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Effect of anisaxin, cecropin-like antimicrobial peptide on *Borrelia burgdorferi* sensu stricto viability

Bachelor thesis

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Annotation

The objective of this thesis is to investigate the efficiency of the antimicrobial peptide anisaxin-1, from the zoonotic nematodes of the *Anisakis* genus, towards *Borrelia burgdorferi* sensu stricto at different concentrations. We aimed to compare its antimicrobial properties with other anti-borrelial agents, including melittin, an antimicrobial agent found in bee venom, and the conventional antibiotics doxycycline, cefoperazone, and daptomycin. It is important to identify a new natural compound that might be used for Lyme disease treatment due to the bacterial ability to develop resistance to the antibiotics. Antimicrobial peptides have been recognised as compounds that do not trigger persistent antimicrobial resistance. The analysis was performed using the live/dead bacterial staining method, fluorescent microscopy, statistical testing, and comparison with previously published data. To test statistical significance among treatments, ANOVA and Tukey-HSD were employed to determine the effect of the anisaxin at different concentrations.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

In České Budejovice, (02.05.2024)

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Mediha Kurbegovic

Contents

1. Abstract

Antimicrobial peptides (AMPs) are produced by various organisms as a part of their innate immune system, and they are effective against a wide range of pathogens. They have become a major point of interest in the search for novel therapeutics because, as it was found, the bacteria cannot develop the classical resistance to them as in case with traditional antibiotics (ATB) and AMPs do not produce undesirable side effects as some ATBs do. AMPs are commonly reported to have activity against various types of bacteria, such as *E. coli* or vancomycin-resistant enterococci, fungi, and viruses with non-membrane or membrane mode of action. In this study, the antimicrobial peptide anisaxin-1 (A-1) from *Anisakis pegreffii*, a parasitic nematode, was used to investigate its antimicrobial efficiency against *Borrelia burgdorferi* sensu stricto spirochetes, a tick-borne pathogen, which is a causative agent of Lyme disease (LD). Previous research has presented the efficacy of A-1 against *E. coli* and other gram-positive and gram-negative bacteria. The A-1 mode of action, as suggested, is through permeabilization of the bacterial cell wall. This study examined how A-1 performs at various concentrations as an antimicrobial agent against *B. burgdorferi*, comparing its efficiency with traditional antibiotics and melittin.

2. Introduction

2.1 *Borrelia burgdorferi* sensu lato and Lyme disease

The spirochetes from *Borrelia burgdorferi* sensu lato complex (*B. burgdorferi* s.l.) are the tick-borne spirochetes that causes the Lyme disease (LD) in humans worldwide. *Borrelia* is transmitted with the saliva of infected *Ixodes* ticks to humans during tick bite and blood feeding. *B. burgdorferi* s.l. are spread out throughout different regions of the world. *B. burgdorferi* sensu lato complex, as it is known today, contains about 23 recognised and well characterized spirochete species described (Rudenko et al. [1]) with different host specificity, infectivity or clinical manifestations of LD [1, 2].

Borrelia exploits the proteins in tick saliva to evade host's immune system, enabling it to effectively enter the host without detection, disseminate further, and cause infection [3-7]. The infection by LD spirochetes causes annular rash, arthritis, carditis and in later stages, encephalopathy [2-5]. The impact of LD on public health was recognized when the Centres for Disease Control and Prevention (CDC, USA) released the statement that about 329,000 new LD cases were diagnosed annually in the United States between 2005-2010, 10 times more than previously reported (Kuehn, 2013; Nelson et al., 2015 [64,65]). This was followed by the recognition that approximately 476,000 cases of LD were diagnosed and treated in the USA annually between 2010-2018 (Kugeler et al., 2021 [66]). The increase of LD cases in Europe was recognized in a resolution of the European Parliament on Lyme disease (Borreliosis) (2018/2774 (RSP)) as well, estimating approximately 850,000 of LD cases every year [67].

Borrelia is a spiral shaped bacterium (Figure 1) of approximately 10-30 µm in length and 0.2-0.3 µm in width [3,4]. Individual cells can vary by length, diameter, or shape. The spirochete possesses a genome structure characterized by a linear chromosome measuring approximately 910 kilobase pairs (kbp) in length. Additionally, they contain a unique set of genetic elements, including 12 linear and 9 circular plasmids, collectively containing up to 600 kbp. This genetic makeup is atypical among bacteria, as well as the fact that they do not encode many genes required for biosynthesis of cell contents [4]. Many of the chromosomal genes of LD spirochetes encode housekeeping proteins and proteins for metabolic function, while genes required for the production of outer surface proteins (Osp) involved in host interactions of the bacterium or vectors are found in plasmids [3,4]. Most Osps function as mediators between bacteria and their environment (host/vector) [4].

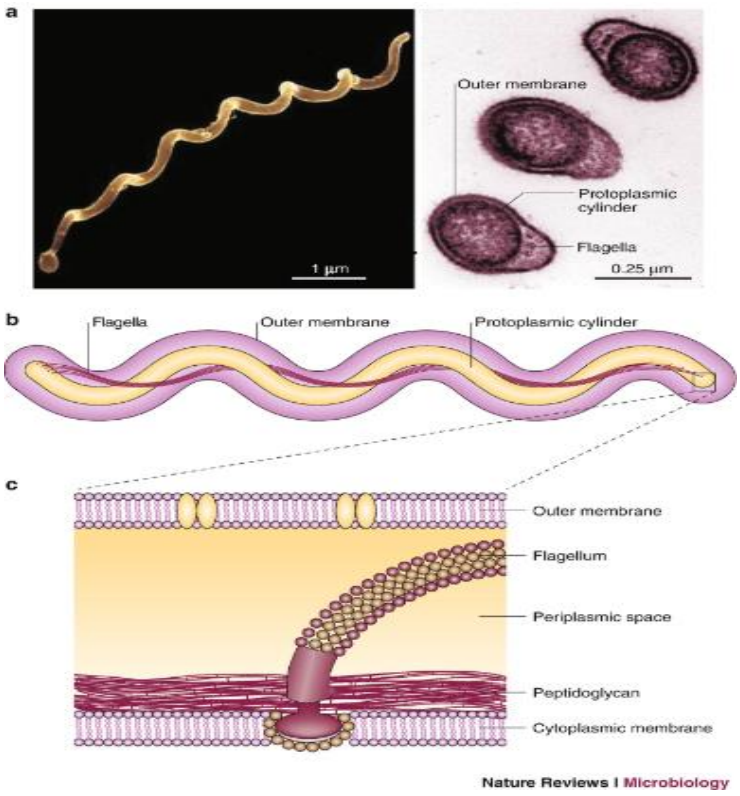

Figure 1: Cross section of *B. burgdorferi* bacterial cells including its membrane structure. In part a) showing the light-microscopy image of the bacterium and transactional schematic of the bacterial cell, b) the protoplasmic space of the flagella inserted into the protoplasmic space, and c) a magnified version of the image in part b) showing the insertion site of the flagellum into the cytoplasmic membrane [51].

Borrelia lacks lipopolysaccharides, and instead *B. burgdorferi* has a diderm cell envelope made up of an outer surface membrane that is covered by a peptidoglycan layer [4]. The bacterium contains about 7-11 flagella that can bend, flex, rotate, and propel the bacterium forward and backwards enabling movement through the viscous media. This motility is essential for the infectious life cycle of the bacterium [4,62].

The bacterium is well-adapted to survive in a variety of different organisms, such as arthropods, vertebrates, and mammals [3-5,7]. The surface modifications of *B. burgdorferi* s.l. are suggested to play a role in host adaptation and further immune evasion mechanism [3-4,7]. Another mechanism of adaptation to the host environment is the alteration of its gene expression [4-5,7]. Infection by these bacteria in humans can be effectively cleared by antibiotic treatment in the early stages of infection. Currently, the most effective treatments for patients with LD are intravenous or oral administration of the following antibiotics: ceftriaxone, doxycycline, or amoxicillin [4,6]. The usual treatment period is about 30 days if it is started early. However, there are multiple cases where bacteria tend to develop persisters which resist the treatment. *In vitro*, antibiotics such as daptomycin, cefoperazone, cefuroxime and doxycycline or their combination are used against persistent agents [8]. Various drug combinations were tested to address borrelial

persisters that exhibit resistance to doxycycline or amoxicillin treatments. Among these combinations, the most promising treatments involved daptomycin in combination with beta-lactams (such as cefoperazone or carbenicillin) or an energy inhibitor (clofazimine) [8].

Another complication is that the bacterium is pleomorphic, *i.e.*, it changes its morphology (formation of biofilms or round bodies) [6] under the pressure of hostile environmental conditions. These morphological changes cause the bacteria to develop a higher persistence to conventional ATB treatment and continue to survive in the host.

Some studies have emerged on how to treat or prevent the spirochete infection more effectively. One of the studies evaluated vaccines that work efficiently in mice, but presently have been no studies on their use in humans. The vaccine that immunized the mice used both recombinant Osp A and Osp B antigens and their effectiveness was evaluated. Results confirmed that in ticks (vector) that were feeding on the immunized mice, the spirochetes were killed regardless of species of *B. burgdorferi* sensu lato [2,9-10]. The mice were protected from infection by the vaccine and this protection did not depend on the region from which the bacteria came from; this means it would be useful against various *B. burgdorferi* s.l. species [9]. Other studies proposed that antimicrobial peptides (AMPs) could be used instead of antibiotics [11,13,17-18,20-21,25-27,28-29,31,50], as they have a non-specific mechanism of action, such as membrane permeabilization or inhibition of protein, DNA or RNA synthesis.

2.2 Antimicrobial peptides (AMPs)

All living organisms produce antimicrobial peptides as part of the innate immune system. The synthesis of AMPs is the response of the immune system to an infection, and AMPs can fight a wide range of pathogens, such as bacteria, viruses, and fungi, commonly through membrane perturbation. To date, approximately 5,000 AMPs have been identified in all eukaryotic and prokaryotic organisms [11,12].

The structure, size, charge, and hydrophobicity of the protein are important for antimicrobial activity and targeting of the peptide. The amino acid length of AMPs ranges from 10 to 100 amino acids [13]. AMPs are frequently encountered as cationic peptides (possessing a positive charge), but some other AMPs have been found to be anionic (possessing a negative charge). Positively charged peptides consist of a hydrophobic character at one end and a hydrophilic character at the other end [12,13]. AMPs display a wide range of diversity in their properties and are classified according to their secondary structures:

- α-helix
- β-sheet (hairpin or looped)
- extended peptides

The α-helix is the predominant and extensively studied secondary structure among AMPs, with approximately 250 peptides (isolated from vertebrates, plants, and invertebrates) adopting this conformation [14]. Amphipathic α-helices exhibit higher activity in contrast to the other structures, although they have not been studied as thoroughly. Positively charged (cationic) α-helix peptides contain high amounts of histidine, lysine, proline, tryptophan, and arginine, while negatively charged (anionic) α-helix peptides are high in glutamic and aspartic acids [14].

β-sheet peptides have 2 β-strands that connect to each other with the aid of disulfide bonding, leading to the formation of a dimer, and contain an arrangement of six cysteine residues that form three disulfide bridges [13,14]. The circular shape of β-sheet peptides is crucial for enabling the antimicrobial activity of β-sheet peptides and the mutations occurring on the cysteines would lead to drastic change leading to deactivation of the antimicrobial activities [13,14]. In some cases, the β-sheet peptides can also be used as antifungal rather than as antimicrobials [12,14].

Loop/hairpin peptide strands can contain a mixture of β-hairpin or could consist of β-sheets and α-helix. They are largely made up of arginine and overproduced proline, which result in hairpin

or looped structures, regardless of their amphipathic nature. Stability, enabled by 1-4 disulfide bridges in the looped or hair-pinned peptides, protects the peptide from proteolytic degradation [14].

The unique structures and charge of AMPs allows them to have different modes of action and therefore can be divided into two groups:

- Membranolytic
- Non-membranolytic

The membranolytic peptides damage the bacterial membrane by membrane permeabilization or liquification of the membrane, thereby causing cell death. The non-membranolytic peptides interact with protein synthesis, enzymes, DNA, RNA and target cellular organelles such as ribosomes. For membranolytic AMPs, their mode of action depends on the peptide structure, the lipid composition of the membrane, the attraction to anionic phospholipids, and the hydrophobic and the amphiphilic properties of the AMP. For both of the AMP groups, any mutations to the structures or sequences of the peptides could lead to the modification of the peptide's activity [12-14, 50].

