ISPITIVANJE *IN VITRO* OSETLJIVOSTI *BORRELIA AFZELII*, UZROČNIKA LAJMSKE BORELIOZE, NA SUPERNATANTE *LACTIPLANTIBACILLUS PLANTARUM* I *SACCHAROMYCES BOULARDII*

ORIGINALNI RAD

ORIGINAL ARTICLE

IN VITRO SUSCEPTIBILITY OF *BORRELIA AFZELII*, THE CAUSATIVE AGENT OF LYME BORRELIOSIS, TO THE SUPERNATANTS OF *LACTIPLANTIBACILLUS PLANTARUM* AND *SACCHAROMYCES BOULARDII*

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SAŽETAK

Uvod: Lajmska borelioza (LB) je multisistemsko oboljenje koje uzrokuju bakterije iz kompleksa *Borrelia burgdorferi* sensu lato, a prenose je ubodom krpelji roda *Ixodes*. Terapija antibioticima zahteva dugotrajno lečenje, dok trenutno na tržištu ne postoji topikalni proizvod za profilaktičku primenu nakon uboda krpelja koji bi sprečio lokalnu infekciju i dalju diseminaciju patogena u sistemsku cirkulaciju.

Cilj rada: Cilj studije bio je da se *in vitro* ispita uticaj supernatanata probiotskih mikroorganizama *Saccharomyces boulardii* i *Lactiplantibacillus plantarum* i njihovih mešavina na rast i pokretljivost *Borrelia afzelii*.

Materijali i metode: Korišćen je soj *B. afzelii*, prethodno izolovan iz krpelja vrste *Ixodes ricinus*, koji je umnožen do koncentracije 1–2 x 10^6 borelija/mL u Barbour-Stoenner-Kelly-H hranljivoj podlozi. Za ispitivanje osetljivosti testiranog soja na probiotske mikroorganizme korišćena je modifikovana bujon mikrodiluciona metoda za borelije. Minimalne inhibitorne koncentracije (MIK) određene su posmatranjem broja i pokretljivosti borelija nakon 72 sata inkubacije pod anaerobnim uslovima u prisustvu supernatanata, a u poređenju sa pozitivnom kontrolom – netretiranom kulturom *B. afzelii*.

Rezultati: MIK je definisana kao najniža koncentracija testiranog supernatanta pri kojoj borelije nisu bile pokretne a njihov broj je bio smanjen u odnosu na pozitivnu kontrolu. Definisane su sledeće MIK vrednosti: medijana 12,5% za *S. boulardii* i 6% za *L. plantarum*, dok je najniža MIK vrednost (medijana 0,8%) definisana za mešavinu supernatanta probiotskih mikroorganizama.

Zaključak: Supernatanti *S. boulardii* i *L. plantarum* pokazali su antimikrobno dejstvo na *B. afzelii*, dok je mešavina ovih supernatanata pokazala efektivniji inhibitorni efekat na rast i pokretljivost borelija. Dobijeni rezultati daju nam osnovu za dalja istraživanja molekularnih mehanizama inhibitornog dejstva probiotskih mikroorganizama na rast borelija.

Ključne reči: Lajmska borelioza, *Borrelia afzelii*, osetljivost, *in vitro*, probiotski mikroorganizmi, minimalne inhibitorne koncentracije

ABSTRACT

Introduction: Lyme borreliosis (LB) is a multisystem disorder caused by bacteria of the *Borrelia burgdorferi* sensu lato complex, transmitted to humans through the bite of *Ixodes* spp. ticks. Standard treatment typically involves prolonged antibiotic therapy. Currently, no topical product is available on the market for prophylactic use following a tick bite.

Aim: The aim of the study was to investigate *in vitro* the effects of supernatants derived from the probiotic microorganisms *Saccharomyces boulardii* and *Lactiplantibacillus plantarum*, and their mixtures, on the growth and motility of *Borrelia afzelii.*

Materials and methods: To test the susceptibility of *B. afzelii* to probiotic microorganisms, a modified broth microdilution method adapted for *Borrelia* species was employed. Minimum inhibitory concentrations (MICs) were determined by evaluating both the number and motility of *B. afzelii* after 72 hours of incubation with supernatants under anaerobic conditions, compared to a positive control consisting of untreated *B. afzelii* culture.

Results: The MIC was defined as the lowest concentration of the tested supernatant at which borrelia were non-motile and their number was reduced compared to the positive control. The following median MIC values were determined: 12.5% for *S. boulardii*, 6% for *L. plantarum*, and the lowest median MIC value of 0.8% was defined for the supernatant mixture of probiotic microorganisms.

Conclusion: The mixture of *S. boulardii* and *Lactiplantibacillus plantarum* exhibited the strongest inhibitory effect on the growth and motility of *B. afzelii*. These results provide a foundation for further investigation into the molecular mechanisms of the inhibitory activity of probiotic microorganisms against *Borrelia* species.

Keywords: Lyme borreliosis, *Borrelia afzelii*, susceptibility, *in vitro*, probiotic microorganisms, minimum inhibitory concentrations

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UVOD

Lajmska borelioza (LB) je zoonoza čije uzročnike ubodom prenose inficirani krpelji roda *Ixodes*. Uzročnici LB su spiralne bakterije iz kompleksa *Borrelia burgdorferi* sensu lato (s.l.) [1,2]. Primarni vektori u Evropi su krpelji vrste *Ixodes ricinus* [3]. Budući da LB predstavlja multisistemsko oboljenje ljudi i životinja, bolest se može ispoljiti različitim kliničkim manifestacijama kao što su: kožne lezije, mišićno-skeletne, neurološke, okularne i/ili kardiovaskularne manifestacije, dok u nekim slučajevima, infekcija prolazi asimptomatski ili čak u kasnijim fazama LB može biti pogrešno dijagnostikovana zbog kompleksnosti kliničkih znakova i simptoma [2,4].

Kompleks *B. burgdorferi* s.l. obuhvata do sada 21 imenovanu vrstu borelija. Dok je u Severnoj Americi glavni uzročnik LB vrsta *Borrelia burgdorferi* sensu stricto (s.s.), u Evropi je prisutan veći diverzitet borelija sa pet potvrđenih patogenih vrsta: *Borrelia afzelii, Borrelia garinii, B. burgdorferi* s.s., *Borrelia spielmanii* i *Borrelia bavariensis*. Vrste *Borrelia lusitaniae*, *Borrelia valaisiana* i *Borrelia bissettii* su takođe izolovane ili detektovane kod ljudi, ali njihov patogeni potencijal nije u potpunosti razjašnjen [2].

