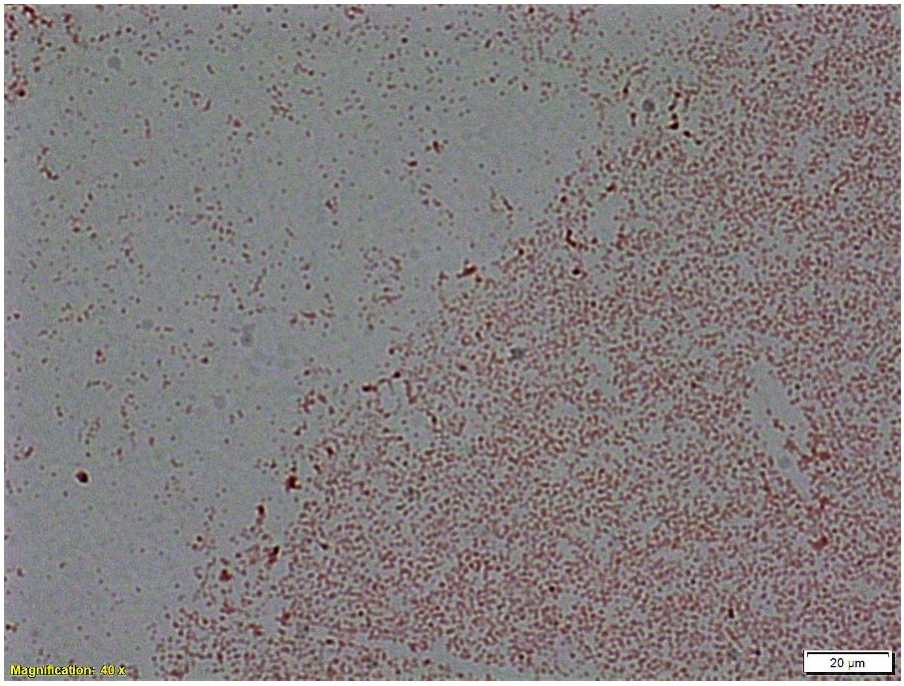

Figure 5. *F. novicida* is gram-negative in its staining morphology. Representative stained microscopic image of *F. novicida*.

3.2.2. Cultivation of amoeba

3.2.2.1. Cultivation of *D. discoideum*

D. discoideum was cultivated in HL5 medium on 25 °C. Since we needed 1 x 10^5 cells/mL, the exact number of cells was counted using Neubauer chamber. Cells were distributed in microtiter plates with 96 wells in HL5: Sorensen (1:1) medium and were left overnight to adhere. One

hour before infection cells were treated with 100 mM methylamine and incubated for 1 hour on 25 °C.

3.2.2.2. Cultivation of *A. castellanii*

A. castellanii was cultivated in ATCC 30234 medium with glucose on 25 °C. Since we needed 1 x 10^5 cells/mL, the exact number of cells was counted using Neubauer chamber. Cells were distributed in microtiter plates with 96 wells in ATCC 30234 medium without glucose and were left overnight to adhere. One hour before infection cells were treated with 100 mM methylamine and incubated for 1 hour on 25 °C.

3.2.2.3. Microscopy determination of amoeba

Amoebae were cultivated in their growth medium and examined under florescence microscopy.

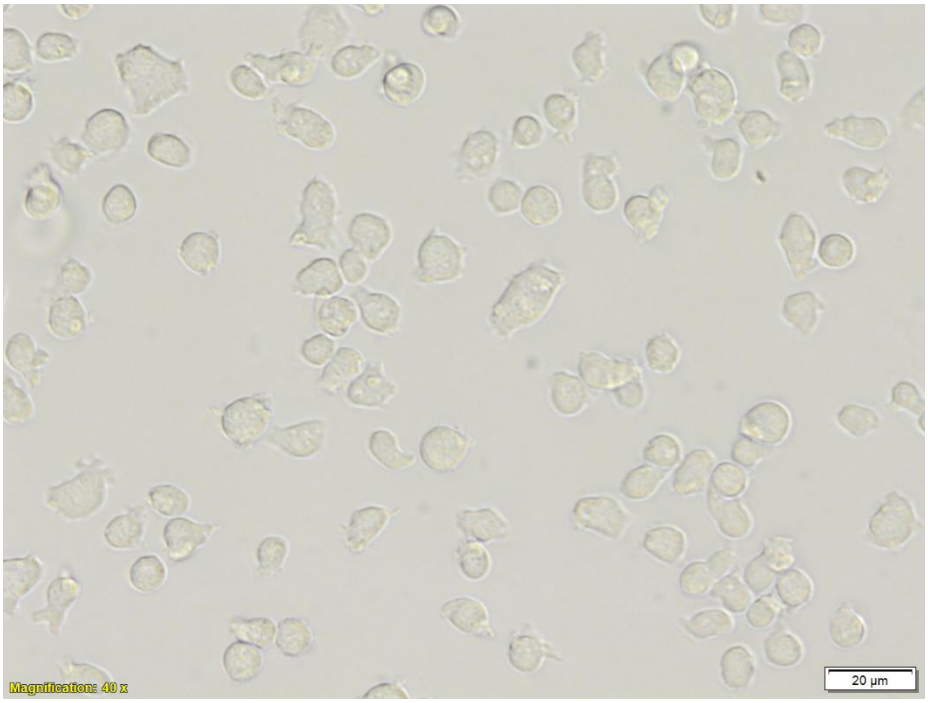

Figure 6. Cultivation of *D. discoideum* cells. Representative microscopy images of *D. discoideum* cells. Scale bar= 20 µm.