Research has shown that the use of antibiotics to combat bacterial infections is slowly starting to be ineffective against infections [12,15-17]. Bacteria have established various ways to resist antibiotics by enzymatic breakdown, alteration of drug-targets, changing membrane fluidity, and active elimination of antibiotics by increasing efflux pump production [15, 4, 18-19]. This ability to resist multiple classes of ATB has caused significant increase in loss of life and economic damage in all countries around the world.

Mycobacterium tuberculosis, vancomycin-resistant enterococci, *Klebsiella pneumoniae*, *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* and many others have been identified to be multidrug resistant, including some bacteria that are fully resistant to any antibiotics currently available. The overuse of last-resort drug colistin in multidrug-resistant pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* resulted in the development of a plasmid-mediated mobilized colistin resistance (MCR-1) gene encoding for its resistance [20]. This increase in bacterial resistance has led to a high demand for alternative treatments against antibiotic-resistant bacteria and led to the exploration of antimicrobial peptides as potential replacements for traditional antibiotics.

AMPs are known for their potent antimicrobial effects and various modes of action, making them a promising option for combating antibiotic-resistant bacteria. They have been found to rapidly kill the pathogens they target, and studies have shown that AMPs work synergistically with ATB [12]. This synergistic activity facilitates the eradication of antibiotic-resistant bacterial strains, as well as biofilm and persisters formation by *Borrelia* spp. As a component of bee venom, melittin was found to be active against *B. burgdorferi in vitro* [18]. However, melittin has high cytotoxicity to human cells and sensitivity to proteolytic degradation by proteases. These problems can be overpassed by encapsulation (nanoparticles), modification of the AMPs structure, and specific release strategies [17,21-22].

The AMPs also have antiviral activities against enveloped viruses such as transmissible gastroenteritis coronavirus (TGEV), herpes simplex virus (HSV) or human immunodeficiency virus (HIV). New research even suggests that it may be possible to use AMPs against severe acute respiratory syndrome coronavirus (SARS-CoV-2), *i.e.*, COVID-19 [23]. Additionally, AMPs could be used in immunisation or as vaccine adjuvants to produce protective immunity or to trigger the immune response during infections. The triggering of the immune response was shown in the formulation consisting of a synthetic adjuvant IC31 containing the AMP KLKL5KLK against a chosen antigen. For example, it showed high potency as an adjuvant for a DNA vaccine against *Myobacterium tuberculosis* [15,18]. Due to the rise in antimicrobial resistance, it is proposed that using AMPs in vaccine formulations could help alleviate this problem.

2.3 AMPs against *Borrelia burgdorferi* spirochetes

Various studies have identified several AMPs as potential candidates against *Borrelia burgdorferi* sensu stricto (*B. burgdorferi*) spirochetes. These AMPs include defensins, cathelicidins, persulcatusin, bee venom and its component melittin, and interleukins [24-33]. The defensins are cationic AMPs, found in many eukaryotic organisms, including ticks, and in vectors of LD spirochetes. Mature defensins contain about 38 to 39 amino acids that include six cysteine residues linked in a recurring pattern of three intramolecular disulfide bridges. The bridges connect in a sequence of Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 [24]. This formation is typical for arthropod defensins and is referred to as the cysteine-stabilised αβ motif, which is crucial for the antimicrobial properties of defensins. Tick defensins, similarly to other AMPs, also cause cell

membrane permeabilization and have been found to show antimicrobial activity against various bacteria, including *B. burgdorferi* [25].

However, it should be noted that persulcatusin, a defensin originating from the vector ticks *Ixodes persulcatus*, did not show activity against the spirochetes [26]. In contrast, *in vitro* investigations have demonstrated that the defensins derived from the tick *Dermacentor variabilis* possess a potent bactericidal effect on the borrelia spirochetes, successfully eliminating all viable cells that could be cultured. Furthermore, the use of a chicken lysozyme in combination with defensin has been shown to enhance its effect [27].

In contrast, cathelicidins that possess the same mode of action but no effect on borrelial cells are found in various mammalian species (pigs, humans and other animals belonging to the order Artiodactyla) [26, 27]. They are usually inactive peptides that are stored in neutrophil secretory granules, activated by proteolysis, and can be released extracellularly or into the phagosome. It was initially named "cathelin" after a polypeptide found in pig leukocytes before cathelicidins were identified [29].

Bee venom, which has been in medicinal use for about 5000 years, was isolated from honeybee (*Apis mellifera*) and has been shown to have activity against gram-negative bacteria such as *Escherichia coli* and *B. burgdorferi* [17]. Melittin is a polypeptide that comprises about 40% to 60% of the dry honeybee venom and is 26 amino acids in length [30]. While it is used against *E. coli*, it acts on the bacterial membrane, inducing pore formation on the surface of the membrane that leads to leakage of cell contents [22]. In contrast to *D. variabilis* defensin activity against borrelia, the 7-day subculture of spirochetes treated with bee venom and melittin reportedly still contain observable viable spirochetes cells [27, 17, 30]. As mentioned before, there are problems with the use of bee venom or melittin for treatment in humans.

Interleukins (ILs) represent a group of cytokines and have been found to be secreted by many types of blood cells, not just leukocytes. Interleukins play an important role in activating and differentiating immune cells, including cell proliferation, and modulation of pro- and anti-inflammatory processes [32, 33]. One of the most notable ILs is the cytokine IL-26, which is a protein consisting of 171 amino acids that belongs to the IL-10 family. It can bind to and form complexes with the DNA of microbes such as *E. coli*, *Staphylococcus aureus*, and *Pseudomonas*. The IL-26 has been shown to induce an amplified immune response against borrelia and may also partially degrade its DNA. It is believed that IL-26 intercalates with DNA without completely

breaking it down. Moreover, activation of human macrophages by IL-26 monomers results in enhanced phagocytosis of borrelial cells [32,33].

2.4 *Anisakis simplex* species complex and their AMPs anisaxins

Anisakis pegreffii is a parasitic nematode that belongs to the *Anisakis simplex* sensu lato species complex that causes fish-borne zoonosis called anisakiasis [34,35]. The life cycle of the nematode involves five stages and various marine organisms (Figure 2). The first two stages involve intermediate hosts (crustaceans and small fish), the third stage involves paratenic hosts (cephalopods and many fish), and in the final stage, toothed whales as definitive hosts [35,36]. Cephalopods and fish infected with third-stage larvae of *Anisakis* spp. can also infect humans as accidental hosts if they are consumed raw or undercooked. However, the nematode is not capable of developing fully in the gastrointestinal environment of humans. As a result, the parasite dies, and the decomposition of the parasite triggers an immune response characterized by the appearance of eosinophilic granulomas [36,37]. The infection is recorded worldwide in at least 39 different countries. The number of cases worldwide was around 76,000 and Japan alone accounted for approximately 20,000 cases of anisakiasis per year [63].

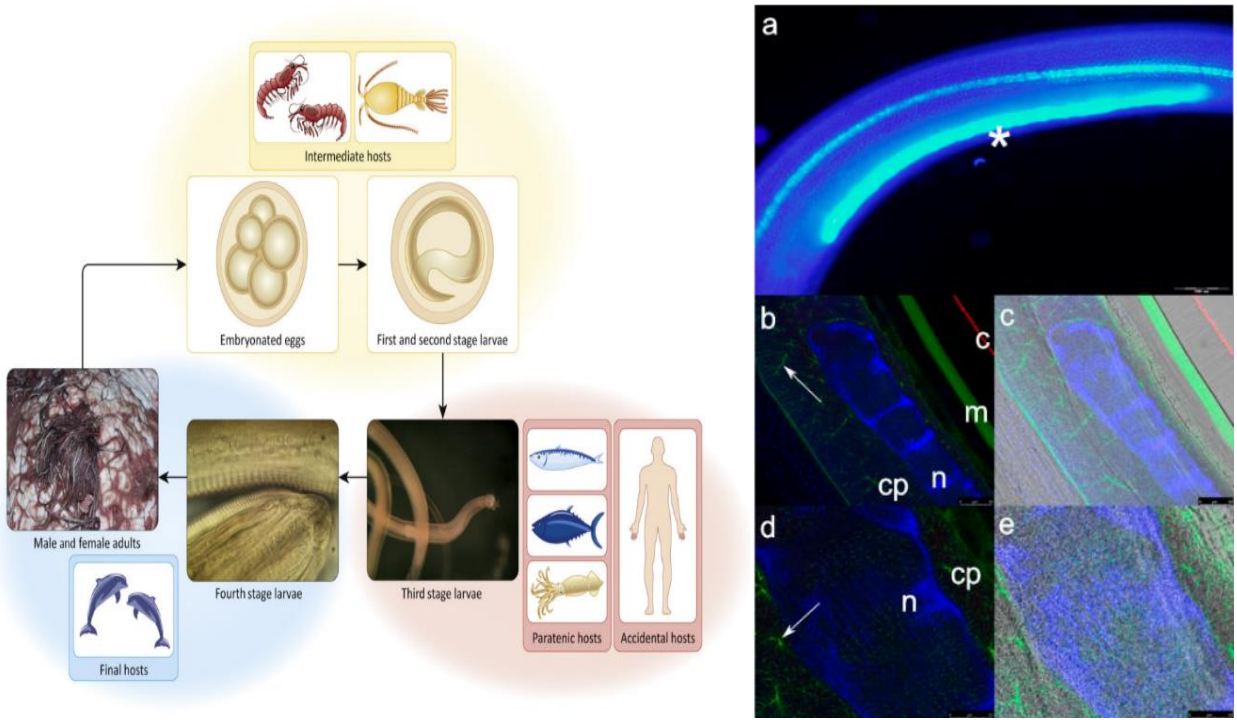

Figure 2: A) Life cycle of *Anisakis pegreffii*, B) the excretory gland of the organism ((a) The entire specimen, when observed as a whole, exhibits a prolonged, rod-shaped nucleus found in the excretory cell (*). (b) Excretory cell constituents: the nucleus (n), cytoplasm (cp), somatic muscles (m), and the cuticle (c). The arrow points to the canaliculi located in the excretory cell cytoplasm. (c) Comparable to (b) with a merged bright field and immunofluorescence. (d) Higher magnification reveals excretory cell nucleus (n), cytoplasm (cp), and canaliculi (arrow). (e) Enhanced view from (d) with merged bright field and immunofluorescence.) [35,38].

Several AMPs from *Anisakis pegreffii* and *A. simplex*, anisaxins, were studied and have been shown to exhibit antimicrobial activity. These peptides are likely to originate from the excretory gland cell of the nematode (Figure 2) [38]. Recent research of anisaxins AMPs (A-1, A-2P, A-2S, A-3 and A-4) showed that they cause membrane permeabilization of some of the gram-negative bacteria [22].

Peptide	Name	Sequence	Charge	H[a]	H[rel b]
		----:----|----:----|----:----|-			
Anisaxin-1	A-1	SWLSKTYKKLENSAKKRIAEGIAIALRGGPR	+6	-2.1	0.31
Anisaxin-2S	A-2S	SWLSKTWKKLENSGKKRIAEGIAIALKGGLR	+6	-1.6	0.30
Anisaxin-2P	A-2P	SWLSKTWKKLENSGKKRIAEGIAIALKGGAR	+6	-1.9	0.35
Anisaxin-3	A-3	SWLSKTAKKLENSAKKRIAEGIAIAIQGGPR	+5	-2.1	0.28
Anisaxin-4	A-4	GWLSKTWKKLENSAKKRIAEGIAIAIRGGPR	+6	-1.8	0.34
As-cecropin [20]	*As*-Cec	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	+5	-2.1	0.29
Melittin [53]	Mel	GIGAVKLVLTTGLPALISWIKRKRQQ-NH_2	+6	0	0.48

a) Hydrophobicity, calculated using the CCS scale
b) Hydrophobic moment relative to a perfectly amphipathic helical peptide of 18 residues. Conserved positions are shaded gray.

Figure 3: The peptide sequences and the physio-chemical properties of different anisaxin AMPs [22].

Anisaxins are cecropin-like α-helical antimicrobial peptides with a slight amphipathic amino acid composition in the mature sequence, at the N-terminus, designated as their cationic region. The mature sequence is the highly conserved part of the peptide, whereas the N- and C-terminal parts tend to be variable regions. Anisaxins have a conserved tryptophan at the second position that was shown to provide extensive antimicrobial activities. It is typical for these AMPs to assume a α-helical conformation in an anisotropic environment, which is present to some extent in highly potent AMPs. The cytotoxicity of anisaxin is low, but *in vivo* they show high instability and rapid degradation [22].