Dok su u većini evropskih zemalja u krpeljima najzastupljenije vrste borelija *B. afzelii, B. garinii* i *B. valaisiana* [5], u Srbiji je najdominantnija vrsta *B. lusitaniae*, praćena vrstama *B. afzelii, B. bavariensis, B. garinii, B. valaisiana* i *B. burgdorferi* s.s. [6,7]. Najčešća klinička manifestacija lajmske bolesti u Srbiji je *erythema migrans*, koja je zabeležena u 93,2% slučajeva, slede neurološke, mišićno-skeletne, i kardiološke manifestaticije, u 2,8%, 2,4%, odnosno 1,1% slučajeva [8].

Generalno, tok LB se može podeliti u tri kliničke faze. Prva faza – rana lokalizovana infekcija – najčešće se manifestuje kao kožna lezija *erythema migrans*, karakteristična crvena promena u obliku koncentričnog kruga na mestu uboda inficiranog krpelja koja se obično javlja sedam dana nakon infekcije. Ova lezija može biti praćena različitim simptomima uključujući groznicu, glavobolju, bolove u mišićima i zglobovima, kao i uvećanjem limfnih čvorova [1,2,9]. Druga faza – rana diseminovana infekcija – razvija se nekoliko nedelja do nekoliko meseci nakon uboda i ako se antibiotska terapija ne sprovede u prvoj fazi, borelije iz primarne kožne lezije mogu da diseminuju i tako zahvate nervni sistem, kožu, zglobove i srce. Treća faza – kasna diseminovana infekcija se javlja nekoliko meseci ili čak nekoliko godina nakon uboda inficiranog krpelja i može dovesti do teških neuroloških, reumatoloških i dermatoloških komplikacija [1,2]. Iako sve patogene vrste borelija mogu izazvati *erythema migrans* i diseminovati u cirkulaciju, *B. afzelii* najčešće ostaje lokalizovana na koži i često se povezuje sa kožnim manifestacijama koje

INTRODUCTION

Lyme borreliosis (LB) is a zoonosis, the causative agents of which are transmitted through the bite of infected ticks of the genus *Ixodes*. The causative agents of LB are spiral-shaped bacteria from the *Borrelia burgdorferi* sensu lato (s.l.) complex [1,2]. The primary vectors in Europe are ticks of the species *Ixodes ricinus* [3]. Since LB is a multisystemic disease affecting both humans and animals, it can present with various clinical manifestations, such as skin lesions, musculoskeletal, neurological, ocular, and/or cardiovascular symptoms. In some cases, the infection may be asymptomatic, while in later stages, LB can be misdiagnosed due to the complexity of its clinical signs and symptoms [2,4].

The *Borrelia burgdorferi* sensu lato (s.l.) complex currently comprises 21 named *Borrelia* species. While in North America the primary causative agent of LB is *Borrelia burgdorferi* sensu stricto (s.s.), Europe exhibits greater *Borrelia* diversity, with five confirmed pathogenic species: *Borrelia afzelii*, *Borrelia garinii*, *B. burgdorferi* s.s., *Borrelia spielmanii*, and *Borrelia bavariensis*. In addition, *Borrelia lusitaniae*, *Borrelia valaisiana*, and *Borrelia bissettii* have also been isolated or detected in humans, although their pathogenic potential has not yet been fully elucidated [2].

While *B. afzelii*, *B. garinii*, and *B. valaisiana* are the most common *Borrelia* species found in ticks across most European countries [5], in Serbia the dominant species is *B. lusitaniae*, followed by *B. afzelii*, *B. bavariensis*, *B. garinii*, *B. valaisiana*, and *B. burgdorferi* s.s. [6,7]. The most common clinical manifestation of Lyme disease in Serbia is erythema migrans, observed in 93.2% of cases, followed by neurological (2.8%), musculoskeletal (2.4%), and cardiac (1.1%) manifestations [8].

In general, the course of LB can be divided into three clinical stages. The first stage-early localized infection-most commonly presents as erythema migrans, a characteristic red skin lesion in the form of a concentric circle that appears at the site of the infected tick bite, typically around seven days after infection. This lesion may be accompanied by a range of symptoms, including fever, headache, muscle and joint pain, and swollen lymph nodes [1,2,9]. The second stage-early disseminated infection-develops several weeks to several months after the tick bite. If antibiotic therapy is not administered during the first stage, *Borrelia* from the primary skin lesion can disseminate throughout the body, potentially affecting the nervous system, skin, joints, and heart. The third stage-late disseminated infection-occurs several months or even years after the bite of an infected tick and can lead to severe neurological, rheumatological, and dermatological complications [1,2]. Although all pathogenic *Borrelia* species se javljaju u trećoj fazi poput *acrodermatitis chronica atrophicans* (ACA) prisutnih isključivo u Evropi dok se *B. burgdorferi* s.s. povezuje sa reumatološkim manifestacijama, posebno lajmskim artritisom, koji je učestaliji u Severnoj Americi nego u Evropi. Vrste *Borrelia garinii* i *B. bavariensis* najčešće uzrokuju neurološke simptome, lajmsku neuroboreliozu, koji se javljaju u drugoj i trećoj fazi lajmske borelioze [2].