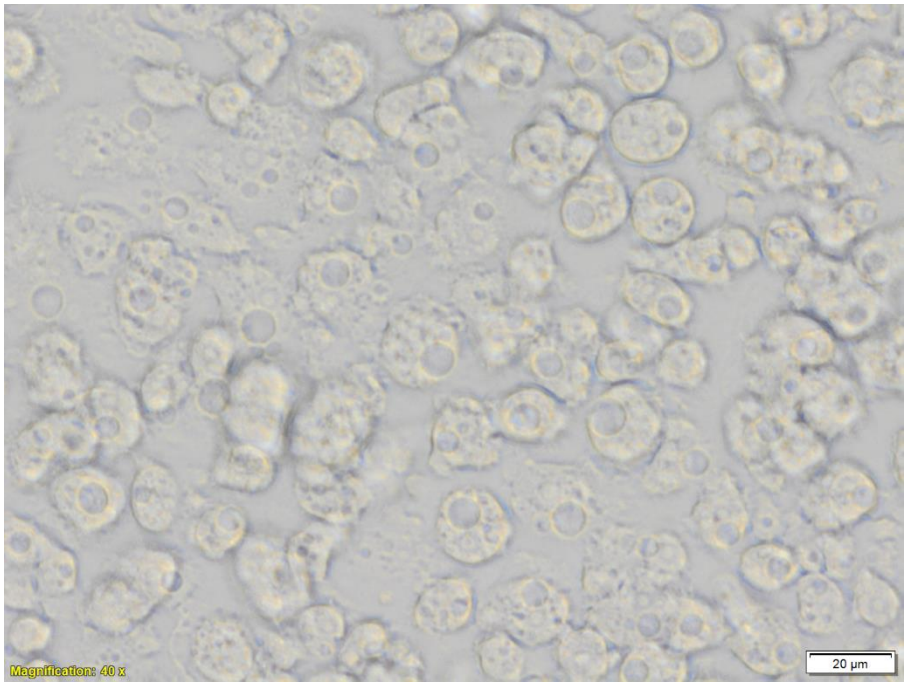

Figure 7. Cultivation of *A. castellanii* cells. Representative microscopy images of *A. castellanii*. Scale bar=20 µm.

3.2.3. Spectrophotometric determination of the number of bacteria

Bacteria were picked up from agar using sterile loop and were stirred in HL5: Sorensen (1:1) and ATCC medium without glucose. Optical density (OD) of samples was measured in cuvette on wavelength 580 nm. Sterile distilled water was used as control. According to bacterial growth curve, $OD_{sample}=1$ refers to concentration of 1 x 10^9 CFU/mL, as determined by previous experiments.

Amoeba *D. discoideum* and *A. castellanii* were infected with *F. novicida* at a multiplicity of infection (MOI) 5, 10 and 100. The MOI of 5, 10 and 100 represents 5, 10 and 100 bacteria inoculated per one cell.

3.2.4. Infection of *D. discoideum* and *A. castellanii*

Cells were allowed to adhere to the bottom of the wells. Cells were infected with 10 µL bacterial suspension (5 x 10^7 CFU/mL, 10^8 CFU/mL and 10^9 CFU/mL) to achieve the dose of 5 x 10^5 CFU/mL, 10^6 CFU/mL and 10^7 CFU/mL. Suspensions were centrifuged on 1200 rpm for 3 minutes and incubated for 1 hour on 27 °C. After incubation cells were washed (2x) with saline to remove extracellular bacteria. After second wash the cells were added HL5: Sorensen (1:1) medium or ATCC 30234 medium without glucose and medium with 100 mM methylamine. This time point was considered as time zero.

Two hours after infection 160 µL of media was removed into another well (extracellular bacteria). The 50 µL of media was taken for measurement of LDH. Cells were treated for 10 minutes with Triton X-100 (0.9 %) to destroy the cell membrane and release the intracellular bacteria. 10-fold dilutions were made and 10 µL of suspension was plated on BCYE agar. Plates were incubated for 2 days on 37 °C. All the steps were repeated twice after 4, 24, 48 and 72 hours after infection.

3.2.5. The CytoTox 96® Non-Radioactive Cytotoxicity Assay

The CytoTox 96® Assay was used to quantitatively measure lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis. The half-life of released LDH from cells into the surrounding medium is about 9 hours. Released LDH in culture supernatants was measured with a 30-minute couple enzymatic assay, which resulted in the conversion of a tetrazolium salt (iodonitro-tetrazolium violet; INT) into red formazan product. The amount of color formed is equal to the number of lysed cells. Visible wavelength absorbance data were collected using a standard 96-well plate reader. To form the CytoTox 96® Reagent, 12 mL of Assay Buffer was defrosted and warmed at room-temperature and was added to a bottle of Substrate Mix. Supernatant samples (50 µL) were transferred to a 96 well plate and 50 µL of Substrate Mix was added to each well. Plate was covered with foil to protect it from light and incubated for 30 minutes at

room temperature. After incubation 50 µL of Stop Solution was added to each well and recorded at the absorbance at 490 nm within 1 hour.

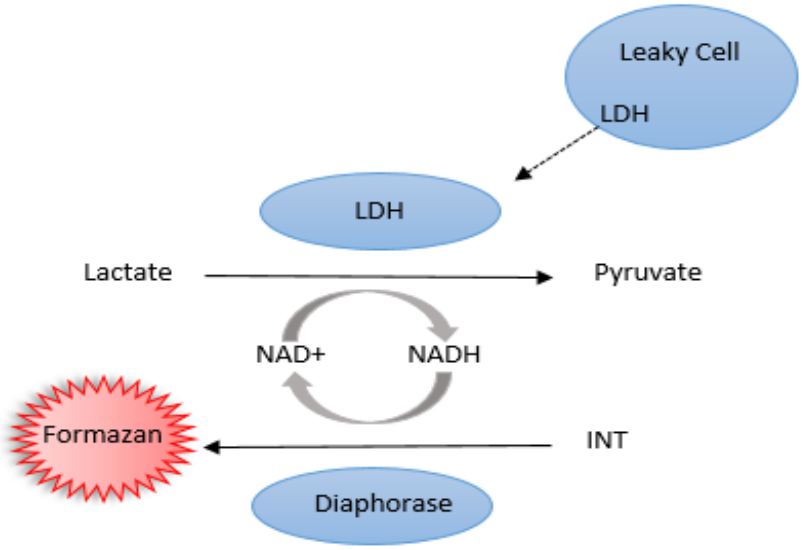

Figure 8. Release of LDH from damaged cells is measured by supplying lactate, NAD+ and INT as substrates in the presence of diaphorase. Generation of a red formazan product is proportional to the amount of LDH released and therefore the number of lysed cells.

Figure 9. The Cytotox 96® Non- Radioactive Cytotoxicity Assay protocol. Supernatant samples were transferred to a 96 well plate and an equal volume of Cytotox 96® Reagent was added to each well and incubated for 30 minutes. Stop Solution was added, and absorbance signal was measured at 490 nm in a plate reader."Retrived from: Promega Corporation, "CytoTox 96 Non-Radioactive Cytotoxicity Assay," Promega Tech. Bull. 163, pp. 1–14, 1999."

4. Results

4.1. Methylamine in concertation of 100 mM inhibits intracellular replication of *F. novicida* in *D. discoideum*

Receptor-mediated phagocytosis seems to be the crucial step for *Francisella* infection and replication in macrophages [31] and amoeba cells [34,35]. Because of apparent importance of membrane receptor-complexes we examined the methylamine, inhibitor of *Francisella* entrance to cells. Amoebae cells were treated with 100 mM methylamine 1 h prior to infection with *F. novicida* at MOI 5, 10 or 100. After infection, supernatant was separated for further examination of extracellular bacteria and cells were washed (x2) with saline and treated with Triton X-100 to release intracellular bacteria. The *F. novicida* growth kinetics was determined by plating suspensions onto BCYE agar at the time point 2, 4, 24, 48, 72 h post infection.

