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ORIGINAL ARTICLE

Reciprocal cooperation of phytochemicals and micronutrients against typical and atypical forms of *Borrelia* sp.

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Keywords
biofilm, *Borrelia* sp., micronutrients, phytochemicals, reciprocal cooperation.

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Abstract

Aims: *Borrelia* sp., a causative pathogenic factor of Lyme disease (LD), has become a major public health threat. Current treatments based on antibiotics often lead to relapse after their withdrawal. Naturally derived substances that could work synergistically to display higher efficacy compared with the individual components may serve as a resource for the development of novel approaches to combat both active and latent forms of *Borrelia* sp.

Methods and Results: Using checkerboard assay, we investigated the anti-borreliae reciprocal cooperation of phytochemicals and micronutrients against two species of *Borrelia* selected as prevalent causes of LD in the United States and Europe. We tested 28 combinations of phytochemicals such as polyphenols (baicalein, luteolin, rosmarinic acids), fatty acids (monolaurin, cis-2-decenoic acid) and micronutrients (ascorbic acid, cholecalciferol and iodine). The results showed that the combinations of baicalein with luteolin as well as monolaurin with cis-2-decenoic acid expressed synergistic anti-spirochetal effects. Moreover, baicalein and luteolin, when combined with rosmarinic acid or iodine, produced additive bacteriostatic and bactericidal effects against typical corkscrew motile spirochaetes and persistent knob/round-shaped forms, respectively. An additive anti-biofilm effect was noticed between baicalein with luteolin and monolaurin with cis-2-decenoic acid. Finally, application of the combination of baicalein with luteolin increased cytoplasmic permeability of *Borrelia* sp. but did not cause DNA damage.

Conclusions: These results show that a specific combination of flavones might play a supporting role in combating *Borrelia* sp. through either synergistic or additive anti-borreliae effects.

Significance and Impact of the Study: Presented here *in vitro* results might help advancing our knowledge and improving the approach to target *Borrelia* sp.

Introduction

Borrelia burgdorferi sensu lato is a group of invasive spirochaetes transmitted by ticks that have the ability to transform into latent persistent forms such as knob/round-shaped bodies (forms) and/or biofilm (Burgdorfer *et al.* 1985; Brorson and Brorson 1997, 1998; Murgia and Cinco 2004; Timmaraju *et al.* 2015). To date, 17 species have been recognized worldwide as a causative pathogenic factor of Lyme disease (LD). They include *B. burgdorferi sensu stricto* and *Borrelia mayonii* (predominantly causing LD in the United States) as well as *Borrelia afzelii* and *Borrelia garinii* (predominantly causing LD in Eurasia) (Lovrich *et al.* 1994; Dryden and Hodgkins 2010; Rudenko *et al.* 2011; Calderaro *et al.* 2014).

It has been proposed that by converting into morphologically divers persistent forms (i.e. spheroplasts/L-forms, cysts/round bodies, granular forms, biofilm-like colonies), active and motile spirochaetes can evade the host's immune system and/or survive long-term in an

unfriendly environment (Hodzic *et al.* 2008; Miklossy *et al.* 2008; Sapi *et al.* 2011; Berndtson 2013). These diverse morphological forms are transmissible, living and immunogenic structures with a low metabolic rate. They are formed in response to any adverse conditions such as changes in temperature, pH, starvation, exposure to antibiotics or attacks from the immune system as a result of the genotypic followed by the phenotypic alterations in spirochaetes (Kersten *et al.* 1995; Aberer *et al.* 1996; Alban *et al.* 2000; Sapi *et al.* 2011). Moreover, it was shown that some forms, i.e. cysts, are able to reconvert to typical motile spirochaetes once the unfavourable conditions cease (Brorson and Brorson 1997, 1998; Gruntar *et al.* 2001). Numerous *in vitro* and *in vivo* and at least one human study demonstrated the existence of knob/round-shaped persisters, although the link at which their presence causes the infection is yet to be established (Hodzic *et al.* 2008; Miklossy *et al.* 2008; Sapi *et al.* 2012; Lantos *et al.* 2014; Feng *et al.* 2016a,b). Another latent form that has proven to exist *in vitro*, and recently in human skin specimens from patients with *Borrelial Lymphocytomas*, is biofilm which, as was shown, is the most challenging dormant form of *Borrelia sp.* to eliminate (Sapi *et al.* 2012, 2016). It is a thin-layered aggregate(s) of bacteria covered with self-produced extracellular polymeric substances composed mostly of polysaccharides, lipids, proteins and nucleic acids (Timmaraju *et al.* 2015; Sapi *et al.* 2016). This organized structure is formed as a protective hiding place for *Borrelia* sp. under harsh conditions in which their elimination either by synthetic and nonsynthetic agents or the host immune system is limited. It was demonstrated that biofilm may contain both typical motile spirochaetes and round-shaped persistent forms of *Borrelia* sp., which is able to resist most severe threats (Timmaraju *et al.* 2015; Sapi *et al.* 2016).