Nakon postavljanja dijagnoze, trajanje i doza antibiotske terapije (oralna ili intravenska) određuju se u zavisnosti od faze bolesti, starosti pacijenta, i njegovog imunološkog statusa, a u terapiji se najčešće koriste doksiciklin, amoksiciklin, ceftriakson, cefuroksim, i azitromicin, u vremenskom periodu od 10 do 30 dana u zavisnosti od oblika i faze bolesti [1]. Najbolja zaštita od LB jeste izbegavanje uboda krpelja i detaljan pregled kože nakon boravka u prirodi. Preventivna upotreba antibiotika nakon uboda krpelja se ne preporučuje iz razloga što nije svaki krpelj inficiran borelijama i neće svaki inficirani krpelj da prenese patogen nakon uboda. U Evropi ne postoje vodiči i/ili smernice za takvu namenu. Takođe, na tržištu do sada nije plasiran topikalni proizvod koji bi se primenjivao na koži nakon uboda krpelja i tako sprečio transfer borelija u kožu. Ne postoji ni preparat koji bi se koristio za lokalni tretman *erythema migrans* u cilju sprečavanja dalje diseminacije borelija u sistemsku cirkulaciju. Za razliku od patogenih vrsta borelija, koje mogu izazvati lokalne i sistemske manifestacije LB i zahtevaju antibiotsku terapiju, probiotski mikroorganizmi su pokazali pozitivne efekte na zdravlje ljudi [10,11]. Određeni sojevi proizvode antimikrobne supstance, poput organskih kiselina, bakteriocina, peptida i polisaharida, koje se tokom kultivacije oslobađaju u supernatante [12]. Probiotski kvasac *Saccharomyces boulardii* za sada nepoznatim mehanizmima, *in vitro* inhibira rast nekih patogenih i uslovno patogenih mikroorganizama poput *Candida albicans*, *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Vibrio cholerae, Salmonella enteritidis* [12,13]. Takođe, određene vrste bakterija mlečne kiseline među kojima je *Lactiplantibacillus plantarum,* sprečavaju kolonizaciju patogena kompeticijom za hranljive materije i adhezivna mesta, modifikacijom uslova sredine koja postaje nepovoljna za rast patogena, proizvodnjom antimikrobnih jedinjenja i modulacijom imunskog odgovora [14,15].

Transformacija bakterija tokom vremena i velika i/ili povećana potrošnja antibiotika širom sveta značajno doprinose porastu antimikrobne rezistencije, što predstavlja ozbiljnu pretnju po javno zdravlje na globalnom nivou. Samo tokom 2019. godine, antimikrobna rezistencija je bila odgovorna za smrt najmanje 1,27 miliona ljudi širom sveta, što jasno pokazuje da predstavlja

can cause erythema migrans and disseminate into the circulation, *B. afzelii* most often remains localized to the skin and is commonly associated with late-stage skin manifestations, such as acrodermatitis chronica atrophicans (ACA), a condition found exclusively in Europe. *Borrelia burgdorferi* s.s. is primarily associated with rheumatological manifestations, particularly Lyme arthritis, which is more frequently observed in North America than in Europe. *Borrelia garinii* and *B. bavariensis* are most often linked to neurological symptoms, including Lyme neuroborreliosis, which may occur during the second and third stages of the disease [2].

Following diagnosis, the duration and dosage of antibiotic therapy, administered either orally or intravenously, are determined based on the stage of the disease, the patient's age, and immune status. The most commonly used antibiotics include doxycycline, amoxicillin, ceftriaxone, cefuroxime, and azithromycin, typically prescribed for a period of 10 to 30 days, depending on the clinical form and stage of the disease [1]. The best protection against Lyme borreliosis (LB) is to avoid tick bites and to carefully examine the skin after spending time outdoors. Prophylactic antibiotic use following a tick bite is not recommended, as not every tick is infected with *Borrelia*, and not every bite from an infected tick results in transmission of the pathogen. Currently, there are no guidelines or recommendations for prophylactic antibiotic use after tick bites in Europe. To date, there is no topical product available on the market that can be applied to the skin after a tick bite to prevent the transmission of *Borrelia*. Similarly, no preparation exists for the local treatment of erythema migrans aimed at preventing further dissemination of *Borrelia* into the systemic circulation. Unlike pathogenic *Borrelia* species, which cause both local and systemic manifestations of LB requiring antibiotic therapy, probiotic microorganisms have demonstrated beneficial effects on human health [10,11]. Certain strains produce antimicrobial substances, such as organic acids, bacteriocins, peptides, and polysaccharides, that are released into the culture supernatant during cultivation [12]. The probiotic yeast *Saccharomyces boulardii* has been shown *in vitro* to inhibit the growth of various pathogenic and opportunistic microorganisms, including *Candida albicans*, *Escherichia coli*, *Salmonella enterica* serovar Typhi, *Shigella dysenteriae*, *Vibrio cholerae*, and *Salmonella enteritidis* [12,13]. Additionally, certain lactic acid bacteria, including *Lactiplantibacillus plantarum*, help prevent pathogen colonization by competing for nutrients and adhesion sites, altering environmental conditions to inhibit pathogen growth, producing antimicrobial compounds, and modulating the immune response [14,15].

ozbiljan rizik za zdravlje ljudi u svim životnim dobima [16]. Zbog ograničenja u lečenju lajmske borelioze, neophodno je razviti bezbedne i delotvorne alternativne pristupe, kao što je topikalni preparat na bazi biološki aktivnih komponenti, koji bi se profilaktički primenjivao nakon uboda krpelja ili na mestu primarne kožne lezije, s ciljem da spreči dalju diseminaciju borelija i time značajno smanji ili eliminiše potrebu za primenom antibiotika.

Cilj ove studije bio je da se *in vitro* ispita rast, razmnožavanje i pokretljivost *B. afzelii* u prisustvu supernatanata *S. boulardii*, *L. plantarum* i njihovih mešavina kao i da se odrede vrednosti njihovih minimalnih inhibitornih koncentracija (MIK).

MATERIJAL I METODE

U studiji je testiran soj *B. afzelii* RS163_11i, prethodno izolovan iz krpelja vrste *I. ricinus* [6], a koji je deo kolekcije izolata Instituta za medicinska istraživanja, Instituta od nacionalnog značaja za Republiku Srbiju, Univerziteta u Beogradu. Soj je čuvan na -80 °C u brucela-glicerol bujonu, rekultivisan u Barbour-Stoenner-Kelly-H (BSK-H) (HiMedia, India) podlozi i inkubiran na 33 °C do postizanja koncentracije borelija od 1–2 x 10^6/mL, dok je za određivanje broja borelija/mL primenjena Neubauer komora za brojanje (Brand Gmbh & Co. KG, Wertheim, Germany) u kombinaciji sa mikroskopiranjem u tamnom polju [17].

Za pripremu supernatanata korišćene su liofilizovane kulture iz komercijalno dostupnog probiotskog preparata, iz kojih su zatim izolovane čiste kulture *S. boulardii* i *L. plantarum* pripremljene na Katedri za mikrobiologiju i imunologiju, Farmaceutskog fakulteta Univerziteta u Beogradu.