Our results showed that methylamine in concentration of 100 mM has a weak influence on extracellular replication of *F. novicida* in *D. discoideum* and differ among different MOI. There was a slight difference in the extracellular growth of bacteria within untreated amoeba cells and those treated with methylamine when comparing different doses of infection where 4 h p.i. (MOI 10 and 100) and 5 h p.i. (MOI 5) was almost 10-fold (Figure 10A,B) and 100-fold (Figure 10C) increase in bacteria in the absence of inhibitor with typical decline in numbers 48 h after infection. In contrast, it is inexplicable why bacteria persisted longer in media with methylamine and continue to multiply 48 h p.i. (Figure 10). Our results correspond to difference between MOI where extracellular bacteria reached up to 10^{11} CFU/mL (Figure 10A), 10^{12} CFU/mL (Figure 10B) and 10^{14} CFU/mL (Figure 10C).

Opposed to that, *F. novicida* did not grow within *D. discoideum* after treatment with 100 mM methylamine, with no detection in any time point, but multiplied to 10^{6} CFU/mL (MOI 5 and 10) and more (MOI 100) in the

absence of methylamine with typical decline in numbers 48 h after infection (Figure 10).This indicates that intracellular multiplication of bacteria was completely inhibited in amoeba by methylamine after added 1 h before infection. There is also a difference among MOI where bacteria (MOI 5 and 10) started to multiply intracellularly at 4 h p.i. (Figure 10A,B) but proliferate from time point 0 when added 100 bacteria per cells (Figure C).

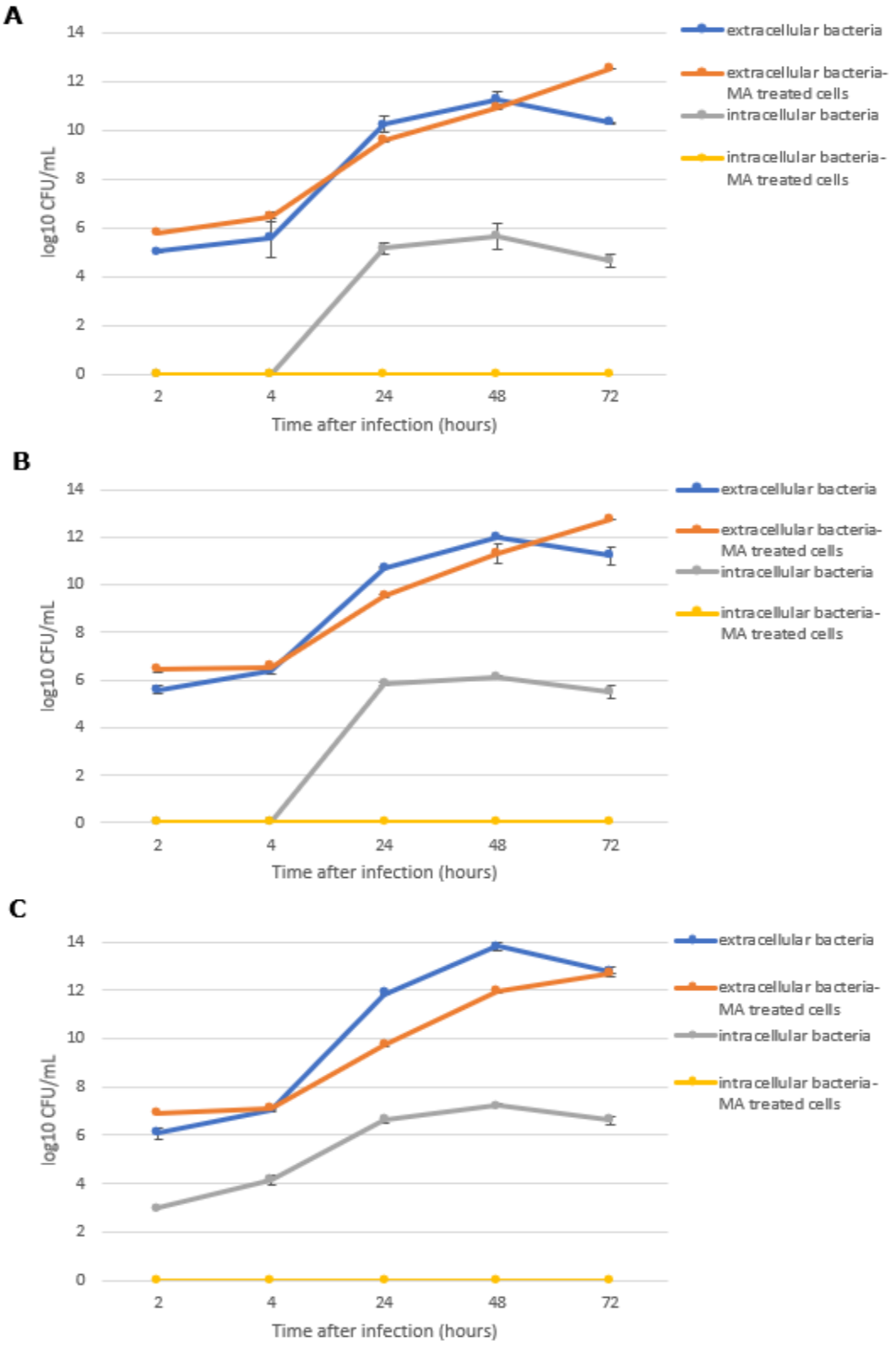

Figure 10. The growth kinetics of *F. novicida* in *D. discoideum* following different doses of infection (A) MOI 5, (B) MOI 10 and (C) MOI 100. The amoeba cells were treated with 100 mM methylamine for one hour followed by infection with *F. novicida* and determination of the number of intracellular or extracellular bacteria on BCYE agar. The error bars represent standard deviations of duplicate samples and results shown are representative of two independent experiments.

4.2. Methylamine in concertation of 100 mM inhibits intracellular replication of *F. novicida* in *A. castellanii*

The extracellular replication of bacteria in the presence of *A. castellanii* was also followed in this study. Although in first 24 h p.i. bacteria replicated in medium with presence of methylamine, there was a significant 10,000-fold (Figure 11A) and more (Figure 11B,C) increase in number of extracellular bacteria in the absence of methylamine which were observed 24 hours after infection. These results imply that inhibitors, such as methylamine, could be toxic for *F. novicida* and therefore have long-term inhibitory effect on extracellular bacterial proliferation under favorable conditions. There was also a variation in extracellular growth kinetics between different MOI with corresponding values 10^{16} CFU/mL for MOI 5 (Figure 11A),10^{18} CFU/mL for MOI 10 (Figure 11B) and more than 10^{18} CFU/mL for MOI 100 (Figure 11C).