In this study, A-1 is applied to cultures of *B. burgdorferi* to evaluate its antimicrobial efficacy. Melittin and ATBs, both known to have a detrimental effect against borrelia, were included in our study and used as controls to further evaluate the impacts of A-1 against LD spirochetes.

3. Materials and Methods

Briefly, for the cultivation of *Borrelia burgdorferi* sensu stricto, a fresh liquid medium was prepared, and the bacteria were left to grow for a set number of days. The bacteria were checked for growth and purity using dark-field microscopy regularly. A dark-field microscope and a Petroff-Hausser counting chamber were used to count the number of cells and then prepare the spirochete samples for the following treatment with AMP and ATBs. The treated samples and the

untreated samples were stained with a LIVE/DEAD™ BacLight™ Bacterial Viability kit according to manufacturer's protocol (ThermoFisher Scientific [39]). This kit permits quick and easy determination of cell viability using two common microscope filters (FITC and TRITC) based on intracellular esterase activity and plasma membrane integrity. The *Borrelia* were then observed under the fluorescent microscope. The images were recorded and fused using Fiji ImageJ software to distinguish between live and dead cells.

3.1 Cultivation of *Borrelia burgdorferi* sensu stricto

3.1.2 Modified Kelly-Pettenkofer (MKP) medium

The spirochetes were cultivated in modified Kelly-Pettenkofer (MKP) liquid medium. The medium was prepared according to the protocol from Ružić-Sabljić et al. [38]. Table 1 lists the amount of ingredients used in the preparation of 200 ml of MKP medium.

Table 1: Reagents and their amounts for the preparation of MKP medium.

Reagents	Amount (g)
CMRL-1066	1.94
Neopeptone	0.6
HEPES	1.2
Citric acid	0.14
D-Glucose	0.6
Pyruvic acid	0.16
N-acetyl glucosamine	0.08
Sodium bicarbonate	0.4

The procedure for the basic MKP medium preparation (200 ml):

All the solid components were dissolved in 100 ml of ddH_2O (by stirring approximately 60 minutes). After complete dissolution, the pH was adjusted to 7.6 with concentrated NaOH (10 N) and the final volume was adjusted to 200 ml. For the preparation of the complete MKP medium, the reagents that were added to the basic medium (200 ml) are listed in the table below.

Table 2: Reagents and their amounts for complete MKP medium.

Reagent	Amount (g)
BSA	2.46
Reagents	Volume (ml)
7% Gelatine	40
Rabbit serum	14.4

Gelatine was prepared by boiling it in the microwave until complete dissolution and cooling before adding it to the mixture. After complete dissolution of all components, the medium was sterilised by filtration, aliquoted into 50 ml centrifuge tubes, and stored at +4°C until use. The storage time of the complete MKP medium should not exceed 1 month. An aliquot (5 ml) of freshly prepared medium was kept at 34°C for several days and then checked under a dark-field microscope to control sterility.

3.1.3 Cultivation

The complete MKP medium was preheated before use to room temperature and then aliquoted into 15 ml tubes with 11 ml of media. The *Borrelia* from the frozen stock (-80°C) was used to seed the culture in fresh MKP medium. The newly prepared culture was incubated at 34°C for a week before being checked under the dark-field microscope.

3.2 Microscopy

Instrumentation	Objectives used
Dark-field microscope: Leica DM 1000 LED (Leica)	x20
Fluorescent microscope: Olympus BX60 (Olympus)	x40

The density of the bacterial working culture was then determined by a Petroff-Hausser counting chamber. For this, 10 µl of the culture was pipetted onto the Petroff-Hausser counting chamber and then the cells were counted at a magnification of x20 in five different squares of the chamber.

The cell density was calculated according to the following formula:

$$\boldsymbol{A \times 1.25 \times 10^6}$$

Formula 1: Calculation of the final concentration of cells in a culture, where A represents the average number of spirochetes in five squares of the chamber, 1.25 is the dilution factor and 10^6 represents the cells per ml.

3.3 Treatment with AMP, melittin, and antibiotics

Before treatment of the borrelia culture, the stock solution of anisaxin A-1 (1.08 mM) was diluted with ddH_2O 1:100. A series of dilutions was made (see section Results below). The 900 µl of diluted spirochete culture (about 10^5-10^6 cells/ml) was aliquoted into 2 ml tubes and then each tube was filled up to 1 ml with the corresponding dilution of either A-1 or melittin and left for 3 days at room temperature. The melittin was used as a positive control.

For the antibiotic treatment of *Borrelia* we followed the protocol from Jie Feng et al. [8] with doxycycline, cefoperazone and daptomycin. The concentration of the stock solution was 10 mg/ml (both for individual antibiotics and their mixture). The antibiotics here were not used to compare with the collected data, but to verify the claimed killing effect of other studies. A single tube containing 1 ml of culture was left untreated (negative control), while the rest were treated, to check the viability of the bacteria. The samples were then incubated, and fluorescence microscopy of the prepared samples was performed after incubation.

3.4 Staining

SYTO™ 9 and propidium iodide (LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit) were used for staining the cells. The staining was preformed according to protocol provided with the kit (ThermoFischer Scientific) [39] with 0.3 µl of dye used for staining of 100 µl of culture (0.3%). After applying the dye, the samples were incubated in a dark room for 15 minutes before fluorescent microscopy. One of the samples was left untreated as a negative control to check the

vitality of the spirochetes. This was done to compare whether cell death was due to the chemical treatment used or was occurring naturally.

3.5 Fluorescent microscopy

Images taken under the fluorescent microscope were taken with a 40x objective and a 20 µm scale was burnt in. The red and green images acquired from microscopy were then subsequently merged using an image processing package Fiji [40]. The images of the negative control were checked first, before processing treated cultures. Using fluorescent microscopy, approximately 4-8 image pairs (one red and one green) were captured for each concertation. In total, approximately 505 images were made.

3.5 Statistics

From the images, the number of dead cells was counted. This was then transformed into percentage data and graphed. Statistical testing and data visualization were performed using the RStudio version: 2021.9.0.351 [41]. The data was arcsine transformed, which handles and spans out the data points of percentages between 0 and 1. Subsequently, the acquired values of the arithmetic mean and confidence limits were back-transformed.

One-way analysis of variance (ANOVA) and Tukey HSD (honestly significant difference) were performed using transformed data. This data is normally distributed, *i.e.*, they are symmetrically spread without skew. ANOVA is a statistical technique used to compare the means of two or more independent groups simultaneously. It enables the measurement of differences between means of multiple groups and provides an overall summary of these differences. By examining the variance within and between groups, ANOVA can determine whether the observed differences in means are statistically significant or simply due to chance [42,43]. To validate the ANOVA results, a Tukey HSD test was performed to identify specific groups that contain significant differences between their means. The test involves computing a critical value based on the sample size, the number of groups, and the level of significance. Then it compares the differences in the means of each group pair to the critical value, and if the difference is greater than the critical value, it indicates a significant difference between those groups [44,45].

Both ANOVA and Tukey HSD have a chosen significance level ($\alpha = 0.05$), and the resulting p-values are used to determine if the results are significant [46-49]. In addition to these parameters, ANOVA also calculates the F-value, which is a ratio of variances between groups and variances

within groups. The F-value is used to test the null hypothesis that the means of all groups are equal, and a large F-value suggests that there is a significant difference between the means of at least two groups [48,49].

4. Aims

The project aims to evaluate the antimicrobial capacity of AMP anisaxin A-1 against *Borrelia burgdorferi* sensu stricto. Specifically, anisaxin A-1 was used to determine whether its treatment of live *Borrelia* affects the viability of the spirochete and if its effect is comparable to that of traditionally used ATB and melittin in the possible treatment of LD.

5. Results

5.1 Cultivation

The SCW-53 strain (*B. burgdorferi* sensu stricto) previously checked for infectivity and dissemination in mice was selected to be used in the experiments. The cultivation was done from a frozen stock of borrelia as described before. In case of the appearance of unexpected culture contamination, the samples were syringe filtrated and re-used after 7 days of recovery.

5.2 Dilution of anisaxin-1 and melittin

After the series of dilutions, the following concentrations obtained were used in further experiments (listed in Table 3):

Table 3: Concentrations of A-1 used in the experiments.

A-1 concentration	Volume (µl)	ddH_2O (µl)
1 µM	9.25	1990.75
5 µM	1.85	1998.15
500 nM	9.25	190.75
50 nM	20	180
5 nM	20	180
500 pM	20	180
50 pM	20	180

The 1 µM and 5 µM dilutions were made directly from the stock solution of A-1 (1.08 mM), while the rest of the concentrations were made using serial dilutions. Each AMP concertation was tested 8 times in total, while 1 µM and 5 µM were tested 4 times.

Melittin dilutions were performed in a similar manner as A-1, but the calculations were made starting from the lower concertation of the stock solution (0.985 mM) (Table 4). According to this table, the higher concentration ranges used in A-1 were excluded from the melittin experimentation.

Table 4: Concentrations of melittin used in the experiments.

Melittin concentration	Volume (µl)	ddH_2O (µl)
500 nM	10.15	189.85
50 nM	20	180
5 nM	20	180
500 pM	20	180
50 pM	20	180

5.2 Microscopy

Using dark-field microscopy, the number of cells was calculated for each experiment. The following dilutions were made from 3-day old cultures (Table 5).

Table 5: Average number of spirochetes found in five squares of the counting chamber and the respective cell concertation.

Number of spirochetes	Concentration (cells/ml)
0.6	$7.5x10^5$
1.2	$1.5x10^6$
2.4	$3.0x10^6$

In the following figures, a collection of images of borrelial cells taken under different types of microscopes is included. Figure 4 contains an image of live and untreated borrelial cells taken

under a dark-field microscope, while in Figures 6-13 are images taken under a fluorescent microscope representing either treated or untreated cells.

Figure 4: A 2-day-old culture obtained from sub-cultivation of *Borrelia burgdorferi* from original bacterial stock; image taken under dark-field microscope (Leica DM 1000 LED).

The Live/Dead staining kit made bacteria fluoresce in a specific colour under the light of the fluorescent microscope. Bacteria with intact membranes (live bacteria) were stained green, while those with damaged membranes (dead) were stained red. The results of fluorescent microscopy showed some effect of anisaxin-1 on *B. burgdorferi*. The results are summarized in the images below.