Serije dvostrukih razblaženja supernatanta *S. boulardii*, *L. plantarum* i njihovih mešavina su pripremljene u BSK-H podlozi. Zapremina svakog testiranog supernatanta i njihove mešavine iznosila je 100 µL (početna maksimalna koncentracija 100%) koja je u opadajućim koncentracijama mešana sa 100 µL kulture ispitivanog soja *B. afzelii,* koncentracije 1–2 x 10^6 borelija/mL, a samostalno služila kao pozitivna kontrola, dok je kao negativna kontrola korišćena BSK-H podloga. Od svakog testiranog probiotskog mikroorganizma (*S. boulardii*, *L. plantarum* i njihove mešavine), pripremljeno je po 10 serijskih dvostrukih razblaženja u opadajućim koncentracijama (od 100% do 0,2%), dok je mešavina supernatanata pripremljena u odnosu 1:1, dodavanjem po 50 µL svakog supernatanta u opadajućim koncentracijama počevši od maksimalne koncentracije, od 100% svakog probiotskog mikroorganizma.

Antibiotik ceftriakson je korišćen kao kontrolni antibiotik u bujon mikrodilucionoj metodi za borelije i te-

Bacterial evolution over time, combined with the widespread and increasing use of antibiotics globally, has significantly contributed to the rise of antimicrobial resistance, a major threat to public health worldwide. In 2019 alone, antimicrobial resistance was responsible for at least 1.27 million deaths globally, underscoring the serious risk it poses to human health across all age groups [16]. Due to the limitations in LB treatment, there is a need to develop safe and effective alternative approaches, such as a topical preparation based on biologically active components that can be applied prophylactically after a tick bite or at the site of the primary skin lesion. The goal is to prevent further dissemination of *Borrelia* infection and thereby significantly reduce or eliminate the need for antibiotic therapy.

The aim of this study was to investigate *in vitro* the growth, reproduction, and motility of *B. afzelii* in the presence of supernatants from *S. boulardii*, *L. plantarum*, and their mixtures, as well as to determine their minimum inhibitory concentration (MIC) values.

MATERIALS AND METHODS

The study tested the *B. afzelii* strain RS163_11i, previously isolated from *I. ricinus* ticks [6], and maintained within the isolate collection of the Institute for Medical Research, National Institute of the Republic of Serbia, University of Belgrade. The strain was preserved at –80 °C in Brucella-glycerol broth and subsequently recultivated in Barbour-Stoenner-Kelly H (BSK-H) medium (HiMedia, India). Cultivation was carried out at 33 °C until a *Borrelia* concentration of 1–2 × 10^6 cells/mL was achieved. Cell counts were determined using a Neubauer counting chamber (Brand GmbH & Co. KG, Wertheim, Germany) under dark-field microscopy [17].

To prepare the supernatants, lyophilized cultures from a commercially available probiotic formulation were used as the source for isolating pure cultures of *S, boulardii* and *L, plantarum*. These isolates were prepared at the Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade.

Two-fold serial dilutions of *S. boulardii*, *L. plantarum*, and their mixed supernatants were prepared in BSK-H medium. For each test, 100 µL of the undiluted supernatant (initial concentration 100%) or its dilution was mixed with 100 µL of the *B. afzelii* culture (1–2 × 10^6 cells/mL). The *B. afzelii* culture without supernatant served as a positive control, while BSK-H medium without the addition of bacteria was used as a negative control. For each tested probiotic microorganism (*S. boulardii*, *L. plantarum*, and their mixtures), ten serial two-fold dilutions were prepared, ranging from 100% to 0.2% concentration. The mixed supernatant was prepared in a 1:1 ratio by combining 50 µL of each instiran u rasponu 8–0,016 μg/mL kao što je prethodno opisano [18].

Antimikrobna aktivnost supernatanata i kontrolnog antibiotika ceftriaksona je ispitivana primenom modifikovane bujon mikrodilucione metode za borelije, s obzirom na to da Evropski komitet za ispitivanje antimikrobne osetljivosti (*European Committee on Antimicrobial Susceptibility Testing* – EUCAST) i Institut za kliničke i laboratorijske standarde (*Clinical and Laboratory Standards Institute* – CLSI) ne definišu uputstva i kriterijume za izvođenje ove metode za borelije, kao ni granične vrednosti MIK za antibiotke [17]. Ukratko, za bujon mikrodilucionu metodu i određivanje MIK vrednosti antibiotika i testiranih supernatanata korišćene su sterilne plastične 96-mikrotitarske ploče. Prva kolona je služila kao pozitivna kontrola (kultura borelija), druga kolona kao negativna kontrola (BSK-H podloga), a od treće do 12. kolone bazeni su punjeni mešavinom borelija i testiranim supstancama u opadajućim koncentracijama [17]. Opseg testiranih koncentracija supernatanata probiotskih mikroorganizama u mikrotitarskim pločama je bio od 50% do 0,1%, za mešavinu probiotskih mikroorganizama od 25% do 0,05%, dok je za kontrolni antibiotik opseg bio od 168 μg/mL do 0,3 μg /mL [17]. Eksperimenti sa testiranim supstancama ponovljeni su dva puta u triplikatu. Nakon postavljanja mikrodilucionog testa, mikrotitarske ploče su inkubirane 72 sata na 33 °C u Gas-Pak posudi (BD GasPak™ EZ Container Systems) sa anaerobnim generatorom i indikatorom. Nakon inkubacije, određene su MIK vrednosti. Preparati su posmatrani mikroskopiranjem u tamnom polju i poređeni su broj i pokreljivost borelija u prisustvu testiranih supernatanata sa pozitivnom kontrolom i kontrolnim antibiotikom. MIK je definisana kao najniža koncentracija testiranog supernatanta probiotskog mikroorganizma (*S. boulardii* i *L. plantarum,* ili njihove mešavine) i antibiotika u kokultivaciji sa *B. afzelii*, pri kojoj borelije nisu bile pokretne ili su bile slabo pokretne, a njihov broj je bio smanjen u odnosu na pozitvnu kontrolu.

STATISTIČKA ANALIZA

Za statističku obradu podataka korišćeni su neparametarski Kruskal-Wallis test i Mann-Whitney test, a podaci su prikazani kao medijana (min-max). Statističke hipoteze su testirane na nivou statističke značajnosti $p = 0,05$.

Rezultati su prikazani tabelarno. Svi podaci su obrađeni u *R* programskom okruženju (R Core Team, 2024).