In untreated cells *F. novicida* multiply intracellularly within *A. castellanii* up to 10^5 CFU/mL with typical decline in numbers after 3 days after infection (Figure 11). Bacteria did not grow in amoebae that have been pretreated with 100 mM methylamine, indicating influence of this inhibitor on receptor-mediated phagocytosis. There is also a difference of intracellular proliferation of bacteria among different doses of infection where with infection dose of 5 or 10 bacteria started multiplication 24 h p.i. (Figure 11A,B) and 4 h p.i. when cells were infected with MOI of 100 (Figure 11C). This signifies that bacteria proliferate earlier when added higher infective doses.

These results demonstrate significance of cell membrane receptors in *F. novicida* infection of *D. discoideum* and *A. castellanii* and consequently illustrate the role of methylamine in inhibition of transglutaminase, thereby inhibiting receptor-dependent phagocytosis and preventing further development of bacterial infection.

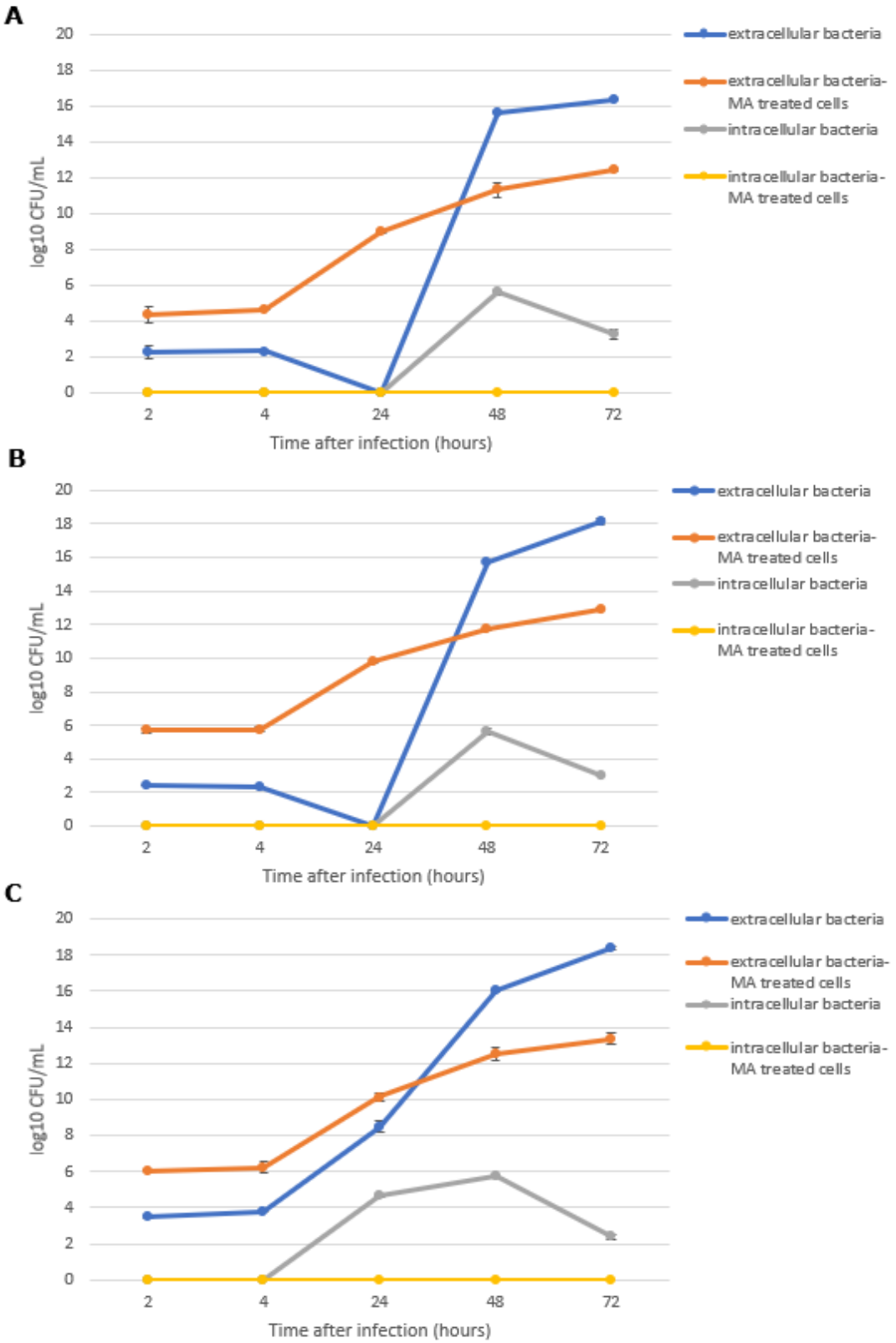

Figure 11. The growth kinetics of *F. novicida* in *A. castellanii* after different dose of infection (A) MOI 5, (B) MOI 10 and (C) MOI 100. The amoeba cells were treated with 100 mM methylamine for one hour followed by infection with *F. novicida* and determination of the number of intracellular or extracellular bacteria on BCYE agar. The error bars represent standard deviations of duplicate samples and results shown are representative of two independent experiments.

4.3. Methylamine does not affect induction of cytopathogenicity in *D. discoideum* cells infected with *F. novicida*

Cytopathogenic response of cells infected with *F. tularensis* are characterized by morphological changes such as lactate dehydrogenase (LDH) release. Induction of cytopathogenicity is essential for escape form phagosome and cytosolic replication of bacteria [59]. We examined the cytotoxicity of methylamine, inhibitor of phagocytosis, by testing the ability of inhibitor to affect the induction of cytopathogenicity (LDH release) in *D. discoideum* and *A. castellanii* infected with *F. novicida*. After treatment with 100 mM methylamine and infection of amoeba cells with *F. novicida*, the supernatants were sampled at 2, 4, 24, 48 and 72 h and examined for the presence of the LDH. The results were reported in comparison to non-treated infected cells and treated with 100 mM methylamine after different doses of infection (MOI 5, 10, 100).