It is a challenge to combat these atypical forms of *Borrelia* sp. as they are less susceptible to the most popular antibiotics used in the treatment of LD or nonsynthetic biocides (Straubinger *et al.* 1997; Hodzic *et al.* 2008; Sapi *et al.* 2011; Embers *et al.* 2012; Feng *et al.* 2014a,b). Data from many *in vitro*, *in vivo* and even human studies confirmed poor efficacy of conventional antibiotic treatments against persistent forms, even upon their long-term administration, in contrast to the active typical motile spirochetal forms (Fallon *et al.* 2008; Hodzic *et al.* 2008; Klempner *et al.* 2013; Feng *et al.* 2014a,b; Shapiro 2014). Therefore, the call for new or more effective approaches for LD in its comprehensive aspects has been steadily growing (Loewen *et al.* 1999; Donta 2002; Hansmann 2009; Sapi *et al.* 2011; Kadam *et al.* 2014). This is especially dictated by the emergent number of patients with relapsing LD symptoms appearing after completion of conventional treatments. The exact mechanism of this phenomenon called PTLDS (post-treatment LD syndrome) still is not clear and awaits further exploration (Klempner *et al.* 2013, Center for Disease Control and Prevention 2014; Johnson *et al.* 2014; Marques *et al.* 2014; Stricker and Johnson 2014; Yu *et al.* 2016).

Naturally occurring compounds have already been viewed as an important source of antimicrobials for clinical applications and are largely untapped source of new chemical entities (Cowan 1999; Ayala *et al.* 2014; Morrison and Hergenrother 2014). A few, in particular, studies have reported about the anti-borreliae potential of several specific plant extracts, polyphenols and fatty acids, and such compounds might serve as a pool of new anti-borreliae agents (Brorson and Brorson 2007; Liebold *et al.* 2011; Goc *et al.* 2015; Theophilus *et al.* 2015). However, these studies together with others that examined synthetic biocides and antibiotics showed that it is still a challenge for them to be effective, when applied individually, to either become therapeutics or be effective against all pleomorphic forms of *Borrelia* sp. (Straubinger *et al.* 1997; Barthold *et al.* 2010; Embers *et al.* 2012; Klempner *et al.* 2013). Studies addressing complex pathologies have shown their improved efficacy when applied in synergistically or, at least additively, designed combinations. Therefore, studying reciprocal cooperation between particular agents effective in killing all morphological forms of *Borrelia* sp. might lead to developing more comprehensive treatment approaches. It would allow for expanding their bacteriostatic and bactericidal spectrum of action, minimizing cytotoxicity and preventing emergence of persisters. The preference of studying and using naturally occurring compounds instead of the synthetic in search for new or more effective anti-bacterial treatments is growing. It is based on the commonly known side effects of antibiotics that so far are either not, or rarely, reported with nonsynthetic compounds. It may additionally benefit the effectiveness of the treatment approach since many of them also possess immunomodulatory activity, and this aspect is suspected to play an important role in LD (Diterich *et al.* 2003; Bockenstedt *et al.* 2012; Berndtson 2013). Therefore, we tested various dual combinations of naturally occurring agents, which were previously identified in our study (Goc *et al.* 2015), for their enhanced anti-borreliae efficacy against active as well as persistent and biofilm forms of two *Borrelia* sp. to identify the most suitable one with the further pharmacological prospects.