REZULTATI

Nakon 72 sata inkubacije na 33 °C određene su vrednosti MIK za *S. boulardii*, *L. plantarum*, njihovu mešavinu kao i za kontrolni antibiotik ceftriakson. Kruskal-Wallis

dividual supernatant at corresponding dilution levels, starting from the maximum concentration of 100% for each strain.

Ceftriaxone was used as the reference antibiotic control in the broth microdilution method for *Borrelia*, and was tested at concentrations ranging from 8 to 0.016 μg/mL, as previously described [18]. The antimicrobial activity of the supernatants and the control antibiotic ceftriaxone was evaluated using a modified broth microdilution method adapted for *Borrelia*, as neither the European Committee on Antimicrobial Susceptibility Testing (EUCAST) nor the Clinical and Laboratory Standards Institute (CLSI) provide standardized guidelines or interpretive criteria for this method, including MIC breakpoints for *Borrelia* spp [17]. Briefly, the broth microdilution method for determining the MIC values of antibiotics and tested supernatants was performed using sterile 96-well microtiter plates. The first column served as a positive control containing *Borrelia* culture, while the second column served as a negative control with BSK-H medium. Wells in columns three through twelve were filled with mixtures of *Borrelia* culture and the tested substances at serially decreasing concentrations [17]. The tested concentration range of probiotic supernatants in the microtiter plates was 50% to 0.1% for individual strains, 25% to 0.05% for the mixed supernatants, and 168 μg/mL to 0.3 μg/mL for the control antibiotic ceftriaxone [17]. Each experiment was performed in duplicate, with three technical replicates per condition. Following the setup of the microdilution assay, the microtiter plates were incubated for 72 hours at 33 °C in a GasPak™ EZ anaerobic container system (BD), equipped with an anaerobic generator and indicator. After incubation, MIC values were determined. Dark-field microscopy was used to assess *Borrelia* cell count and motility, and results were compared between samples treated with the tested supernatants, the positive control (untreated *Borrelia* culture), and the control antibiotic. MIC was defined as the lowest concentration of the tested probiotic supernatants (*S. boulardii*, *L. plantarum*, or their mixture) or antibiotics during co-cultivation with *B. afzelii* at which the *Borrelia* cells were either non-motile or showed significantly reduced motility, accompanied by a decrease in their number compared to the positive control.

STATISTICAL ANALYSIS

Statistical analysis was performed using the non-parametric Kruskal-Wallis and Mann-Whitney tests. Data were presented as median (minimum–maximum), and statistical significance was determined at a *p*-value threshold of 0.05.

test je pokazao da postoji statistički značajna razlika između MIK vrednosti testiranih supernatanata (*S. boulardii, L. plantarum* i njihovih mešavina) (p = 0,01). Mann-Whitney test je pokazao da postoji statistički značajna razlika između MIK vrednosti supernatanta *S. boulardii* i mešavine supernatanata (p = 0,02) kao i između supernatanta *L. plantarum* i mešavine supernatanata (p = 0,01) dok statistički značajna razlika ne postoji između supernatanata *S. boulardii* i *L. plantarum* (p = 0,68). Statističkom analizom je pokazano da mešavina testiranih probiotskih supernatanata efikasnije inhibira rast i razmnožavanje *B. afzelii* u odnosu na pojedinične probiotske supernatante.

Najefikasniji supernatant u inhibiciji rasta, razmnožavanja i pokretljivosti *B. afzelii* bila je mešavina supernatanata probiotskih mikroorganizama pri MIK vrednosti medijane od 0,8%. Rezultati su predstavljeni u Tabeli 1.

The results are presented in tabular form. All data analyses were conducted using the R programming environment (R Core Team, 2024).

RESULTS

After 72 hours of incubation at 33 °C, MIC values were determined for *S. boulardii*, *L. plantarum*, their mixture, and the control antibiotic ceftriaxone. The Kruskal-Wallis test revealed a statistically significant difference among the MIC values of the tested supernatants (*S. boulardii*, *L. plantarum*, and their mixture) (p = 0.01). Wilcoxon test showed significant differences between the MIC of *S. boulardii* and the mixture (p = 0.02), as well as between *L. plantarum* and the mixture (p = 0.01). No statistically significant difference was observed between the MIC values of the supernatants of *S. boulardii* and *L. plantarum* (p = 0.68). Statistical analysis demonstrated that the mixture of the tested probiotic supernatants inhibited the growth and reproduction of *B. afzelii* more effectively than the individual supernatants alone.

The most effective supernatant in inhibiting the growth, reproduction, and motility of *B. afzelii* was the mixture of probiotic supernatants, with a median MIC value of 0.8%. The results are presented in Table 1.

Tabela 1. Raspon i medijana vrednosti minimalnih inhibitornih koncentracija (MIK) (%) supernatanta *Saccharomyces boulardii, Lactiplantibacillus plantarum* i njihovih mešavina na vrstu *Borrelia afzelii*

Table 1. Range and median values of minimum inhibitory concentrations (MIC, %) of supernatants from *Saccharomyces boulardii, Lactiplantibacillus plantarum*, and their mixtures against *Borrelia afzelii*

Vrsta supernatanta / *Type of supernatant*	Raspon MIK (%) vrednosti / *MIC (%) range*	Medijana MIK (%) / *Median MIC (%) value*
Saccharomyces boulardii	0.8% – 50%	12.5%
Lactiplantibacillus plantarum	1.6% – 25%	6%
Mešavina supernatanta / *The mixture of supernatants*	0.2% – 3.1%	0.8%

Pored definisanih MIK vrednosti za supernatante *S. boulardii*, *L. plantarum* i njihovu mešavinu, pri nižim koncentracijama supernatanata probiotskih mikroorganizama uočena je povećana pokretljivost i broj borelija u odnosu na pozitivnu kontrolu. Prisustvo supernatanta *S. boulardii*, *L. plantarum* i njihovih mešavina, uticalo je stimulativno na broj i pokretljivost borelija u odnosu na pozitivnu kontrolu dok je *B. afzelii* bila najpokretljivija a njena brojnost najviše povećana u odnosu na pozitivnu kontrolu u prisustvu mešavine supernatanata pri vrednosti medijane od 0,1%. Rezultati su predstavljeni u Tabeli 2.