5.2.1 Results of A-1 treatment

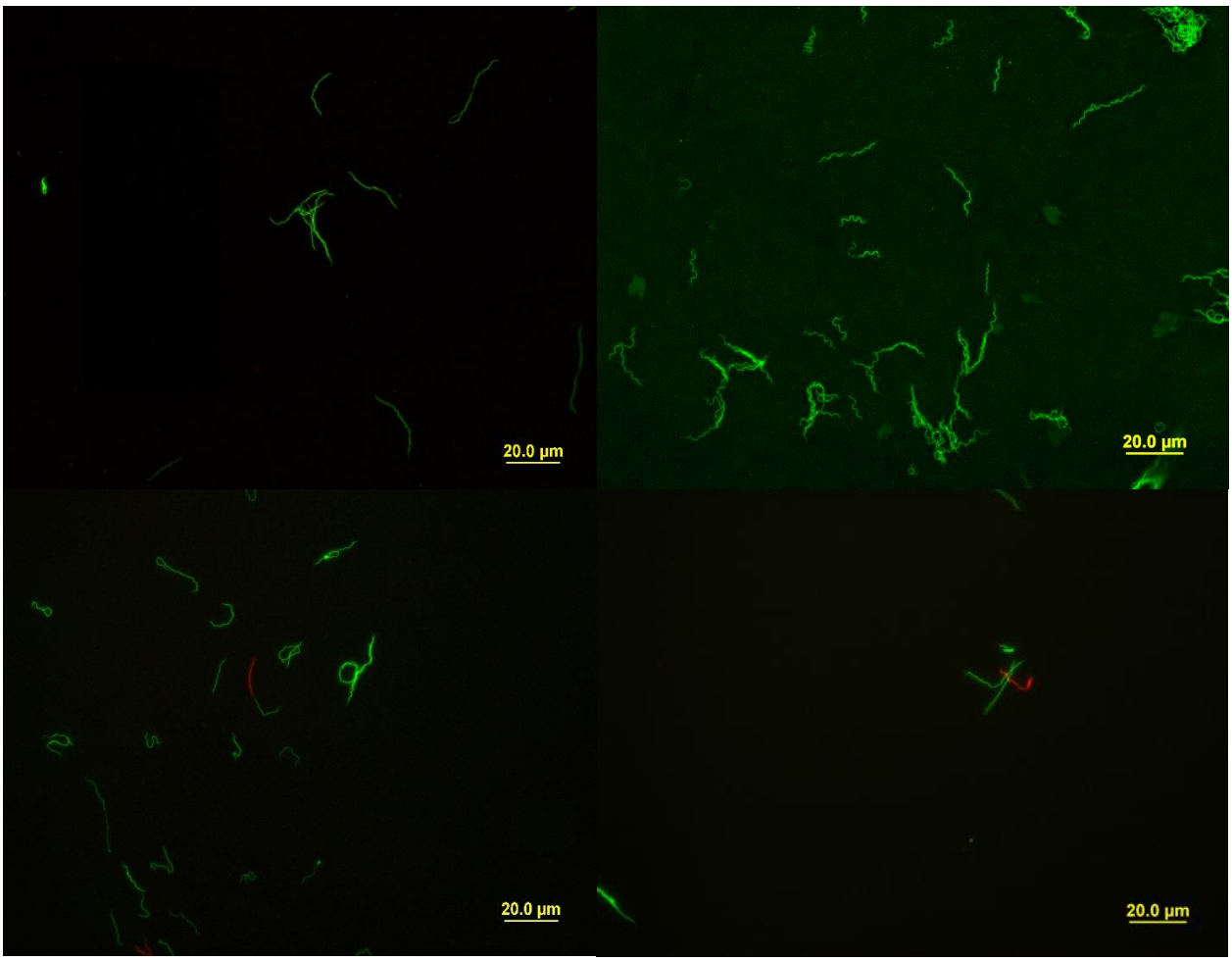

Figure 5: Untreated (negative) control cultures of *Borrelia burgdorferi* tested to confirm whether the bacterial culture used was viable and able to survive under given physiological conditions. The expected cell death is negligible here. Cells that stain red are considered dead and those that stain green are alive.

The negative control cultures under the fluorescent microscope display high motility and mostly fluoresce green, as it would be expected from a live culture. The live/dead ratio is 96.98% alive to 3.01% dead cells. Dead cell count should not exceed 10% for bacteria that have been taken out of the exponential phase of growth, so that any cell death observed here is attributed to physiological death.

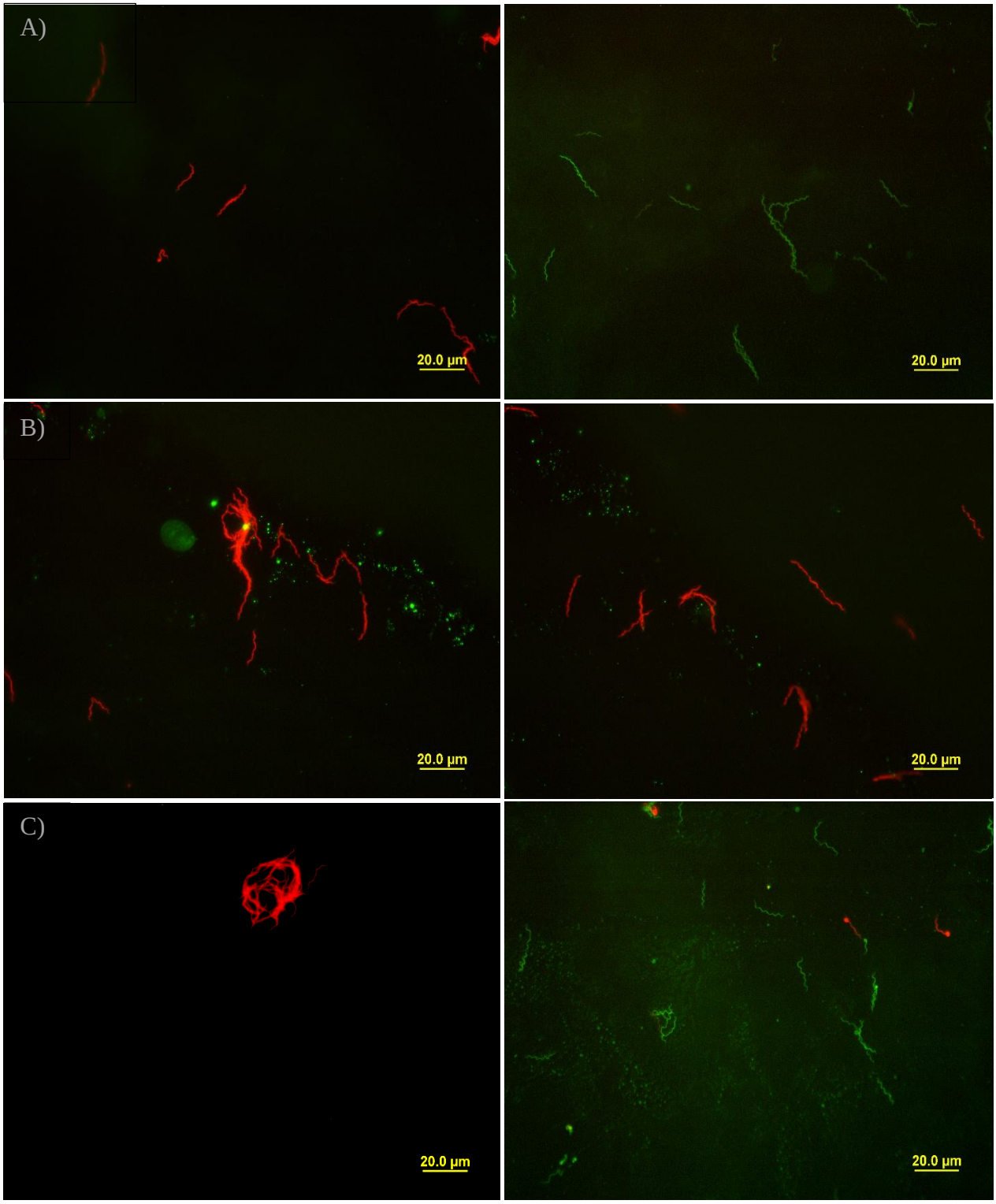

Figure 6: Images from fluorescent microscopy showing from left to right: A) *B. burgdorferi* cell cultures treated with 50 pM A-1, B) 500 pM and C) 5 nM A-1. Cells that stain red are considered dead and those that stain green are alive. The unlabelled images on the right are from the same concentrations as the images on the left.

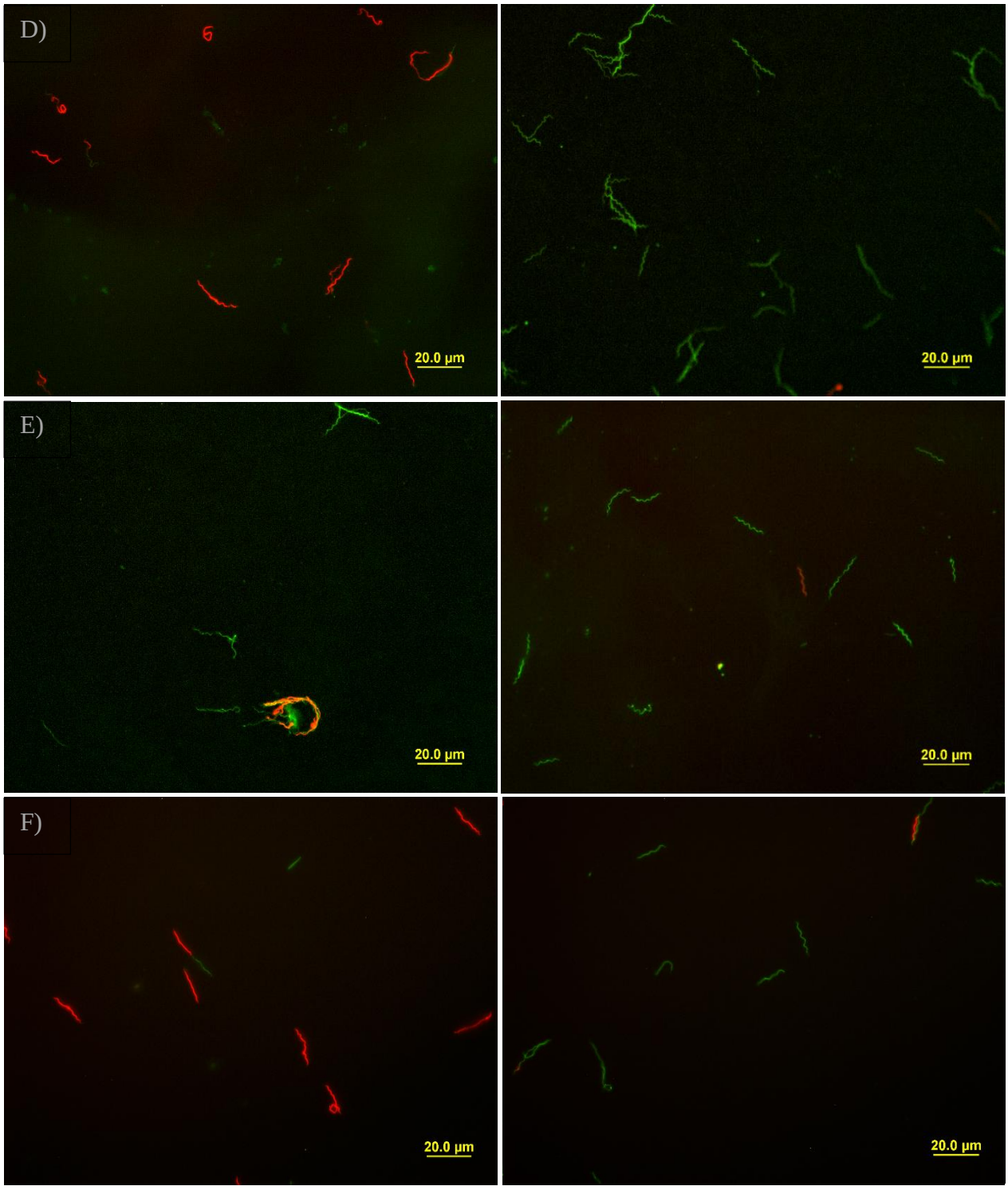

Figure 7: Images from fluorescent microscopy showing from left to right *Borrelia burgdorferi* cell cultures treated with: D) 50 nM; E) 500 nM and F) 1 µM of A-1 AMP. Cells that stain red are considered dead and those that stain green are alive. The unlabelled images on the right are from the same concentrations as the images on the left.

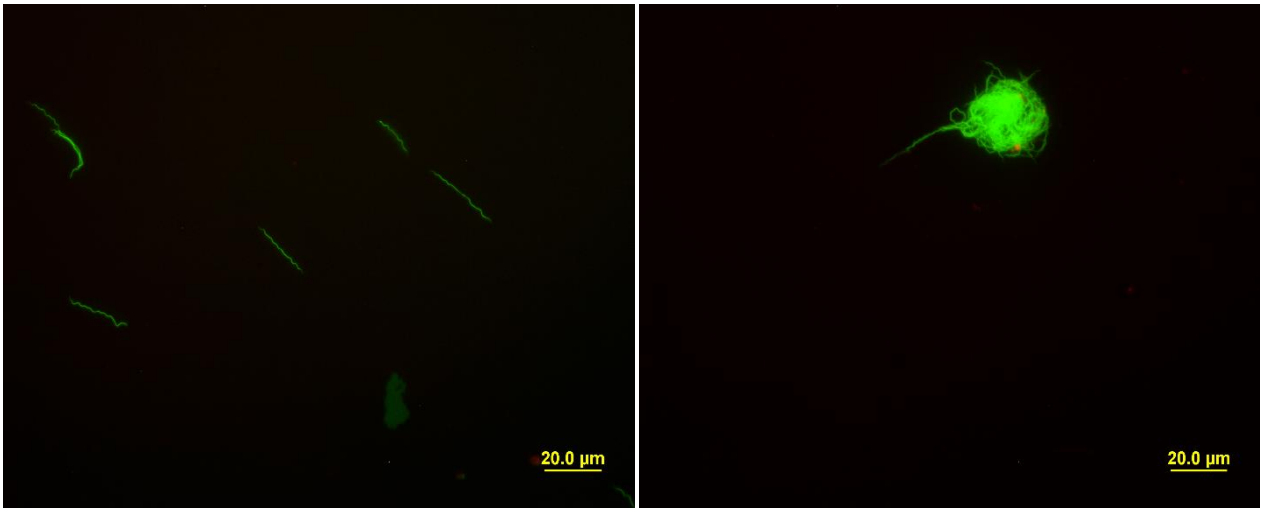

Figure 8: Images of 5 µM A-1 AMP treated *Borrelia burgdorferi* cell cultures. Cells that stain red are considered dead and those that stain green are alive.