Materials and methods

Test compounds

The compounds such as baicalein, cholecalciferol (vitamin D_3), ascorbic acid (vitamin C), cis-2-decenoic acid,

daptomycin, doxycycline and cefoperazone, with the purity between 90% and 98% according to the manufacturer, were purchased from Sigma (St Louis, MO). Luteolin and rosmarinic acid, with the purity between 97% and 99% according to the manufacturer, were obtained from Tocris Bioscience (Bristol, UK). Kelp with standardized iodine content (i.e. 150 μg ml^{-1} as a 100% of daily value) was from World Organic Ltd. (Auckland, New Zealand), whereas monolaurin (Lauricidin®), as a pure sn-1 monolaurin (glycerol monolaurate) derived from coconut oil, was from Med-Chem Laboratories, Inc., (Goodyear, AZ).

Preparation of test compounds for susceptibility testing

A stock solution (10–100 μg ml^{-1}) of each compound (depending on solubility of the substance) was prepared by suspending individual test compounds in absolute ethanol and sterilized by 0·22 μm syringe filtration. All stock solutions were stored in aluminium foil-wrapped tubes at −20°C. Due to the bactericidal effect of a high percentage of ethanol, its added amount to the growth medium was kept below 0·4% (v/v). The maximal ethanol content in the preliminary experiment was established as 0·5% (v/v) (data not shown). The appropriate amount of each stock solution was then added to 1·8 ml sterile screw-cap test tubes containing 1 ml of BSK-H complete medium to yield final concentrations of 0–500 μg ml^{-1} for all compounds. As a negative control, ethanol at 0·1–0·4% (v/v) was applied.

Test micro-organisms

Two *Borrelia* species, *B. burgdorferi* and *B. garinii*, were tested in their three morphological forms: typical motile spirochaetes, knob/round-shaped persistent forms and biofilm. Low passage isolates of the B31 strain of *B. burgdorferi* and the CIP103362 strain of *B. garinii* were obtained from the American Type Culture Collection (Manassas, VA). The B31 strain is an isolate from *Ixodes dammini*, whereas the CIP103362 strain is an isolate from *Ixodes ricinus*. The stocks of both species were cultured in commonly used conditions, i.e. Barbour-Stoner-Kelly H (BSK-H) medium supplemented with 6% rabbit serum (Sigma) without antibiotics at 33°C with 5% CO_2, in sterile screw-cap 15-ml polypropylene test tubes with or without gentle shaking.

Preparation of test micro-organisms for susceptibility testing

Both strains of *Borrelia* sp. were prepared for testing according to Sapi *et al.* (2011). Briefly, the strains were activated from original cryobank vials and inoculated into 10-ml BSK-H complete medium and maintained at 33°C with 5% CO_2. Generation of homogeneous cultures (having only typical motile spirochaete form) of tested *Borrelia* sp. were obtained by maintaining inoculums in a shaking incubator at 33°C and 250 rev min^{-1}, where there is no biofilm formation (Sapi *et al.* 2011). Generation of biofilm-like colonies of tested *Borrelia* sp. was attained by incubation of inoculums in four-well chambers (BD Biosciences, Sparks, MD) coated with collagen type I from rat tail for up to 1 week without shaking at 33°C with 5% CO_2.

Evaluation of cooperation of tested combinations of agents against active and latent forms of *Borrelia* sp.