MIK vrednost za testirani kontrolni antibiotik na *B. afzelli* iznosio je 0,016 µg/mL (odnosno 0,3 µg/mL u mikrotitarskim pločama) što je u skladu sa prethodno

In addition to the defined MIC values for the supernatants of *S. boulardii*, *L. plantarum*, and their mixture, lower concentrations of these probiotic supernatants were associated with increased *Borrelia* motility and cell numbers compared to the positive control. The presence of supernatants from *S. boulardii*, *L. plantarum*, and their mixture stimulated both the number and motility of *Borrelia* compared to the positive control. Notably, *B. afzelii* exhibited the greatest increase in

Tabela 2. Koncentracije (%) i vrednosti medijana (%) supernatanata *Saccharomyces boulardii, Lactiplantibacillus plantarum* i njihovih mešavina koje su stimulativno delovale na rast i pokretljivost vrste *Borrelia afzelii*

Table 2. Concentrations (%) and median values (%) of *Saccharomyces boulardii, Lactiplantibacillus plantarum*, and their mixed supernatants that exhibited a stimulatory effect on the growth and motility of *Borrelia afzelii*

Vrsta supernatanta / *Type of supernatant*	Raspon koncentracija (%) / *Concentration range (%)*	Medijana (%) / *Median value (%)*
Saccharomyces boulardii	0.1% – 6.3%	0.4%
Lactiplantibacillus plantarum	0.1 % – 3.1%	0.2%
Mešavina supernatanta / *The mixture of supernatants*	0.1%	0.1%.

objavljenim rezultatima i njegovom dokazanom inhibitirnom dejstvu na rast i razmnožavanje borelija.

DISKUSIJA

Saccharomyces boulardii je jedini eukariotski mikroorganizam koji se koristi u farmaceutskoj industriji u svrhu probiotika. Za sada su poznata dva mehanizma pomoću kojih *S. boulardii* deluje na patogene: vezivanjem za bakterije i njihove toksine, čime se olakšava njihova eliminacija i lučenje specifičnih proteina koji imaju ulogu u degradaciji toksina i/ili ćelijskih elemenata patogena [19]. Studija Rajkowske i Kunicka-Styczyńske [20] pokazala je da *S. boulardii* vezuje enteropatogene bakterije (*E. coli, S. enterica var. Typhimurium, Staphylococcus aureus, Campylobacter jejuni* i *Enterococcus faecalis*) olakšavajući njihovu eliminaciju iz organizma. Uočeno je da *S. boulardii* značajno smanjuje broj ćelija *Listeria monocytogenes*, *Pseudomonas aeruginosa* i *S. aureus*, a pored toga smanjuje i stopu rasta *L. monocytogenes* i *P. aeruginosa*.

Jedan od mehanizama kako *L. plantarum* kao i ostale bakterije mlečne kiseline utiču na patogene jeste proizvodnja različitih antimikrobnih jedinjenja, kao što su bakteriocin, vodonik peroksid, organske kiseline, aceton i masne kiseline [21,22]. Antimikrobni peptidi-bakteriocini, deluju baktericidno ili inhibiraju rast srodnih bakterija, uključujući patogene sojeve. Poseduju niz pozitivnih svojstava, a neka od njih su dejstvo protiv patogena rezistentnih na antibiotike, uz odsustvo ili nisku toksičnost prema eukariotskim ćelijama [23]. Na primer, plantaricin PLNC8 αβ-bakteriocin, koji proizvodi *L. plantarum* je pokazao antimikrobno dejstvo prema paradontalnom patogenu *Porphyromonas gingivalis* izazivanjem deformacije spoljašnje membrane i lize ćelija.

Upravo zbog pomenutog antimikrobnog dejstva oba probiotska mikroorganizma, polazna hipoteza je podrazumevala mogućnost postojanja antibakterijskog efekta, a glavni cilj studije je bio ispitati njihov inhibitorni efekat na uzročnika LB, bakteriju *B. afzelii, in vitro,* koji do sada nije ispitivan. Poređenje naših rezultata, sa rezultatima prethodnih istraživanja nije u potpunosti adekvatno, jer je korišćena drugačija metodologija sa različitim test mikroorganizmima. Takođe, u okviru naše studije dobijeni su prvi preliminarni rezultati za borelije primenom bujon mikrodilucione metode, kojom do sada nisu bili testirani pomenuti probiotski mikroorganizmi. U studiji iz 2012. godine je metodom difuzije u agaru testiran antimikrobni potencijal supernatanta *S. boulardii* protiv *S. aureus*, *E. coli*, *C. albicans* i *Aspergillus niger* [12]. Njihovi rezultati su prikazani kao zone inhibicije (u milimetrima), što nije direktno uporedivo sa rezultatima dobijenim u našoj

motility and abundance in the presence of the mixed supernatants at a median concentration of 0.1%. These results are presented in Table 2.

The MIC value of the tested control antibiotic for *B. afzelii* was 0.016 µg/mL (corresponding to 0.3 µg/mL in microtiter plates), consistent with previously published data and confirming its established inhibitory effect on the growth and reproduction of *Borrelia*.

DISCUSSION

Saccharomyces boulardii is the only eukaryotic microorganism used as a probiotic in the pharmaceutical industry. To date, two mechanisms of its action against pathogens have been identified: it binds to bacteria and their toxins, promoting their elimination, and it secretes specific proteins involved in the degradation of toxins and/or pathogenic cellular components [19]. A study by Rajkowska and Kunicka-Styczyńska [20] demonstrated that *S. boulardii* binds to enteropathogenic bacteria (*E. coli*, *S. enterica* serovar Typhimurium, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Enterococcus faecalis*), thereby facilitating their elimination from the body. The study also showed that *S. boulardii* significantly reduces the number of *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *S. aureus* cells, and additionally slows the growth of *L. monocytogenes* and *P. aeruginosa*.

One of the key mechanisms by which *L. plantarum* and other lactic acid bacteria affect pathogens is the production of a range of antimicrobial compounds, including bacteriocins, hydrogen peroxide, organic acids, acetone, and fatty acids [21,22]. Antimicrobial peptides known as bacteriocins exhibit bactericidal activity or inhibit the growth of related bacteria, including pathogenic strains. They possess several advantageous properties, notably their effectiveness against antibiotic-resistant pathogens and their low or negligible toxicity to eukaryotic cells [23]. For example, the bacteriocin plantaricin PLNC8 αβ, produced by *L. plantarum*, has demonstrated antimicrobial activity against the periodontal pathogen *Porphyromonas gingivalis* by inducing outer membrane deformation and cell lysis.