The cultures treated with A-1 show mostly viable cells, with the live/dead ratio (Table 7) remaining inconsistent across various concentration ranges. Notably, at 5 µM, a slightly higher percentage of live cells was observed compared to other concentrations, with a ratio of 87.30% live to 12.70% dead. In contrast, the average live-to-dead ratios for the other concentrations were approximately 70.80% to 29.65%. To further interpret the data, statistical tests were conducted to provide a more comprehensive analysis of the results presented in Figure 14.

5.2.2 Results of melittin treatment

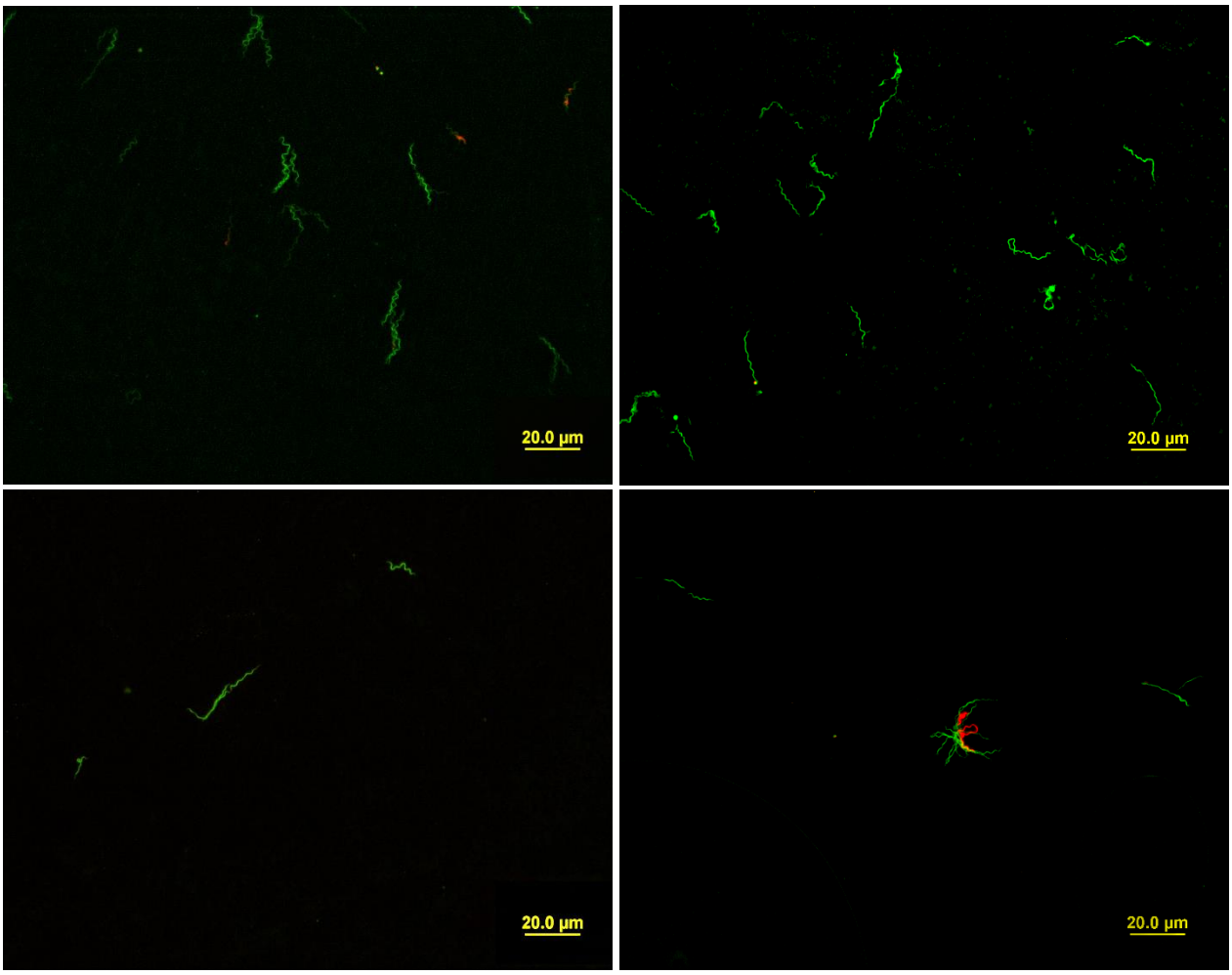

Figure 9: Untreated (negative) control cultures of *Borrelia burgdorferi* tested to confirm whether the bacterial culture used was viable and able to survive under given physiological conditions. The expected cell death is negligible here. Cells that stain red are considered dead and those that stain green are alive.

Melittin negative controls were showing similar survival to those of A-1. They contained 90.99% live cells and 9% dead cells on average. Again, as expected for any living culture, no more than a few cells were observed to be dead in all the experiments.

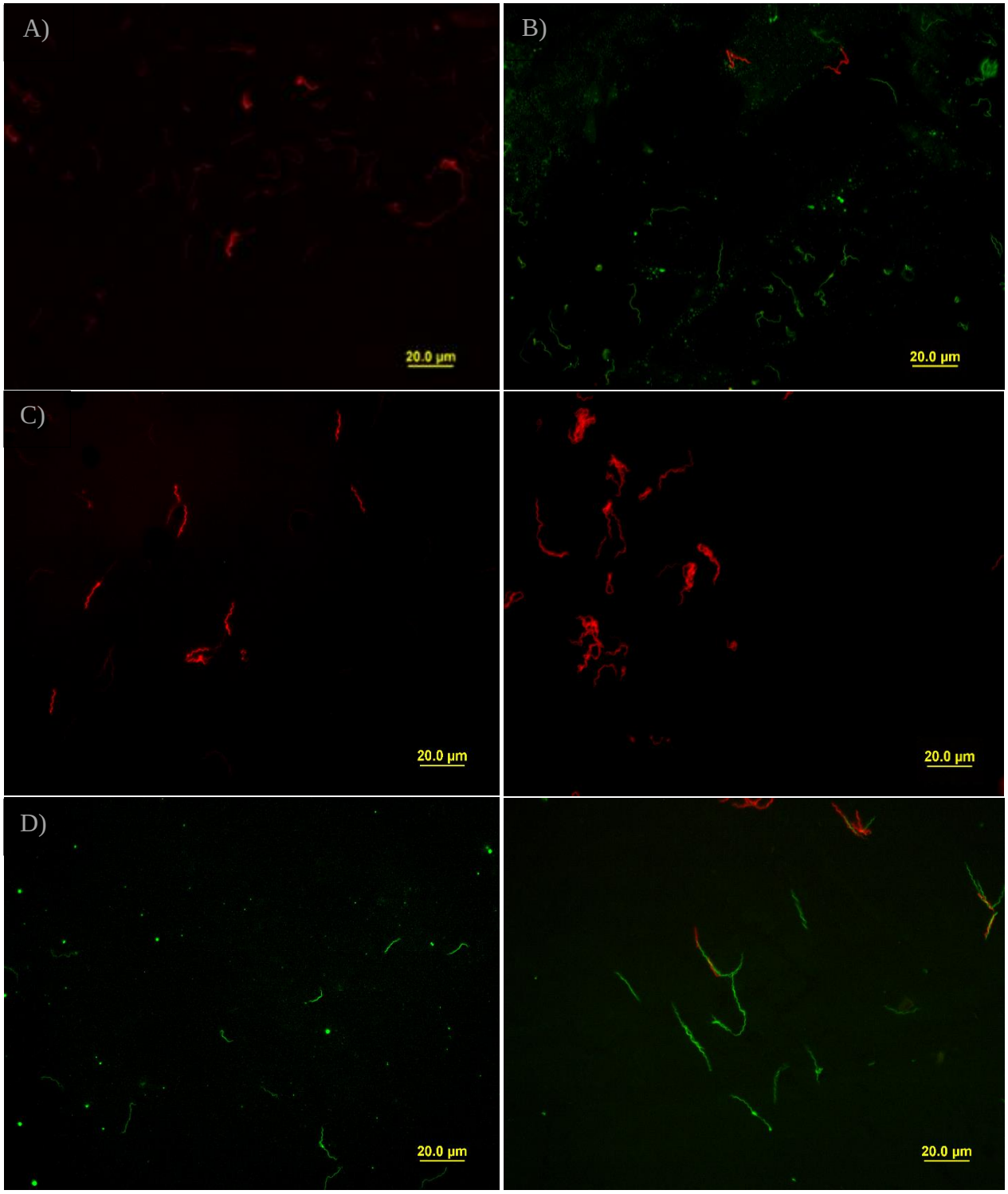

Figure 10: Images from fluorescent microscopy showing from left to right: A) 50 pM, B) 500 pM, C) 5 nM and D) 50 nM melittin treated *B. burgdorferi* cell cultures. Cells that stain red are considered dead and those that stain green are alive. The unlabelled images on the right are from the same concentrations as the images on the left.

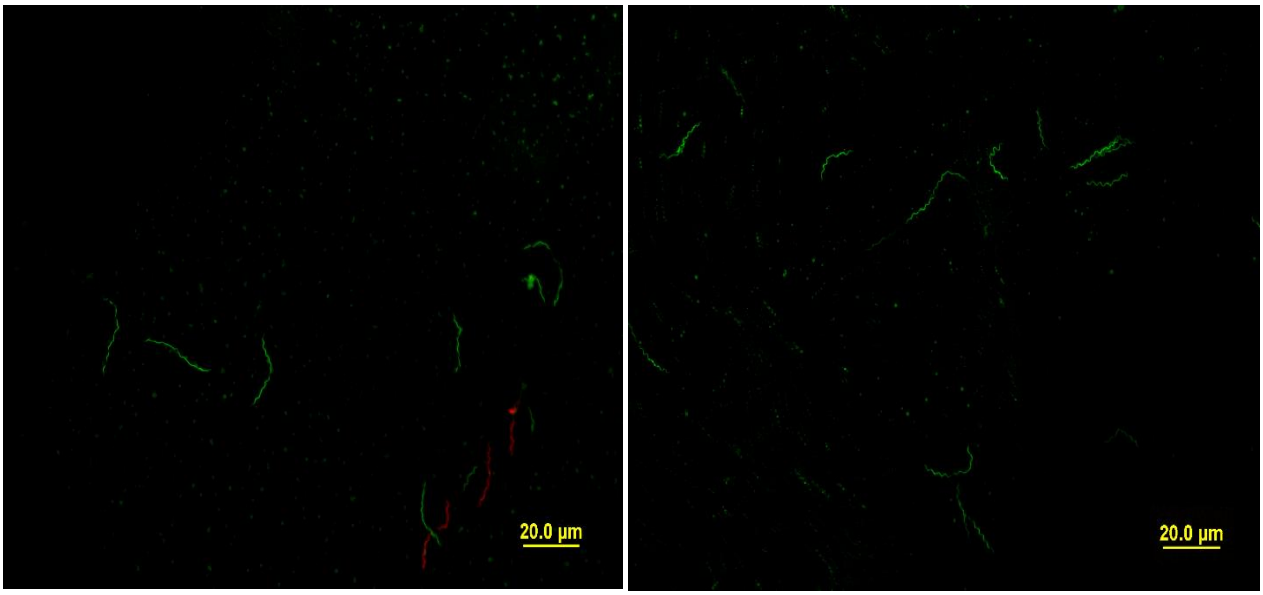

Figure 11: Images showing 500 nM melittin treated *B. burgdorferi* cell cultures. Cells that stain red are considered dead and those that stain green are alive.

The melittin treated cultures were problematic and later proved to be unreliable. The melittin used for the experiment appears to have been inactive and did not show much activity against borrelia as expected. The images here are part of the preliminary acquired data and show an increased killing effect at lower concentrations (explained in Section 6). The dead/live percentage of melittin treatment is summarized in Table 6.

Table 6: The average % of dead *B. burgdorferi* cells per melittin concentration used.