Methylamine did not induce significant reduction of LDH release from infected *D. discoideum*. With infection dose of 5, at 2 and 4 h after infection the LDH levels in supernatant of untreated cells differed from that of cells treated with 100 mM MA (2 h p.i. P= 0.032, 4 h p.i. P= 0.007), whereas 24, 48 and 72 h p.i. there was no significant difference in comparison to treated and untreated cells, which is consistent with results of the extracellular growth (Figure 12A). At infection dose of 10, LDH levels of untreated cells did not differ much from that of treated with MA with significant difference 2 and 72 h (2 h p.i. P= 0.028, 72 h p.i. P= 0.025) (Figure 12B). When cells were infected with MOI 100, the LDH release did not differ from untreated in comparison to MA treated cells with only significance at 2 and 24 h post infection (2 h p.i. P=0.038, 24 h p.i. P= 0.010) (Figure 12C). The LDH release from uninfected cells treated with MA was correlating in comparison with *F. novicida* infected cells treated with MA among all doses of infection (MOI 5, 10 and 100) (unpublished data).

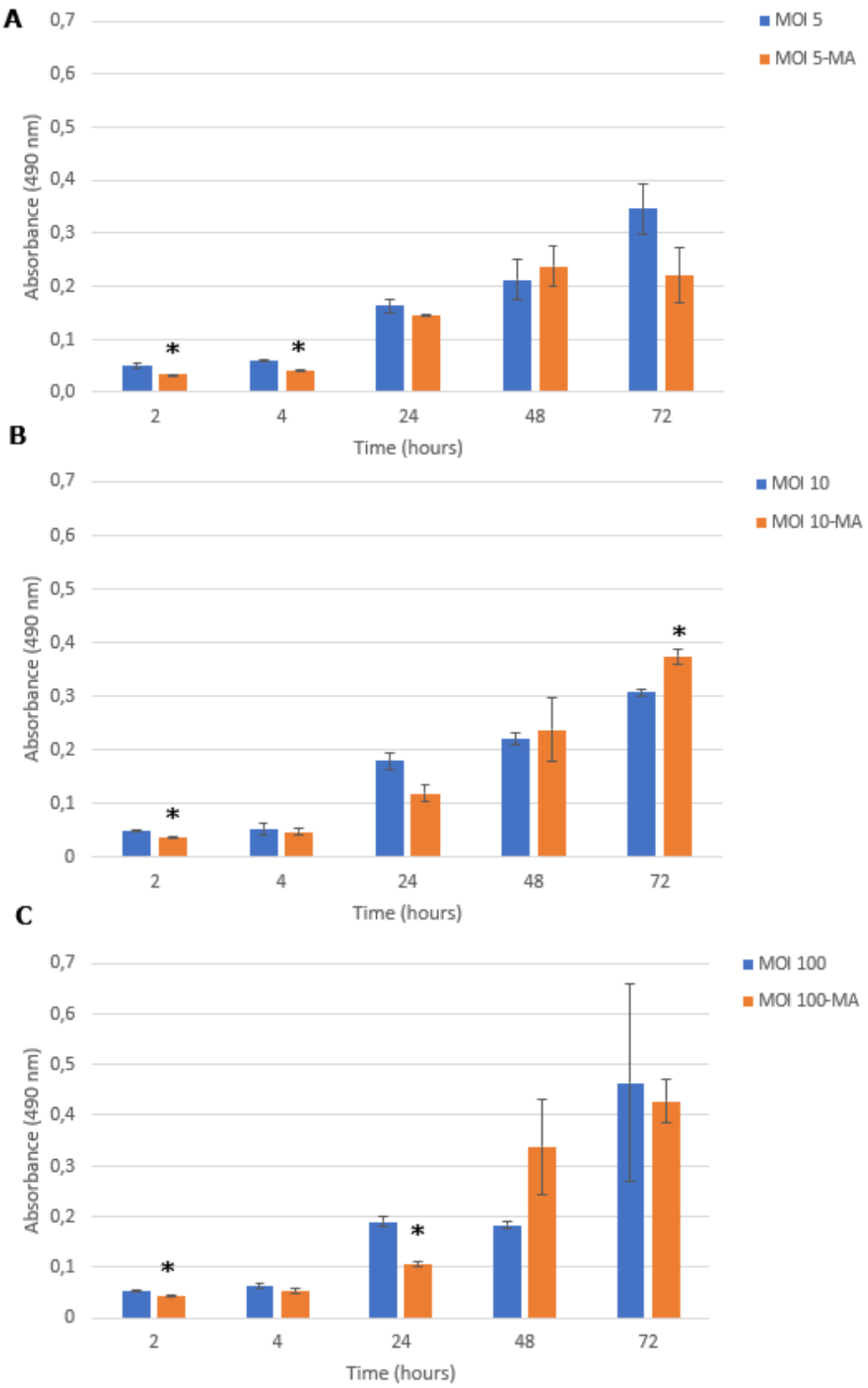

Figure 12. The LDH release from *D. discoideum* cells after pretreatment with 100 mM methylamine and different dose of infection (A) MOI 5, (B) 10 and (C) 100. Culture supernatants of infected D. discoideum cells were sampled and tested for LDH activity at 2, 4, 24, 48 and 72 h after infection. The error bars represent standard deviations. Statistical significance was determined using Student's t test. $P < 0.05$ was considered significant in comparison to the untreated cells *.

4.4. Methylamine does not affect induction of cytopathogenicity in *A. castellanii* cells infected with *F. novicida*

The LDH levels in supernatant of infected and untreated *A. castellanii* did not differ from those treated with 100 mM methylamine. At the infection dose 5, there was no significant difference between LDH release of untreated cells and treated in any time point of infection (Figure 13A). When cells were infected with an MOI of 10, LDH levels in supernatant did not vary much among treated and untreated cells with significant difference at 24 and 72 h after infection (24 h p.i. P= 0.022, 72 h p.i. P= 0.0505) (Figure 13B). In the infection dose of 100 cells per bacteria, the LDH release did not differ between untreated and MA treated cells with only one significance at 48 h after infection (48 h p.i. P= 0.041) (Figure 13C). Increasing MOI from 5 to 100 comparatively follow enhancement trend in LDH levels in supernatant due to increase in extracellular and intracellular growth of bacteria.