In light of the previously described antimicrobial properties of both probiotic microorganisms, the initial hypothesis suggested their potential antibacterial effect. The main objective of this study was to examine, for the first time, their inhibitory activity against *Borrelia afzelii*, the causative agent of LB, *in vitro*. However, comparisons with earlier studies are limited due to differences in methodology and the microbial strains tested. Additionally, this study provides the first preliminary results on *Borrelia* using the broth microdilution method, which had not previously been applied to the studiji, s obzirom na razlike u primenjenoj metodologiji, korišćenim test organizmima i rezultatima koji su u našem slučaju predstavljeni procentima. Antimikrobna aktivnost supernatanta *L. plantarum* potvrđena je metodom tačkaste difuzije u agaru protiv sojeva *E. coli*, *L. monocytogenes*, *E. faecalis* i *P. aeruginosa*, pri čemu je veličina zone inhibicije korišćena kao pokazatelj intenziteta antimikrobnog delovanja [24]. U eksperimentu u kojem je primenjena bujonmikrodiluciona metoda [23], kao i u našoj studiji, korišćena je serija dvostrukih razblaženja različitih tipova plantaricina koji su bili predmet testiranja. Za razliku od navedenog rada, u našem ispitivanju korišćeni su celokupni supernatanti probiotskih mikroorganizama i *B. afzelii* iz kompleksa *B. burgdorferi* s.l., pri čemu je primenjena modifikovana mikrodiluciona metoda. S druge strane, bujon mikrodiluciona metoda za borelije nije standardizovana, tj. EUCAST i CLSI kao što je prethodno pomenuto, ne definišu smernice za izvođenje bujon mikrodilucione metoda za borelije, čak ni za ispitivanje osetljivosti borelija na antibiotike. S tim u vezi treba pomenuti da se ne definišu ni granične vrednosti MIK za antibiotike kada su u pitanju bakterije iz kompleksa *B. burgdorferi* s.l. Pored MIK koje smo utvrdili za ispitivane probiotske mikroorganizme, važan deo rezultata čine i podaci o procentualnim vrednostima koje su pokazale stimulativan uticaj na brojnost i pokretljivost borelija. Ovi nalazi predstavljaju prvi korak u razumevanju kako određeni probiotski sojevi mogu uticati na rast i ponašanje borelija u veštačkim uslovima. Time se otvara mogućnost daljeg usavršavanja BSK-H (Hi-Media) podloge – standardnog medijuma za njihovu kultivaciju, s ciljem da se ona učini efikasnijom za izolaciju i kultivaciju borelija iz različitih bioloških uzoraka. Iako je Sigma BSK-H podloga skupa, Hi-Media, koja ima isti sastav, predstavlja jeftiniju alternativu koja može značajno smanjiti troškove ispitivanja. Efikasnost podloge je ključna, jer uspešnost izolacije i kultivacije borelija zavisi upravo od njenog kvaliteta. U ovom kontekstu, prethodna istraživanja, poput studije Ružić-Sabljić i saradnika [25], pokazala je da Hi-Media podloga bolje stimuliše rast borelija. Naša istraživanja su dodatno potvrdila ove nalaze, pokazujući da dodavanje supernatanata probiotika borelijama utiče na njihov bolji rast i povećanu pokretljivost, čime se otvara nova perspektiva za primenu probiotika u optimizaciji laboratorijskih uslova za njihov rast. S druge strane, dobijene MIK vrednosti za supernatante *S. boulardii* i *L. plantarum* i njihovu mešavinu na *B. afzelii* (Tabela 1), predstavlja prvi do sada dobijen rezultat na borelijama sa ovim supernatantima, što pruža osnov za dalja istraživanja supernatanata i njihovog inhibitornog efekta na rast, razmnožavanje i pokretljivost borelija.

probiotic microorganisms in question. In a 2012 study, the antimicrobial potential of *S. boulardii* supernatant was evaluated against *S. aureus*, *E. coli*, *C. albicans*, and *Aspergillus niger* using the agar diffusion method [12]. Their results were expressed as zones of inhibition (in millimeters), which are not directly comparable to the findings of our study due to differences in methodology, the test organisms used, and the way results were reported-in our case, as percentages. The antimicrobial activity of *L. plantarum* supernatant was previously confirmed using the agar point diffusion method against *E. coli*, *L. monocytogenes*, *E. faecalis*, and *P. aeruginosa*, with the size of the inhibition zone serving as an indicator of antimicrobial intensity [24]. In the experiment where the broth microdilution method was used [23], as in our study, a series of two-fold dilutions of various types of plantaricins were tested. However, unlike that study, our research employed whole supernatants of probiotic microorganisms and *B. afzelii* from the *B. burgdorferi* s.l. complex, using a modified microdilution method. On the other hand, the broth microdilution method for *Borrelia* is not standardized; as previously mentioned, neither EUCAST nor CLSI provides guidelines for performing this method on *Borrelia*, including antibiotic susceptibility testing. It is important to note that MIC breakpoint values for antibiotics have not been established for bacteria within the *B. burgdorferi* s.l. complex. Alongside the MIC values we determined for the tested probiotic microorganisms, a significant part of our results includes percentage data indicating a stimulatory effect on the number and motility of *Borrelia*. These findings represent an initial step toward understanding how specific probiotic strains may influence the growth and behavior of *Borrelia* under controlled, *in vitro* conditions. This opens up the possibility for further optimization of the BSK-H medium (Hi-Media), the standard substrate for *Borrelia* cultivation, with the goal of enhancing its efficiency for isolating and growing *Borrelia* from various biological samples. While the Sigma BSK-H medium is expensive, Hi-Media offers a more affordable alternative with the same composition, potentially reducing testing expenses significantly. The effectiveness of the medium is critical, as successful isolation and cultivation of *Borrelia* depend heavily on its quality. In this regard, previous research, such as the study by Ružić-Sabljić et al. [25], demonstrated that the Hi-Media medium more effectively promotes the growth of *Borrelia*. Our research further supports these findings by demonstrating that the addition of probiotic supernatants to *Borrelia* enhances their growth and motility, thus opening new possibilities for using probiotics to optimize laboratory cultivation conditions. Moreover, the MIC values obtained for the supernatants of *S. boulardii*, *L. plantarum*, and their