(A) Reciprocity of test compounds against typical motile spirochaetes and persistent forms of studied *Borrelia* sp. was performed using the culturing method approach as reported previously (Feng *et al.* 2015; Theophilus *et al.* 2015). Briefly, 1·8 ml sterile screw-cap test tubes containing 1-ml BSK-H complete medium, supplemented with the tested combination of agents, were inoculated with 2×10^6 spirochaetes per millilitre of the homogenous bacterial suspension (3-day culture in logarithmic phase) or 1×10^7 spirochaetes per millilitre of the homogenous bacterial suspension (8-day culture in stationary phase). Samples were set up with increasing concentrations of active phytochemicals (0–500 μg ml^{-1}) or micronutrients (0–88 μg ml^{-1}), according to checkerboard format. These ranges were selected based on results from our earlier studies (used as a single dose, not in combination with a partner agent) (Goc *et al.* 2015). The tubes were then incubated at 33°C with 5% CO_2, and growth and viability were monitored at regular intervals for up to 72 h. Cooperation was evaluated using a Petroff-Hausser counting chamber with a dark-field microscopy, BacLight bacterial viability assay with fluorescent microscopy (Eclipse E600; Nikon, Melville, NY), and/or SYBER Green I/IP staining with spectrofluorometry, where green fluorescence determined live forms and red fluorescence the dead forms, as standard procedures. Control cultures were treated with a combination of 10 μg ml^{-1} doxycycline, 10 μg ml^{-1} daptomycin and 10 μg ml^{-1} cefoperazone as a positive control, and ethanol (0·1–0·4% v/v) a s a negative control, respectively. FICIs and FIBIs were calculated as standard calculations (Privett *et al.* 2010; Gopal *et al.* 2014):

$$\mathrm{FIC} = \frac{\text{MIC for agent in combination}}{\text{MIC for agent alone}}$$

$$\mathrm{FBC} = \frac{\mathrm{MBC_{90}}\text{for agent in combination}}{\mathrm{MBC_{90}}\text{for agent alone}}$$

The experiment was repeated three times for each species and each tested compound combination.

(B) Reciprocity of test compounds against biofilms of studied *Borrelia* sp. was evaluated using a qualitative method based on crystal violet staining (Sapi *et al.* 2011). Briefly, 1×10^7 spirochaetes per millilitre of the homogeneous culture were inoculated into four-well chambers coated with collagen type I from rat tail and incubated at 33°C with 5% CO_2 for up to 1 week. Earlier studies in our laboratory have documented a lack of antifungal carryover using this procedure (Goc *et al.* 2015). Once the biofilm was established, all chambers were supplemented with the tested combinations of agents and incubated at 33°C for up to 72 h. Control wells were treated with a combination of 10 μg ml^{-1} doxycycline, 10 μg ml^{-1} daptomycin and 10 μg ml^{-1} cefoperazone as a positive control and ethanol (0·1–0·4 v/v) as a negative control, respectively. All wells were fixed with 500 μl of cold methanol-formalin (1 : 1) for 30 min and stained with 1 ml of crystal violet (0·1%) for 10 min. The biofilms were then carefully washed three times with 1× PBS (phosphate-buffered saline), and 1 ml of methanol was added to each well to extract a dye which was measured at 595 nm using a spectrophotometer (Spectra Max 340; Molecular Device, Sunnyvale, CA). Cooperation was evaluated according to checkerboard format and the percentage of biofilm eradication (BE%) was calculated as BE% = (1−(OD_{959} of cells treated with agent A and agent B/OD_{959} of untreated control)) × 100%. Also, fractional eradication concentration indexes FECIs (adapted from the FICI/FBCI equation reported by Elion *et al.* 1954) were calculated as standard calculations, where EC_{50} is an effective concentration causing at least 50% of biofilm eradication:

$$\mathrm{FEC} = \frac{\mathrm{EC_{50}for\ agent\ in\ combination}}{\mathrm{EC_{50}for\ agent\ alone}}$$

The experiment was repeated three times for each species and each compound combination. Fractional inhibitory concentration indexes (FICIs), fractional bactericidal concentration indexes (FBCIs) and fractional eradication of biofilm concentration indexes (FECIs) were calculated by combining two FIC, FBC or FEC values and interpreted them as follows: synergy = FICI/FBCI/FECI of ≤0·5; antagonism = FICI/FBCI/FECI >4·0; additive = 0·5 < FICI/FBCI/FECI<1·0, and indifferent (no interaction) = 1·0 < FICI/FBCI/FECI < 4·0 (Dawis *et al.* 2003; Jeong *et al.* 2010; Gopal *et al.* 2014).