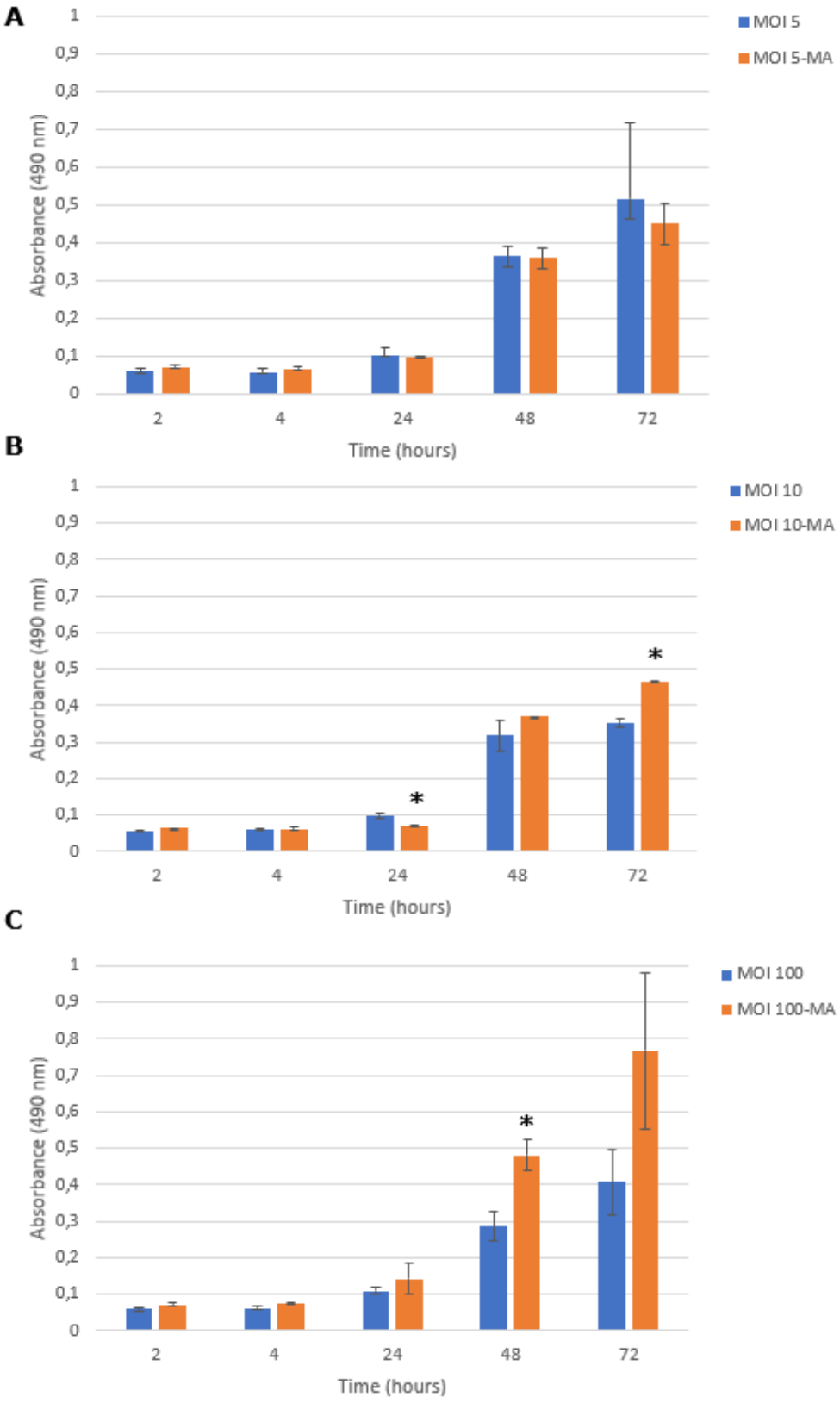

Figure 13. The LDH release from *A. castellanii* cells after pretreatment with 100 mM methylamine and different dose of infection (A) MOI 5, (B) 10 and (C) 100. Culture supernatants of infected *A. castellanii* cells were sampled and tested for LDH activity at 2, 4, 24, 48 and 72 h after infection. The error bars represent standard deviations. Statistical significance was determined using Student's t test. P < 0.05 was considered significant in comparison to the untreated cells *.

5. Discussion

Receptor-mediated phagocytosis appear to be necessary for *Francisella* entry and intracellular replication within macrophages [31], arthropod-derived [33] and amoebae cells [34,35]. Therefore may studies have been carried out in contest to examine the importance of inhibitors, such as methylamine [51,52,55,57,58] and cytochalasin D [50,51,52], on their influence on receptor-dependent endocytosis and pinocytosis. To study *Francisella* infection a less virulent strain *F. novicida* was used due to many similarities to type A strain, such as infectivity, intracellular life cycle and genome sequence [31]. Because of attenuation of this strain in healthy humans, and high infectivity in laboratory mice, makes *F. novicida* safer and more tractable mechanism for understanding *F. tularensis* infection [17]. Considering *F. novicida* association with aquatic environment emphasize significance of water-borne transmission animals, such as mosquitoes and amoebae, in providing survival and proliferation of bacteria in water. This suggests that such animals serve as the potential long-term aquatic reservoir of the bacteria in nature [18]. Thereby *A. castellanii*, a water-borne amoeba [34,43], and social amoeba, *D. discoideum*, with many resemblance with *Francisella*-macrophages infection [35], seems to present as a useful models in examination of *F. tularensis* infection mechanism for aquatic environment.

D. discoideum amoebae provide ethically unproblematic and cost-effective models to measure bacterial virulence and host resistance with human pathogens such as *Mycobacterium*, *Pseudomonas* and *Legionella*. This amoeba has also been used as a host model to screen for anti-virulence compounds in *Mycobacterium*, such as inhibitors of mycobacterial sliding motility, in order to develop new antibacterial treatments [60]. This system has also been used for identification of compounds inhibiting *Pseudomonas* infectivity [61]. Our previous experiments have indicated that methylamine in concertation of 10 μM, 1mM and/or 10 mM incompletely inhibit *F. novicida* entry in *D. discoideum* when added 1 hour before and 3 hours

after infection but does not affect intracellular replication of bacteria (unpublished data). Due to these findings we increased the concentration to 100 mM MA which resulted in complete inhibition of intracellular growth of *F. novicida* in *D. discoideum* with no CFU detection in any time point. Opposite of that, bacteria reached up to 10^6 CFU/mL and more in the absence of inhibitor with typical decline in numbers 48 h after infection. This indicates that methylamine completely blocks receptor-mediated phagocytosis of *F. novicida* in *D. discoideum* and consequently entirely inhibit intracellular multiplication of bacteria when added 1 h before infection. When exanimating extracellular growth of bacteria in presence of inhibitor our results showed a slight difference in the extracellular multiplication among untreated cells and those treated with methylamine. Bacterial increase in 10-fold at 4 h p.i. and 100-fold at 5 h p.i. in the lack of inhibitor indicating that methylamine could potentially be toxic to *F. novicida* and has a weak influence on bacterial proliferation under favorable conditions. These results contrast with *L. pneumophila* infection whereas 100 mM methylamine was not toxic and had no effect on extracellular multiplication of bacteria [52]. Regarding intracellular and extracellular multiplication of *F. novicida* when untreated with inhibitor we can observe typical decline in numbers 48 h after infection. In contrast, it is unexplainable why bacteria persisted longer in media with methylamine and continue to multiply 48 h p.i.