Ako se uzmu u obzir mogući značaj dobijenih MIK rezultata probiotskih mikroorganizama i potencijalne smernice daljeg istraživanja, treba razmotriti dalje aspekte istraživanja – formulaciju topikalnog (profilaktičkog) preparta, koji bi se primenjivao nakon uboda krpelja i čime bi se sprečila oralna i/ili intravenska primena antibiotika. Trenutno lek za LB koji bi se koristio topikalno ili profilaktički ne postoji. Ipak, 2017. godine je sprovedena pilot klinička studija u kojoj su ispitanici, nakon uboda krpelja topikalno nanosili preparat 10% azitromicina u formi gela [26]. Rezultati ove studije nisu bili zadovoljavajući za preporuku azitromicin-gela u profilaktičke svrhe. Još jedna studija bavila se sličnom tematikom. Naime, postoje dokazi o efikasnosti topikalne primene kreme azitromicina u cilju prevencije nastanka LB. Ipak, efikasnost u pružanju 100% zaštite od infekcije nakon uboda krpelja je za sada ispitivana samo na miševima [27].

Sve ovo ukazuje na potrebu za razvojem topikalnog preparata sa drugačijom formulacijom i mehanizmom delovanja. S tim u vezi ideja o mogućnosti zamene antibiotika prirodnim proizvodima, koji ne izazivaju neželjene efekte i uzrokuju manji rizik od daljeg razvoja antimikrobne rezistencije, i ujedino doprinose smanjenju ili racionalizaciji njihove upotrebe, postaje sve relevantnija. Formulacija potencijalnog topikalnog probiotskog preparata, na bazi testiranih probiotskih mikroorganizama, mogla bi značajno doprineti prevenciji *erythema migrans*, kao i mogućem sprečavanju širenja borelija sistemskom cirkulacijom. U našem istraživanju, pokazano je da se pri određenim koncentracijama supernatanta probiotskih mikroorganizama brojnost i pokretljivost borelija smanjuje, ali bez ispitanih molekularnih mehanizama delovanja. Zbog toga bi u daljim istraživanjima trebalo proučavati komponente supernatanata kao i molekularne mehanizme kojima supernatanti (celi) i/ili njihove komponente inhibiraju rast i pokretljivost uzročnika LB – *B. afzelii*. Upravo iz tog razloga se dalja istraživanja u svrhu formulacije topikalnog preventivnog probiotskog preparata na bazi ispitivanih probiotskih mikroorganizama ostavljaju kao otvoreno polje za buduća istraživanja.

ZAKLJUČAK

Ovim istraživanjem je pokazano da supernatanti *S. boulardii* i *L. plantarum* imaju antimikrobno dejstvo na *B. afzelii*. Kombinacija supernatanata ova dva probiotska mikroorganizma imala je najefikasnije dejstvo pri njihovoj mešavini (MIK vrednost medijana 0,8%), što ih čini potencijalnim kandidatima za dalje istraživanje. Takođe, primećena je stimulacija rasta i veća pokretljivosti borelija pri nižim koncentracijama supernatanata, odnosno njihovoj mešavini, pri koncentraciji vrednosti

mixture against *B. afzelii* (Table 1) represent the first such data reported for *Borrelia*. These results provide a foundation for future studies on the inhibitory effects of probiotic supernatants on the growth, reproduction, and motility of *Borrelia*.

Considering the potential significance of the obtained MIC results for probiotic microorganisms and future research directions, additional aspects warrant attention-specifically, the development of a topical (prophylactic) formulation to be applied after a tick bite, aimed at preventing the need for oral and/or intravenous antibiotic treatment. Currently, no topical or prophylactic drug for LB exists. However, in 2017, a pilot clinical study investigated the topical application of a 10% azithromycin gel following tick bites [26]. The results were unsatisfactory, and the study did not support recommending azithromycin gel for prophylactic use. Another study explored a similar approach, providing evidence for the effectiveness of topical azithromycin cream in preventing LB However, this protective effect, achieving 100% prevention of infection after a tick bite, has so far only been demonstrated in mice [27].

All of this highlights the need to develop a topical preparation with a different formulation and mechanism of action. In this context, the prospect of replacing antibiotics with natural products, which do not cause side effects and pose a lower risk of promoting antimicrobial resistance, becomes increasingly relevant. Developing a topical probiotic formulation based on the tested probiotic microorganisms could play a significant role in preventing erythema migrans and potentially inhibiting the systemic spread of *Borrelia*. Our research demonstrated that at certain concentrations, probiotic microorganism supernatants reduce the number and motility of *Borrelia*, although the underlying molecular mechanisms were not investigated. Therefore, future studies should focus on identifying the active components within these supernatants and elucidating the molecular pathways through which the whole supernatants and/or their components inhibit the growth and motility of *B. afzelii*, the causative agent of LB. This highlights a promising area for further research aimed at developing a topical probiotic preventive formulation based on the tested probiotic strains.

CONCLUSION

This study demonstrated that the supernatants of *S. boulardii* and *L. plantarum* exhibit antimicrobial activity against *B. afzelii*. The mixed supernatants of these two probiotic microorganisms showed the strongest effect, with a median MIC value of 0.8%, highlighting their potential for further investigation. Interestingly, at lower concentrations, specifically around a median medijane 0,1%, što takođe zahteva dodatna ispitivanja. Potencijalni smerovi daljih istraživanja uključuju analizu sastava supernatanata i proučavanje molekularnih mehanizama kojima bi se objasnila inhibicija rasta, razmnožavanja i pokretljivosti *B. afzelii*. To bi dalje iniciralo pre svega istraživanja za razvoj topikalnog profilaktičkog preparata koji bi se primenjivao nakon uboda krpelja i koji bi sprečavao pojavu *erythema migranas*. Samim tim bi se mogla smanjiti diseminacija borelija u sistemsku cirkulaciju, rizik od kompikacija koje sa sobom nosi LB, kao i upotreba antibiotika.

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of 0.1%, the supernatants and their mixture stimulated *Borrelia* growth and increased motility, indicating the need for additional research to better understand these effects. Potential directions for future research include analyzing the composition of the supernatants and investigating the molecular mechanisms underlying the inhibition of *B. afzelii* growth, reproduction, and motility. Such studies would primarily pave the way for developing a topical prophylactic preparation to be applied after a tick bite, aimed at preventing the onset of erythema migrans. This approach could help reduce the systemic dissemination of *Borrelia*, lower the risk of LB-related complications, and minimize the reliance on antibiotics.

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