Concentration	Live (%)	Dead (%)
500 nM	56.03	43.96
50 nM	77.77	22.22
5 nM	33.33	66.66
500 pM	91.03	8.96
50 pM	16.04	83.95

5.2.3 Antibiotic treatment

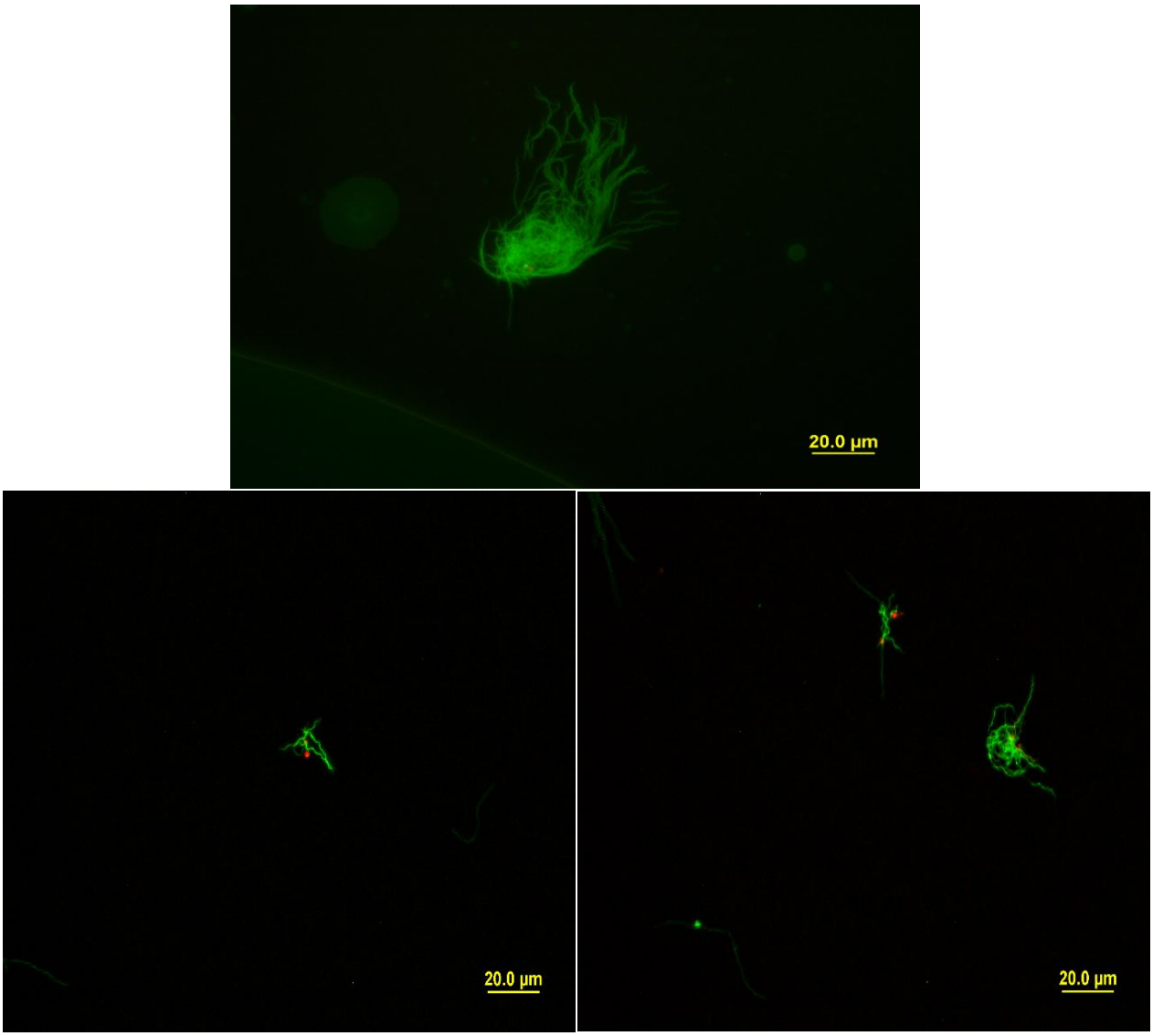

Figure 12: Untreated (negative) control cultures of *Borrelia burgdorferi* tested to confirm whether the bacterial culture used was viable and able to survive under given physiological conditions. The expected cell death is negligible here. Cells that stain red are considered dead and those that stain green are alive.

The antibiotics negative control (untreated) cultures were healthy and alive comparable to the negative controls of other treatments. The highest live/dead ratio among the three cultures was 98.2% live cells and 1.62% dead cells.

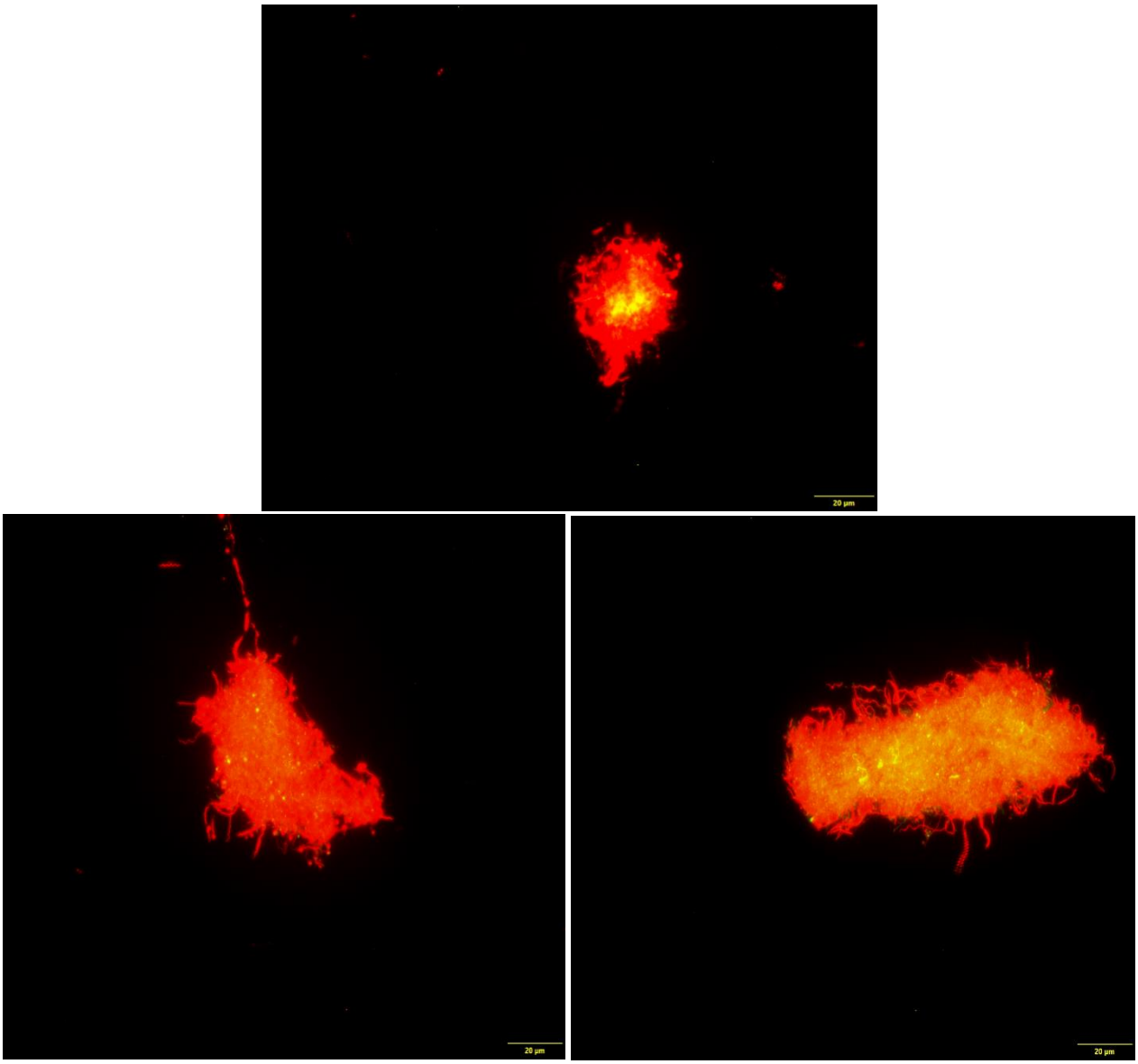

Figure 13: *Borrelia burgdorferi* cultures treated with an antibiotic mixture (doxycycline, cefoperazone, and daptomycin). The high level of cell death here was expected. Cells that stain red are considered dead and those that stain green are alive.

The antibiotic treatment is known to be highly effective against spirochetes. This is further confirmed by the observed cells being stained red in Figure 13. The live/dead count for the antibiotically treated culture was around 5.24% live cells to 94.5% dead cells. The mixture of the three antibiotics was used, as it was shown to be most effective against spirochetes. The final concentration of the antibiotic mixture used was 50 µg/ml.

5.3 Statistical analyses

The plot below shows the confidence intervals of each level (concentration of A-1) and the line connecting each point connects the means of each level. The y-axis displays cell death in percentages from 0-100%.

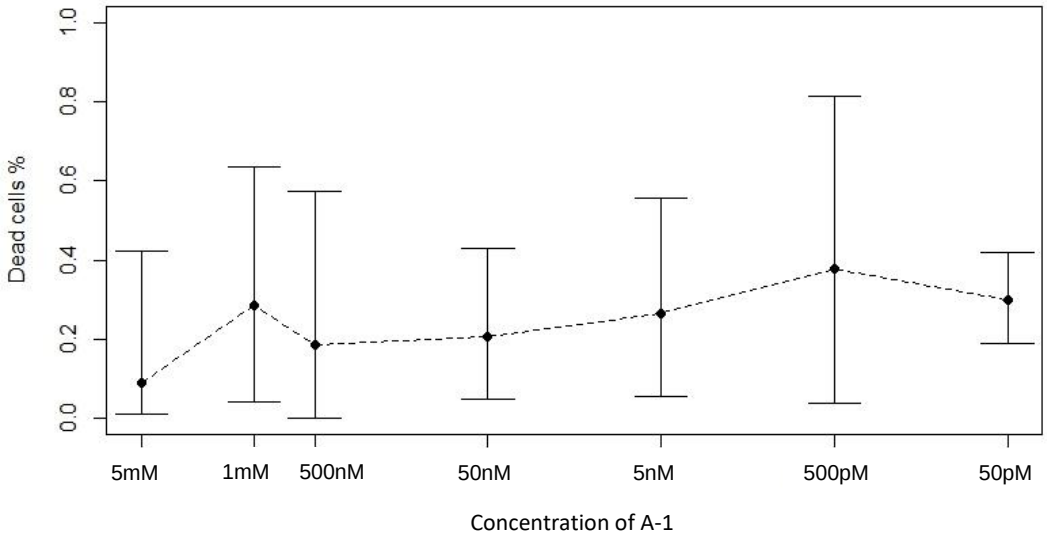

Figure 14: Graphical representation of *B. burgdorferi* dead cells (%, shown on the y-axis) that were treated with different A-1 concentrations (5 µM to 50 pM, shown on the x-axis). The lines above and below the points on the graph depict the confidence intervals, while the points themselves represent the mean value of each concentration. The x-axis, which on the left side contains the concentrations labelled as mM, represents the micro molar rather than the milli molar concentration.

The chosen significance level for testing is $\alpha = 0.05$. According to ANOVA, it rejects the null hypothesis (F-value: 0.481; p-value: 0.818), and the post hoc Tukey HSD test as well, confirming that there are no significant differences between levels as the p-values are too high. Based on tests conducted within the chosen concentration ranges, it has been determined that A-1 does not have a significant effect on the viability of the spirochetes. The following table contains the averaging of the results from all experiments conducted for each concentration range of A-1.

Table 7: Containing average % of live/dead *B. burgdorferi* cells depending on A-1 concentration used.

Concentration	Live cells (%)	Dead cells (%)
500 nM	77.77	22.23
50 nM	74.76	25.23
5 nM	69	31
500 pM	60.27	39.73
50 pM	69.80	30.19
5 µM	87.30	12.70
1 µM	70.86	29.15

6. Discussion

Due to the lack of effective antibiotics in the treatments of possible *B. burgdorferi* infection, interest in the use of natural amtimicrobial peptides (AMPs) has increased. The AMPs have been found to have a non-specific mechanism of action, proving to work well against antibiotic resistant bacteria. AMPs of anisakids have been proven to be active against gram-negative bacteria, such as *E. coli* [22], therefore, one of them was chosen to test its antimicrobial activity against LD spirochetes.