Cellular permeability

The membrane permeabilization was performed as previously described by Shen *et al.* (2012) by measuring the release of a UV-absorbing material using UV-VIS spectrophotometer (Spectra Max 340; Molecular Device). Culturing method was performed as previously described (Feng *et al.* 2015; Theophilus *et al.* 2015). Briefly, 1×10^6 (in logarithmic phase) and 1×10^7 spirochaetes per millilitre of the homogenous bacterial suspension (in stationary phase), respectively, were inoculated into each 1·8 ml sterile screw-cap test tube containing 1 ml BSK-H complete medium supplemented with the tested combination of agents. The tubes were then incubated at 33°C with 5% CO_2, and viability was monitored at regular intervals for up to 72 h. After treatment, samples (1·0 ml) were taken at appropriate time intervals, filtered through a sterile nitrate cellulose membrane (0·22 μm), and OD_{260} value of the supernatant was measured followed by calculation of percentage of the extracellular UV-absorbing materials released by cells. All measurements were done in triplicate.

Measurement of DNA base lesions

The number of base lesions was determined using the DNA Damage Quantification Colorimetric Assay kit (Oxford Biomedical Research, Oxford, MI) accordingly to the manufacturer's protocol. Briefly, 1×10^6 (in logarithmic phase) and 1×10^7 spirochaetes per millilitre (in stationary phase) of the homogeneous culture, respectively, were inoculated into each 1·8 ml sterile screw-cap test tube containing 1 ml BSK-H complete medium, supplemented with the tested combination of agents. The tubes were then incubated at 33°C with 5% CO_2. Next, 0·5 μg ml^{-1} of isolated DNA using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI) was mixed with an equal volume of 10 mmol l^{-1} biotinylated aldehyde reactive probe (ARP) reagent and incubated for 1 h at 37°C. The DNA-ARP product was precipitated, washed three times with 70% ethanol and re-suspended in Tris-EDTA buffer to final concentration of 0·5 μg ml^{-1}. The DNA-ARP product was left for binding to the wells of a 96-well microplate overnight at 37°C. Then, all wells were washed again four times with TPBS (137 mmol l^{-1} NaCl, 2·7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_3HPO_4, 2 mmol l^{-1} KH_2PO_4, 0·5% Tween 20, pH 7·4). In the meantime, the HRP-streptavidin conjugate was diluted to 0·5 μg ml^{-1} in assay buffer (0·15 mol l^{-1} NaCl, 10 mmol l^{-1} Na_3HPO_4, 1·5 mmol l^{-1} KH_2PO_4, 2·5 mmol l^{-1} KCl, 5 mg ml^{-1} BSA, 0·1% Tween, pH 7·5), and 100 μl of it was added to each well followed by incubation for 1 h at room temperature. After incubation, the wells were washed four times with TPBS, and 100 μl of substrate was added to each well and incubated again for 1 h at 37°C. In the end, the reaction was quenched with 100 μl of mol l^{-1} sulphuric acid, and the reaction was monitored at 450 nm. The number of ARPs (DNA base lesions) per 10^5 bp DNA was determined using a standard curve. All experiments were done in triplicate.

Statistical analysis

All the data are presented as means ± SD ($n = 3$). The ANOVA and/or Student's two-tailed t test was used to determine statistically significant differences set at 0·05 levels. Statistical analysis was performed using GRAPHPAD software.

Results

Identification of the type of cooperation of test compounds in dual combinations against typical motile and persistent forms of *Borrelia* sp

The effects of tested dual combinations of phytochemicals and micronutrients on growth and viability of typical motile and persistent forms of *B. burgdorferi* (B31 strain) and *B. garinii* (CIP103362 strain) (Fig. 1), together with calculated FICIs and FBCIs, and MICs and MBCs values are presented in Tables 1–3, respectively. Performed screening indicated synergistic cooperation (FICI $\leq$ 0·5) between baicalein and luteolin against typical motile spirochaetes and knob/round-shaped persisters as well as between monolaurin and cis-2-decenoic acid against typical motile spirochaetes. Additive effect (0·5 < FICI < 1·0) was observed for baicalein or luteolin partnered with rosmarinic acid and iodine, respectively. All other tested combinations revealed no interaction. No antagonistic interactions were noticed. The combination of cholecalciferol (vitamin D_3) with ascorbic acid (vitamin C) did not reach neither MIC, MBC_{90} and EC_{50} mark, which did not allow performing correct calculation.