Recent studies have shown the ability of methylamine to inhibit intracellular replication of *L. pneumophila* in amoeba *H. vermioformis* with a dose-dependent effect and complete inhibition of multiplication of bacteria when used 100 mM [52]. This inhibitor also greatly reduces entry and intracellular replication of *L. pneumophila* in *A. castellanii* in early stages of infection when added 1 h before infection in concentration of 100 mM [51]. Considering *L. pneumophila* and *F. tularensis* similarities of life cycle in protozoa cells [36] we assumed methylamine would have similar inhibitory effect in *Francisella* entry and multiplication in amoeba such as

A. castellanii, as mentioned before. Our results confirmed that methylamine in concentration of 100 mM completely inhibit entry and intracellular replication of *F. novicida* within *A. castellanii*. Bacteria did not grow in presence of inhibitor with no detection in any time point. When untreated, bacteria grow intracellularly to greater than 10^5 CFU/mL with typical decline in numbers after 3 days of infection thereby pointing out the influence of methylamine on receptor-mediated phagocytosis. In contrast to *D. discoideum*, water-borne *A. castellanii* showed meaningful difference in extracellular multiplication of *F. novicida* when cells were treated with inhibitor. Although in first 24 h p.i. bacteria persisted longer and multiplied greater in medium with methylamine, there was a significant 10,000-fold and more increase in number of extracellular bacteria in the absence of inhibitor observed 24 hours after infection. The same number of bacterial CFU and decline in number after 3 days post infection was also presented in *L. pneumophil*a extracellular multiplication without presence of methylamine [52]. These results affirm that inhibitor like methylamine could be toxic for *F. novicida* and therefore have long-term inhibitory effect on extracellular multiplication of bacteria under favorable conditions.

One study pointed out that several levels of bacterial infection of the amoebae affect the total number of bacterial CFU when infected with virulent *L. pneumophila* [51]. To determine whether different doses of bacterial infection of *D. discoideum* and *A. castellanii* had an influence on bacterial kinetics of the assay, several MOIs were tested with *F. novicida*. When multiplied extracellularly bacteria showed almost no difference after treatment with inhibitor within different MOI but were influenced in the absence of methylamine with almost 10-fold increase with MOI 5 and 10 and 100-fold increase with MOI. The intracellular replication of bacteria in untreated cells was differentiate among MOIs whereas for MOI 5 and 10 bacteria started to multiply intracellularly at 4 h p.i. but proliferate from time point 0 with MOI 100. These results showed that a lower concentration of bacterial inoculum was considered as a lower concentration of

intracellular and extracellular bacteria in the absence of methylamine indicating that different levels of *F. novicida* infection of *D. discoideum* influenced a total number of bacterial CFU only when cells were not treated with inhibitor.

There was also a variation in extracellular growth kinetics of *F. novicida* in *A. castellanii* when untreated with methylamine between different MOI with corresponding values 10^{16} CFU/mL for MOI 5,10^{18} CFU/mL for MOI 10 and more than 10^{18} CFU/mL for MOI 100. When replicating intracellularly the number of bacteria differed among levels of infection whereas bacteria started proliferation at 24 h p.i. with MOI 5 and 10 and at 4 h p.i. when infected with MOI 100. *F. novicida* reached up to 10^6 CFU/mL when grown intracellularly at MOI 100, which correspond to results of intracellular replication of *L. pneumophila* in *A. castellanii* [51]. A lower concentration of bacterial inoculum was also considered as a lower concentration of intracellular and extracellular bacteria showing that different MOIs of *F. novicida* also affect number of bacteria in *A. castellanii* in the absence of methylamine with no effect on treated cells.

At last, the effect of multiplicity of infection (MOI) were examined in order to characterize the ideal model and provide productive infection, an optimal dose of infection for intracellular bacterial growth whereas bacterial proliferation does not cause a cell death.

It has been indicated that concentrations of methylamine higher than 250 mM in assay medium caused amoebae encystation within 24 h [52]. To determine whether methylamine in concentration of 100 mM affect the induction of cytopathogenicity in *D. discoideum* and *A. castellanii* cells infected with *F. novicida* LDH release assay was made. Our results showed that methylamine did not induce significant reduction of LDH release from infected *D. discoideum*. Infection dose of 5, at 2 and 4 h p.i. resulted in difference among the LDH levels of untreated and MA-treated cells (2 h p.i. P= 0.032, 4 h p.i. P= 0.007). In contrast, at 24, 48 and 72 h p.i. there was no notable difference between treated and untreated cells, which is

consistent with results of the extracellular growth. At infection dose 10, LDH release of untreated cells did not differ much from treated one with significant difference only 2 and 72 h (2 h p.i. P= 0.028, 72 h p.i. P= 0.025). When infected with dose of 100 cells per bacteria, LDH levels did not differentiate among treated and untreated cells with significance 2 and 24 h post infection (2 h p.i. P=0.038, 24 h p.i. P= 0.010). The LDH releasee from *F. novicida* infected cells treated with MA was correlating in comparison with uninfected cells treated with MA for all doses of infection (MOI 5, 10 and 100) (unpublished data) which is in accordance to the intracellular growth of bacteria.

The LDH levels in supernatant of infected and untreated *A. castellanii* cells did not differ from those treated with 100 mM methylamine. At MOI 5, there was no notable difference between LDH release of treated and untreated cells in any time point. With MOI 10, LDH levels did not vary much with significant difference 24 and 72 h after infection (24 h p.i. P= 0.022, 72 h p.i. P= 0.0505). When cells were infected with MOI of 100, the LDH release did not differentiate among untreated and MA treated cells with only one significance at 48 h after infection (48 h p.i. P= 0.041). This correspond to results from study that indicated that lower concentrations of methylamine were not toxic for amoebae or monocytes [52]. Increasing MOI from 5 to 100 comparatively follow enhancement trend in LDH levels in supernatant due to increase in extracellular and intracellular growth of bacteria.

These results indicate that there is no significance difference of LDH levels in supernatant between treated and untreated cells. This demonstrate that methylamine does not affect inadequately the *D. discoideum* and *A. castellanii* cells and can be used as *F. novicida* entry inhibitor without being toxic for cells. These results also confirm statement from previous studies which claimed that methylamine in concentrations lover than 250 mM are not toxic to amoebae [52].