This is the first and preliminary research on anisaxin efficiency against *Borrelia in vitro*. The live/dead bacterial staining method with fluorescence microscopy was used to verify the viability of cells after anisaxin treatment. The testing showed a large spread of the data in the graph (Figure 14) and resulted in a nonsignificant ANOVA and Tukey HSD inferring that the different concentrations of anisaxin did not show significant differences among themselves. This strongly suggests that the concentrations tested in this study did not show an effect on the viability of *Borrelia*. However, it might not necessarily mean that A-1 is not able to affect the viability of spirochetes if used at different concentrations and maybe used under different experimental conditions.

The study of Roncevic et al. [22] on anisaxins (A-1, A-2S, A-2P, A-3 and A-4) revealed their broad-spectrum antibacterial activity against gram-negative bacteria, particularly against *E. coli*, *K. pneumoniae*, and *A. baumannii*, with minimal inhibitory concentrations (MIC) and

minimum bactericidal concentrations (MBC) ranging from 0.5 µM to 0.25 µM. The study also states that the effect of anisaxins on other gram-positive and gram-negative bacteria is comparable to the effects found on *E. coli*. The study proposes that anisaxins kills *E. coli* by permeabilizing the cell membrane, with this effect occurring at a minimum inhibitory concentration (MIC) of 0.5 µM, affecting around 85% to 90% of cells. The sub-MIC concentrations initially displayed effects similar to the reported MIC, but a decrease in the percentage of affected cells was noted after exposure exceeding 15 minutes.

When we compared the results of the effect of A-1 against *E. coli* with current findings in the case of *B. burgdorferi* at a concentration of 1 µM, it was observed that its activity was significantly lower against spirochetes than against *E. coli*, resulting in approximately 30% dead cells versus 85%. Concentrations lower than 1 µM did not show any notable increase in the number of dead cells, showing at the highest ~40% of dead cells (Table 7).

Although melittin was shown to be significantly active against spirochetes by other studies [17,21-22,30], it was not possible to confirm or reproduce these results in the current work. One of the explanations might be the possible degradation of melittin under the experimental conditions that were selected in our project. Due to this factor, the results presented in Table 6 are biased.

Lastly, the effect of the three antibiotics, doxycycline, cefoperazone, daptomycin, and their combination, against *B. burgdorferi* were evaluated using the protocol published earlier. Available research data on the use of these antibiotics individually to treat borrelial cultures have shown that their activity is high in 3-day log phase cultures in which replicating forms of *Borrelia* were the most prevalent. In the case of culture older than 7 days, where replicating forms may change their morphology, the above showed a marked decrease in their antimicrobial activity [8]. This is because viable spirochetes might develop antibiotic persistence, producing round bodies, cysts, or biofilms. However, using the combination of all three antibiotics revealed a significant improvement of antimicrobial activity against the spirochetes. In the same paper, a full eradication of borrelial cells was observed in the 7-day subcultures [8]. This was also confirmed in our experiments and in a 7-day culture showing spirochete eradication, as shown in Figure 13.

Comparing the antimicrobial activity of the ATB mixture with the results of the A-1 effect on the viability of *Borrelia* cells, it can be suggested that the effect of A-1 is not so significant, as expected, based on previous studies with the use of A-1 in other bacteria. However, doxycycline, cefoperazone and daptomycin are antibiotics, so their effect on bacteria differs from that of natural antimicrobial peptides, such as A-1.

In general, the mechanism of action of antibiotics involves binding to specific proteins, whereas AMPs (depending on the type) affect proteins, DNA, RNA, and cell membranes. For antibiotics such as cefoperazone which are a class of β-lactams, the mode of action involves binding to specific penicillin-binding proteins (PBPs) that are located within the bacterial membrane and inhibit the final stage of cell wall synthesis [52-53]. While doxycycline is a type of tetracycline antibiotic and allosterically binds to the 30S prokaryotic ribosomal subunit of bacteria. This halts the synthesis of essential proteins for bacterial viability [53-55].

However, when it comes to daptomycin, which is a type of a cyclic peptide, the mechanism of action is not well understood and there is not a single mechanism that is fully accepted for daptomycin. The proposed modes of action for daptomycin is either facilitated by calcium binding, by altering the membrane fluidity, or influencing the membrane potential [53, 56-61].

A cecropin-like A-1 is one of the four anisaxins observed to possess characteristics that could provide potent antimicrobial activity (the net charge of almost all anisaxin peptides was found to be +6, and only A-3 being +5). The respective anisaxin peptide sequences can be found in Figure 3. The proposed mechanism of action for these peptides is membrane permeabilization that occurs in treated *E. coli* cells found in the study by Roncevic et al. [22]. A similar mechanism of action was found in melittin, which is a component of honeybee venom (*Apis mellifera*) [21-22]. Melittin consists of a 26 amino acid long chain and has been sequenced. It possesses various activities (such as biological, pharmacological, toxicological, even strong surface activity on cell lipid membranes, antibacterial and antifungal) as well as possible tumour inhibiting characteristics [30]. Studies have found that melittin can effectively clear spirochetes in cultures in their different alternative morphological forms, but this effect could not be confirmed [17, 21].

Live/dead bacterial staining has been tested under various bacteria in various conditions and has been shown to be a reliable method for the detection of the viability of bacterial cultures. The kit stains bacteria based on the state of the membrane, which means that bacteria with damaged

membranes stain red (dead cells) and green with intact membranes (living cells). Although it stains bacteria green/red, it has been found to miss false-positives or -negatives. In some cases, it is possible that the observed red cells could recover from compromised membranes and reproduce normally, while those that stain green with intact membranes may not be able to reproduce in nutrient rich media [39]. One way to circumvent the issues of staining kits is to perform a recultivation of the treated cultures. In this case, the treated samples are transferred into fresh MKP media and left to grow for 7 days, then using a dark-field microscope and a counting chamber to assess the number of bacteria present in the recultivated sample. If the samples were observed to consist of only dead cells using the staining kit, then after the recultivation process, it should also show that there are no recovered spirochetes under dark-field microscopy. However, if any of these "dead" cells were to recover during the recultivation then a population of regrown bacteria would be observed. This method is not exact for quantitative tests, but it could show whether bacteria reproduction has been significantly impacted compared to a culture that is normally developing [8]. This would be a valuable feature of the method in qualitative testing of the efficacy of natural AMPs, such as anisaxins, against LD spirochetes.

Although there are experiments that show a slightly improved killing effect on the borrelial cells, currently there is not enough data altogether to support the hypothesis that anisaxin A-1 is effective against *B. burgdorferi* sensu stricto at any concentration range tested in this project.

7. Conclusion

The effects of different concentrations of anisaxin A-1 on the viability of *B. burgdorferi* sensu stricto *in vitro* were tested. No significant effect of A-1 on *Borrelia* spirochete viability was observed at selected and used concentrations of peptide (from 50 pM to 5 µM). The experimental conditions that were selected for presented study do not reveal any anti-borrelial activity of anisaxin A-1, opposed to the use of traditional antibiotics or their combination against LD spirochetes. The results of live *Borrelia* with melittin were not reliable and inconclusive, which might not necessarily be related to the biological properties of melittin.

The results of our study suggest the establishment of better experimental conditions for the trials, as well as testing of other anisaxins, such as A-2S, A-2P, etc., to determine their effect on the viability of Lyme disease spirochetes. Functional assessment of *Anisakis* spp. AMPs could provide better understanding of its active sites and determine the location, mechanism, and its effect on the bacterial viability.

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9. References

[1] Rudenko, N., & Golovchenko, M. (2021). Sexual transmission of Lyme borreliosis? The question that calls for an answer. Tropical Medicine and Infectious Disease, 6(2), 87. https://doi.org/10.3390/tropicalmed6020087.

[2] Rudenko, N., Golovchenko, M., Grubhoffer, L., & Oliver, J. H. (2011). Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. Ticks and Tick-borne Diseases, 2(3), 123–128. https://doi.org/10.1016/j.ttbdis.2011.04.002.

[3] Pal, U., & Fikrig, E. (2003). Adaptation of *Borrelia burgdorferi* in the vector and vertebrate host. Microbes and Infection, 5(7), 659–666. https://doi.org/10.1016/s1286-4579(03)00097-2.

[4] Lyme borreliosis (2022). Hunfeld, K.-P. & Gray, J. Lyme borreliosis. In Springer eBooks. https://doi.org/10.1007/978-3-030-93680-8.

[5] Anderson, C. C., & Brissette, C. A. (2021). The brilliance of borrelia: mechanisms of host immune evasion by Lyme disease-causing spirochetes. Pathogens, 10(3), 281. https://doi.org/10.3390/pathogens10030281.

[6] Rudenko, N., Golovchenko, M., Kybicova, K., & Vancova, M. (2019). Metamorphoses of Lyme disease spirochetes: phenomenon of borrelia persisters. Parasites & Vectors, 12(1). https://doi.org/10.1186/s13071-019-3495-7.

[7] Wolcott, K. A., Margos, G., Fingerle, V., & Becker, N. S. (2021). Host association of *Borrelia burgdorferi* sensu lato: a review. Ticks and Tick-Borne Diseases, 12(5), 101766. https://doi.org/10.1016/j.ttbdis.2021.101766.

[8] Feng, J., Auwaerter, P. G., & Zhang, Y. (2015). Drug combinations against *Borrelia burgdorferi* persisters *in vitro*: eradication achieved by using daptomycin, cefoperazone and doxycycline. PLOS ONE, 10(3), e0117207. https://doi.org/10.1371/journal.pone.0117207.

[9] Fikrig, E., Telford, S. R., Wallich, R., Chen, M., Lobet, Y., Matuschka, F. R., Kimsey, R. B., Kantor, F. S., Barthold, S. W., Spielman, A., & Flavell, R. A. (1995). Vaccination against Lyme disease caused by diverse *Borrelia burgdorferi*. Journal of Experimental Medicine, 181(1), 215–221. https://doi.org/10.1084/jem.181.1.215.

[10] Richer, L., Brisson, D., Melo, R., Ostfeld, R. S., Zeidner, N. S., & Gomes-Solecki, M. (2014). Reservoir targeted vaccine against *Borrelia burgdorferi*: a new strategy to prevent Lyme disease transmission. The Journal of Infectious Diseases, 209(12), 1972–1980. https://doi.org/10.1093/infdis/jiu005.

[11] Cotton, S., Donnelly, S., Robinson, M. W., Dalton, J. P., & Thivierge, K. (2012). Defense peptides secreted by helminth pathogens: antimicrobial and/or immunomodulator molecules? Frontiers in Immunology, 3. https://doi.org/10.3389/fimmu.2012.00269.

[12] Michel, A. (2013). Antimicrobial peptides. Pharmaceuticals, 6(12), 1543–1575. https://doi.org/10.3390/ph6121543.

[13] Zhang, Q., Ye, Z., Meng, Y., Hong, X., Shao, G., Ma, J., Cheng, X., Li, J., Kang, J., & Fu, C. (2021). Antimicrobial peptides: mechanism of action, activity and clinical potential. Military Medical Research, 8(1). https://doi.org/10.1186/s40779-021-00343-2.

[14] Nayab, S., Aslam, M., Rahman, S. U., Sindhu, Z. U. D., Sajid, S., Zafar, N., Razaq, M., Kanwar, R., & Amanullah, (2022). A review of antimicrobial peptides: its function, mode of action and therapeutic potential. International Journal of Peptide Research and Therapeutics, 28(1). https://doi.org/10.1007/s10989-021-10325-6.

[15] Arranz-Trullén, J., Lu, L., Pulido, D., Bhakta, S., & Boix, E. (2017). Host antimicrobial peptides: the promise of new treatment strategies against tuberculosis. Frontiers in Immunology, 8. https://doi.org/10.3389/fimmu.2017.01499.

[16] Haney, E. F., & Hancock, R. E. W. (2013). Peptide design for antimicrobial and immunomodulatory applications. Biopolymers, 100(6), 572–583. https://doi.org/10.1002/bip.22250.

[17] Socarras, K. M., Theophilus, P. a. S., Torres, J. P., Gupta, K., & Sapi, E. (2017). Antimicrobial activity of bee Venom and melittin against *Borrelia burgdorferi*. Antibiotics, 6(4), 31. https://doi.org/10.3390/antibiotics6040031.