With regard to the MIC parameter, synergistic anti-spirochetal cooperation of baicalein with luteolin allowed for the reduction of their individual MIC values from 150 to 37·5 μg ml^{-1} for baicalein and for luteolin from 125 μg ml^{-1} (for *B. burgdorferi*) and 150 μg ml^{-1} (for *B. garinii*) to 31·25 μg ml^{-1}. Similarly, synergy between monolaurin and cis-2-decenoic acid in their anti-spirochetal activity allowed for the reduction of their individual MIC values from 100 to 25 μg ml^{-1} for monolaurin and for cis-2-decenoic acid from 125 μg ml^{-1} (for *B. burgdorferi*) and 250 μg ml^{-1} (for *B. garinii*) to 31·25 μg ml^{-1}.

With regard to the MBC_{90} parameter, the values were reduced from 250 to 62·5 μg ml^{-1} for baicalein, luteolin, monolaurin and cis-2-decenoic acid, respectively, for typical motile spirochaetes and from 250 to 62·5 μg ml^{-1} for baicalein and luteolin for persisters.

The dual combinations of phytochemicals and micronutrients tested against biofilms of *B. burgdorferi* and *B. garinii*, together with calculated FECIs, and supported by obtained EC_{50} values, are presented in Table 4. Neither synergistic nor antagonistic interactions were observed for all the tested combinations with respect to

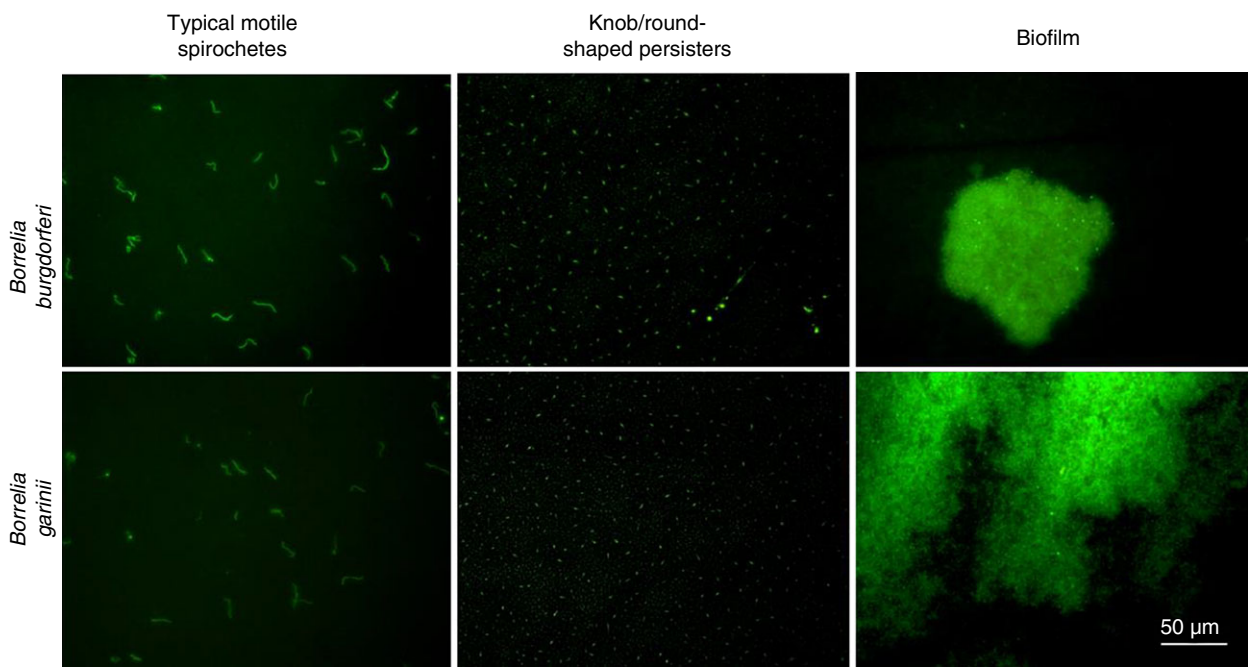

Figure 1 Representative images of different untreated morphological forms of *Borrelia burgdorferi* B31 strain and *Borrelia garinii* CIP103362 strain strained with SYTO9 dye. Images were taken at 200× magnification. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Reciprocal cooperation of phytochemicals and micronutrients against growth of typical motile spirochaetes of *Borrelia burgdorferi* and *Borrelia garinii*