6. Conclusion

F. tularensis has shown a major difference in lifestyle within macrophages and various protozoa cells. Within macrophages bacteria escape form FCV and continue replication in cytosol, while in *A. castellanii F. tularensis* replicate in intact vacuoles. Nevertheless, it has been shown that some amoebae, such as *D. discoideum*, occur in lifestyle more related to macrophages with similarities in phagosomal maturation and acidification and consequently escape and replication in cytosol. However, the molecular aspects of *F. tularensis* entry in both protozoa and mammalian cells are similar. Bacteria enters cells by receptor-mediated phagocytosis followed by extensive replication and lyses of host cell. Our results showed that if receptors are blocked with methylamine, inhibitor of phagocytosis, then *F. novicida* cannot be engulfed by cells and continue replication. These results were demonstrated in two different protozoa cells, a water-borne *A. castellanii* and social amoeba *D. discoideum*, with inhibitory effect of methylamine in both. We have also indicated that a lower concentration of bacterial inoculum (MOI) was represented as a lower concentration of intracellular and extracellular bacteria in the absence of methylamine. This implies that different levels of *F. novicida* infection of *D. discoideum* and *A. castellanii* influenced a total number of bacterial CFU only in the absence of inhibitor. To establish safety and non-toxicity of methylamine in concentration of 100 mM to amoebae cells LDH release test were made. The results imply that methylamine can be used as *F. novicida* entry inhibitor without being toxic for *D. discoideum* and *A. castellanii* due to no significance difference in LDH levels in supernatant among treated and untreated cells. Further studies will be important to identify the mode of action and molecular target of methylamine, since phagocytosis represent only way for bacterial entrance to cells. To conclude, our study has demonstrated influence of methylamine on receptor-mediated phagocytosis, crucial step for bacterial entry into cells, in protozoa with

different lifestyle in which similarities to macrophages could enable future analysis of metabolic inhibitors in higher evolutionary species.

7. References

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Biotechnology MSc student that prospects to research in Microbiology and Molecular Biology. Holds BSc in Biotechnology and Drug Research, with a thesis in Medical Microbiology and Bacteriology. Has been a Researcher at the Department of Microbiology and Parasitology at the Faculty of Medicine of the University of Rijeka and a Drug Research Intern at Jadran Galenski Laboratory (JGL).

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WORK EXPERIENCE

2017–2017 Researcher

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Conducting research for my postgraduate thesis, on the influence of methylamine and infectious doses on the results of infection of *Dictyostelium discoideum* and *Acanthamoeba castellanii* with *Francisella novicida*. The purpose of the research is to find out more about how organic compounds such as methylamine can affect or inhibit *F. novicida* infection in amoeba. Under the supervision of Prof M. Santic. Techniques: bacterial culture, cell culturing, bacterial inoculation and incubation, amoebae cell infection, spectrophotometric method for the determination of a bacterial cell counts, LDH release test.

10/2016–11/2016 Immunology Teaching Assistant

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Prepared laboratory experiments for the Immunology course at the Department of Biotechnology. Demonstrated them to undergraduate students, six hours per day during one month, while coaching and helping them to actively contribute to the course.

10/2015–09/2016 Researcher

Elena Ivek, Rijeka (Croatia)

Conducted research for my undergraduate thesis, on the effect of methylamine on adhesion of *Francisella novicida* on *Dictyostelium discoideum*. Under the supervision of Prof M. Santic. Techniques: bacterial culture, cell culturing, bacterial inoculation and incubation.

06/2016–07/2016 Drug Research Intern

Jadran Galenski Laboratory, Rijeka (Croatia)

Learned techniques: calibrating, measuring pH (pH meter), optical density (densitometer), an index of refraction (refractometer), determining total osmolality of aqueous solutions (Single-Sample Freezing Point Osmometer), determination of samples colour and clarity, processing of experimental data to test bioequivalence for a generic drug approval according to the guidelines.

03/2017–04/2017 Actor, Tailor

Department of Biotechnology | University of Rijeka, Rijeka (Croatia)

Participated in a series of theatrical plays to raise awareness of the immunology field to children.

europass Curriculum vitae

EDUCATION AND TRAINING

2016–2018

MSc in Biotechnology in Medicine

Department of Biotechnology, University of Rijeka, Rijeka (Croatia)

Systems Biomedicine, Nanomedicine, Cell Therapy, Tissue Engineering, Computational Design of Biologically Active Molecules, Molecular Biotechnology, Methods in DNA Technologies, Green Chemistry, Antiviral Drugs

2017–2017

Pathophysiology Summer School

University of Rijeka and St. Cloud State University, Rijeka (Croatia)

Pathophysiology of the Current Public Health Issues and Diseases

2013–2016

BSc in Biotechnology and Drug Research

Department of Biotechnology, University of Rijeka, Rijeka (Croatia)

Cellular and molecular biology, Immunology, Medical Microbiology, Basics of Molecular Medicine, Biology of Stem Cells, Biochemistry, General Chemistry, Analytical chemistry, Organic Chemistry, Physical Chemistry, General Toxicology, Pharmacognosy and natural products,

2009–2013

High school "Second Gymnasium", Varaždin (Croatia)

2001–2009

Primary school "IV Primary School Varaždin ", Varaždin (Croatia)

PERSONAL SKILLS

Mother tongue(s): Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C1	C1	C1	C1
German	B1	B1	B1	B1	B1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

Good communication skills gained through my experience as a sales worker

Excellent contact skills with children gained through my experience as a volunteer in projects "Traveling Scientists"

Organisational / managerial skills

Excellent organizational skills, commitment and ability to work in a team gained through my experience as a project manager of Student carnival group "Kampus".

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem solving
Independent user	Proficient user	Basic user	Independent user	Independent user

Digital skills - Self-assessment grid

Driving licence AM, B

ADDITIONAL INFORMATION

Conferences 8thInternational Conference on Tularaemia, Opatija 2015
Assistant Conference Organizer, Attendance Certificate

Memberships The Student Union of Biotechnology at the University of Rijeka (USBRi)
Student project “Traveling scientists”, Rijeka
2014/2015/2016
Active Volunteer

Projects The Student Union of Biotechnology at the University of Rijeka (USBRi)
Student carnival group “Kampus”, Rijeka
2017/2018
Project Manager

Honours and awards Varazdin City student scholarship for excellent success | Varazdin, Croatia
Provided during 2014 to 2018.

Honours and awards 2nd Place at the Case Study Competition | Jadran Galenski Laboratory | Rijeka, Croatia | 2017
For solving regulatory problems to make a change in two different products from JGL

Publications Ivek, E. (2016): The effect of methylamine on adhesion of *Francisella novicida* on *Dictyostelium discoideum* cells, Bachelor’s thesis, Department of Biotechnology, Rijeka.