[18] Rima, M., Rima, M., Fajloun, Z., Sabatier, J., Bechinger, B., & Naas, T. (2021). Antimicrobial peptides: a potent alternative to antibiotics. Antibiotics, 10(9), 1095. https://doi.org/10.3390/antibiotics10091095.

[19] Bacterial enzymes and antibiotic resistance. (2018, December 1). PubMed. https://pubmed.ncbi.nlm.nih.gov/30713760.

[20] Mwangi, J., Hou, X., Lai, R., & Zhang, Z. (2019). Antimicrobial peptides: new hope in the war against multidrug resistance. Zoological Research, 40(6), 488–505. https://doi.org/10.24272/j.issn.2095-8137.2019.062.

[21] Lubke, L., & Garon, C. F. (1997). The antimicrobial agent melittin exhibits powerful in vitro inhibitory effects on the Lyme disease Spirochete. Clinical Infectious Diseases, 25(s1), S48–S51. https://doi.org/10.1086/516165.

[22] Rončević, T., Gerdol, M., Mardirossian, M., Maleš, M., Cvjetan, S., Benincasa, M., Maravić, A., Gajski, G., Krce, L., Aviani, I., Hrabar, J., Trumbić, Ž., Derks, M. G., Pallavicini, A., Weingarth, M., Zoranić, L., Tossi, A., & Mladineo, I. (2022). Anisaxins, helical antimicrobial peptides from marine parasites, kill resistant bacteria by lipid extraction and membrane disruption. Acta Biomaterialia, 146, 131–144. https://doi.org/10.1016/j.actbio.2022.04.025.

[23] Maleki, M. S. M., Rostamian, M., & Madanchi, H. (2021). Antimicrobial peptides and other peptide-like therapeutics as promising candidates to combat SARS-CoV-2. Expert Review of Anti-infective Therapy, 19(10), 1205–1217. https://doi.org/10.1080/14787210.2021.1912593.

[24] Kalenik, B. M., Góra-Sochacka, A., & Sirko, A. (2018). B-defensins – Underestimated peptides in influenza combat. Virus Research, 247, 10–14. https://doi.org/10.1016/j.virusres.2018.01.008.

[25] Wu, J., Zhou, X., Chen, Q., Chen, Z., Zhang, J., Yang, L., Sun, Y., Wang, G., Dai, J., & Feng, T. (2022). Defensins as a promising class of tick antimicrobial peptides: a scoping review. Infectious Diseases of Poverty, 11(1). https://doi.org/10.1186/s40249-022-00996-8.

[26] Isogai, E., Isogai, H., Okumura, K., Hori, H., Tsuruta, H., & Kurebayashi, Y. (2010). Tertiary structure-related activity of tick defensin (persulcatusin) in the taiga tick, *Ixodes persulcatus*. Experimental and Applied Acarology, 53(1), 71–77. https://doi.org/10.1007/s10493-010-9379-3.

[27] Chrudimská, T., Čeřovský, V., Slaninová, J., Rego, R. O. M., & Grubhoffer, L. (2014). Defensin from the ornate sheep tick Dermacentor marginatus and its effect on Lyme borreliosis spirochetes. Developmental and Comparative Immunology, 46(2), 165–170. https://doi.org/10.1016/j.dci.2014.04.005.

[28] Isogai, E., Isogai, H., Takahashi, K., Kobayashi-Sakamoto, M., & Okumura, K. (2009). Antimicrobial activity of three tick defensins and four mammalian cathelicidin-derived synthetic peptides against Lyme disease spirochetes and bacteria isolated from the midgut. Experimental and Applied Acarology, 49(3), 221–228. https://doi.org/10.1007/s10493-009-9251-5.

[29] Sambri, V., Marangoni, A., Giacani, L., Gennaro, R., Murgia, R., Cevenini, R., & Cinco, M. (2002). Comparative *in vitro* activity of five cathelicidin-derived synthetic peptides against *Leptospira*, *Borrelia* and *Treponema pallidum*. Journal of Antimicrobial Chemotherapy, 50(6), 895–902. https://doi.org/10.1093/jac/dkf220.

[30] Chen, J., Guan, S., Sun, W., & Han, F. (2016). Melittin, the major pain-producing substance of bee venom. Neuroscience Bulletin, 32(3), 265–272. https://doi.org/10.1007/s12264-016-0024-y.

[31] Vaillant A.A.J. & Qurie, A. (2022, August 22). Interleukin. StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK499840/.

[32] Hirsh, J., Kositangool, P., Shah, A., Radwan, Y., Padilla, D. C., Barragan, J., & Cervantes, J. (2020). IL-26 mediated human cell activation and antimicrobial activity against *Borrelia burgdorferi*. Current Research in Microbial Sciences, 1, 30–36. https://doi.org/10.1016/j.crmicr.2020.05.003.

[33] Tengvall, S., Fru, K., & Lindén, A. (2015). Interleukin-26: an emerging player in host defense and inflammation. Journal of Innate Immunity, 8(1), 15–22. https://doi.org/10.1159/000434646.

[34] Mladineo, I., & Hrabar, J. (2020). *Anisakis pegreffii*. Trends in Parasitology, 36(8), 717–718. https://doi.org/10.1016/j.pt.2020.03.004.

[35] Audicana, M. T. (2022). Anisakis, Something is moving inside the fish. Pathogens, 11(3), 326. https://doi.org/10.3390/pathogens11030326.

[36] Mladineo, I., Charouli, A., Jelić, F., Chakroborty, A., & Hrabar, J. (2023). *In vitro* culture of the zoonotic nematode *Anisakis pegreffii* (Nematoda, Anisakidae). Acta Naturae, 16(1). https://doi.org/10.1186/s13071-022-05629-5.

[37] Mladineo, I., Hrabar, J., Smodlaka, H., Palmer, L., Sakamaki, K., Keklikoglou, K., & Katharios, P. (2019). Functional ultrastructure of the excretory gland cell in zoonotic anisakids (Anisakidae, nematoda). Cells, 8(11), 1451. https://doi.org/10.3390/cells8111451.

[38] Ružić-Sabljić, E., & Strle, F. (2004). Comparison of growth of *Borrelia afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto in MKP and BSK-II medium. International Journal of Medical Microbiology, 294(6), 407–412. https://doi.org/10.1016/j.ijmm.2004.08.002.

[39] LIVE/DEAD BaCLight Bacterial Viability Kit Protocol | Thermo Fisher Scientific - IE. (n.d.). https://www.thermofisher.com/cz/en/home/references/protocols/cell-and-tissue-analysis/protocols/live-dead-baclight-bacterial-viability-protocol.html.

[40] Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinévez, J., White, D. J., Hartenstein, V., Eliceiri, K. W., Tomančák, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods, 9(7), 676–682. https://doi.org/10.1038/nmeth.2019.

[41] RStudio Team (2021). RStudio: Integrated Development Environment for R. RStudio, PBC, Boston, MA URL: http://www.rstudio.com/.

[42] ANOVA Test: Definition, Types, Examples, SPSS - Statistics How to. (2024, January 4). Statistics How To. https://www.statisticshowto.com/probability-and-statistics/hypothesis-testing/anova/.

[43] Miller, R. G., Jr. (1997). Beyond ANOVA: Basics of Applied Statistics. CRC Press.

[44] Tukey, J. W. (1984). The collected works of John W. Tukey. Chapman & Hall.

[45] How to calculate statistical significance. (2019, March 2). Sciencing. https://sciencing.com/how-to-calculate-statistical-significance-12286080.html.

[46] Alpha Level (Significance Level): What is it? - Statistics How To. (2024, January 9). Statistics How To. https://www.statisticshowto.com/probability-and-statistics/statistics-definitions/what-is-an-alpha-level/.

[47] Gonick, L. (2005). The Cartoon Guide to Statistics.

[48] F statistic/F value: Definition and how to run an F-Test. (2024, January 5). Statistics How To. https://www.statisticshowto.com/probability-and-statistics/f-statistic-value-test/.

[49] Archdeacon, T. J. (1994). Correlation and regression analysis: A historian's guide. Univ of Wisconsin Press.

[50] Reddy, K. J., Yedery, R. D., & Aranha, C. (2004). Antimicrobial peptides: premises and promises. International Journal of Antimicrobial Agents, 24(6), 536–547. https://doi.org/10.1016/j.ijantimicag.2004.09.005.

[51] Margos, G., Hepner, S., & Fingerle, V. (2022). Characteristics of *Borrelia burgdorferi* sensu lato. In Springer eBooks (pp. 1–29). https://doi.org/10.1007/978-3-030-93680-8_1.

[52] Jones, R.N., & Barry, A.L. (191983b). CefOperaZone: a review of its antimicrobial spectrum, B-lactamase stability, enzyme inhibition, and other in vitro characteristics. Clinical Infectious Diseases, 5(Supplement 1), S108–S126. https://doi.org/10.1093/clinids/5.supplement_1.s108.

[53] DrugBank Online | Database for drug and drug target info. (n.d.). DrugBank. https://go.drugbank.com/.

[54] Patel, R. S. (2023, May 22). Doxycycline hyclate. StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK555888/.

[55] Patel, R. S. (2023, May 22). *Doxycycline hyclate*. StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK555888/.

[56] Karas, J. A., Carter, G. P., Howden, B. P., Turner, A. M., Paulin, O. K. A., Swarbrick, J., Baker, M. A., Li, J., & Velkov, T. (2020). Structure–Activity relationships of daptomycin lipopeptides. Journal of Medicinal Chemistry, 63(22), 13266–13290. https://doi.org/10.1021/acs.jmedchem.0c00780.

[57] Allen, N. E., Hobbs, J. N., & Alborn, W. E. (1987). Inhibition of peptidoglycan biosynthesis in gram-positive bacteria by LY146032. Antimicrobial Agents and Chemotherapy, 31(7), 1093-1099. https://doi.org/10.1128/aac.31.7.1093.

[59] Grein, F., Müller, A., Scherer, K. M., Liu, X., Ludwig, K. C., Klöckner, A., Strach, M., Sahl, H., Kubitscheck, U., & Schneider, T. (2020). Ca2+-Daptomycin targets cell wall biosynthesis by forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids. Nature Communications, 11(1). https://doi.org/10.1038/s41467-020-15257-1.

[60] Alborn, W. E., Allen, N. E., & Preston, D. A. (1991). Daptomycin disrupts membrane potential in growing Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 35(11), 2282–2287. https://doi.org/10.1128/aac.35.11.2282.

[61] Allen, N. E., Alborn, W. E., & Hobbs, J. N. (1991). Inhibition of membrane potential dependent amino acid transport by daptomycin. Antimicrobial Agents and Chemotherapy, 35(12), 2639–2642. https://doi.org/10.1128/aac.35.12.2639.

[62] Sultan, S. Z., Manne, A., Stewart, P. E., Bestor, A., Rosa, P. A., Charon, N. W., & Motaleb, M. A. (2013). Motility is crucial for the infectious life cycle of Borrelia burgdorferi. Infection and Immunity, 81(6), 2012–2021. https://doi.org/10.1128/iai.01228-12.

[63] Shamsi, S., & Barton, D. P. (2023). A critical review of anisakidosis cases occurring globally. Parasitology Research, 122(8), 1733–1745. https://doi.org/10.1007/s00436-023078819.

[64] Kuehn, B. M. (2013). CDC estimates 300 000 US cases of Lyme disease annually. JAMA(Chicago, Ill.), 310(11), 1110. https://doi.org/10.1001/jama.2013.278331.

[65] Nelson, C., Saha, S., Kugeler, K. J., Delorey, M. J., Shankar, M., Hinckley, A. F., & Mead, P. S. (2015). Incidence of Clinician-Diagnosed Lyme Disease, United States, 2005–2010.Emerging Infectious Diseases, 21(9), 1625–1631. https://doi.org/10.3201/eid2109.150417.

[66] Kugeler, K. J., Schwartz, A. M., Delorey, M. J., Mead, P. S., & Hinckley, A. F. (2021). Estimating the frequency of Lyme disease diagnoses, United States, 2010–2018. Emerging Infectious Diseases, 27(2), 616–619. https://doi.org/10.3201/eid2702.202731.

[67] Wiśniewska et al. (2018). Motion for a resolution on Lyme disease (Borreliosis) | B8-0514/2018 | European Parliament. © European Union, 2018 - Source: European Parliament. https://www.europarl.europa.eu/doceo/document/B-8-2018-0514_EN.html.