Tested combination	*Borrelia burgdorferi*							*Borrelia garinii*				
	MIC* alone		MIC in combination		FIC			MIC in combination		FIC		
	Agent (μg ml^{-1})		Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FICI	Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FICI
Agent A+Agent B	A	B										
Baicalein+Luteolin	150	125†/150‡	37·5	31·25	0·25	0·25	0·5	37·5	31·25	0·25	0·21	0·46
Baicalein+RA	150	150	75	75	0·5	0·5	1·0	75	75	0·5	0·5	1·0
Baicalein+Iodine	150	5	75	2·5	0·5	0·5	1·0	75	2·5	0·5	0·5	1·0
Luteolin+RA	125†/150‡	150	62·5	75	0·5	0·5	1·0	75	75	0·5	0·5	1·0
Luteoiln+Iodine	125†/150‡	5	62·5	2·5	0·5	0·5	1·0	75	2·5	0·5	1·0	1·0
Monolaurin+CDA	100	125†/250‡	25	31·25	0·25	0·25	0·5	25	31·25	0·25	0·13	0·38

MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; RA, rosmarinic acid; CDA, cis-2-decenoic acid.
*Published from Goc *et al.* (2015).
†Value for *B. burgdorferi*.
‡Value for *B. garinii*.

Table 2 Reciprocal cooperation of phytochemicals and micronutrients against viability of typical motile spirochaetes and knob/round-shaped persisters of *Borrelia burgdorferi*

Tested combination	Typical motile spirochaetes							Knob/round-shaped persisters				
	MBC* alone		MBC_{90} in combination		FBC			MBC_{90} in combination		FBC		
	Agent (μg ml^{-1})		Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FBCI	Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FBCI
Agent A + Agent B	A	B										
Baicalein+Luteolin	250	250	62·5	62·5	0·25	0·25	0·5	62·5	62·5	0·25	0·25	0·5
Baicalein+RA	250	250	125	125	0·5	0·5	1·0	125	125	0·5	0·5	1·0
Baicalein+Iodine	250	15	125	7·5	0·5	0·5	1·0	125	7·5	0·5	0·5	1·0
Luteolin+RA	125	150	125	125	0·5	0·5	1·0	125	125	0·5	0·5	1·0
Luteoiln+Iodine	125	5	125	7·5	0·5	0·5	1·0	125	7·5	0·5	0·5	1·0
Monolaurin+CDA	250	250	62·5	62·5	0·25	0·25	0·5	250	250	1·0	1·0	2·0

MBC_{90}, minimal bactericidal concentration causing at least 90% of killing; FBC, fractional bactericidal concentration; FBCI, fractional bactericidal concentration index; RA, rosmarinic acid; CDA, cis-2-decenoic acid.
*Published from Goc *et al.* (2015).

their efficacy. Performed screening indicated additive effects ($0{\cdot}5 < \text{FECI} < 1{\cdot}0$) between flavones, such as baicalein and luteolin, and between fatty acids, such as monolaurin and cis-2-decenoic acid. All other tested combinations revealed no interaction. The results show that additive anti-biofilm effect of flavones allowed for the reduction of the EC_{50} values from 750 to 250 μg ml^{-1} for baicalein and from 350 to 87·5 μg ml^{-1} for luteolin. For fatty acids, the EC_{50} values were reduced from 750 to 250 μg ml^{-1} for monolaurin and from 800 to 200 μg ml^{-1} for cis-2-decenoic acid. Obtained anti-borreliae concentrations were valid for both tested species of *Borrelia*.

Kinetic evaluation and anti-biofilm efficacy of selected combination of flavones

Since baicalein and luteolin were able to synergistically eliminate typical motile spirochaetes and knob/round-shaped persisters as well as additively biofilms of both tested *Borrelia* sp., we performed kinetic evaluation of their bactericidal effects. The results showed that baicalein in combination with luteolin, used at concentrations fulfilling the FBCI $\leq$ 0·5 requirement, significantly eliminated spirochaetes and persisters of *B. burgdorferi* and *B. garinii* in a time-dependent manner, reaching MBC_{50} mark after 24 h and MBC_{90} mark after 72 h (Figs 2a,b

Table 3 Reciprocal cooperation of phytochemicals and micronutrients against viability of typical motile spirochaetes and knob/round-shaped persisters of *Borrelia garinii*

Tested combination	Typical motile spirochaetes							Knob/round-shaped persisters				
	MBC* alone		MBC_{90} in combination		FBC			MBC_{90} in combination		FBC		
	Agent (μg ml^{-1})		Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FBCI	Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FBCI
Agent A+Agent B	A	B										
Baicalein+Luteolin	250	250	62·5	62·5	0·25	0·25	0·5	62·5	62·5	0·25	0·25	0·5
Baicalein+RA	250	250	125	125	0·5	0·5	1·0	125	125	0·5	0·5	1·0
Baicalein+Iodine	250	15	125	7·5	0·5	0·5	1·0	125	7·5	0·5	0·5	1·0
Luteolin+RA	125	150	125	125	0·5	0·5	1·0	125	125	0·5	0·5	1·0
Luteoiln+Iodine	125	5	125	7·5	0·5	0·5	1·0	125	7·5	0·5	0·5	1·0
Monolaurin+CDA	250	250	62·5	62·5	0·25	0·25	0·5	250	250	1·0	1·0	2·0

MBC_{90}, minimal bactericidal concentration causing at least 90% of killing; FBC, fractional bactericidal concentration; FBCI, fractional bactericidal concentration index; RA, rosmarinic acid; CDA, cis-2-decenoic acid.
*Published from Goc *et al.* (2015).

and 3a). The combination of these flavones executed ~30–70% improved bactericidal effect compared with the effects of each flavone alone.

The selected combination of baicalein with luteolin, used at concentration fulfilling the $0{\cdot}5 <$ FECI $< 1{\cdot}0$ requirement, after 72 h markedly eliminated biofilms of *B. burgdorferi* and *B. garinii* with EC_{50} mark as well as showed enhanced ~30–35% anti-biofilm action compared with each flavone alone (Figs 2c,d and 3a).

The combination of antibiotics (10 μg ml^{-1} daptomycin, 10 μg ml^{-1} doxycycline and 10 μg ml^{-1} cefoperazone) that was recently identified to be effective against both typical motile spirochaete and knob/round-shaped persistent forms of *B. burgdorferi,* and used here as a positive control, was shown to behave in a similar manners against typical motile spirochaetes and knob/round-shaped persistent forms, without significant anti-biofilm effect (Feng *et al.* 2015; Theophilus *et al.* 2015).

Evaluation of selected combination of flavones on cellular permeability

The cellular permeability measured by UV-absorbing release materials interpret to be mostly DNA, RNA and metabolites (Shen *et al.* 2012), as demonstrated in Fig. 4, showed to be significantly augmented after treatment of spirochaetes (logarithmic phase) and persistent forms (stationary phase) of *B. burgdorferi* and *B. garinii* with the combination of baicalein and luteolin, at concentration shown in our study to be bactericidal, respectively. Compared with control (containing both flavones without bacteria), there was a ~twofold increase, whereas compared with each of the flavones used alone, a ~1·5-fold increase was noticed. Although we observed increased UV-absorbing release materials after baicalein and luteolin treatment alone, which markedly was induced than control (containing each of the flavones, respectively, without bacteria) ($P < 0{\cdot}01$), the absorbance values of baicalein plus luteolin of treated group of cells were also significantly higher than those of baicalein and luteolin treated alone ($P < 0{\cdot}01$).

Evaluation of DNA damage by selected combination of flavones

To determine whether baicalein and luteolin in combination and alone can damage *Borrelia* sp. DNA, strain B31 and CIP103362 (from the logarithmic and the stationary phase) were treated with these flavones, at concentration shown here to be bactericidal, and the isolated DNA was assayed for AP sites (DNA base lesions). The results shown in Fig. 5 demonstrate that in all cases the numbers of AP sites per 10^5 bp DNA were equivalent, indicating that the addition of these compounds alone or in combination did not increase the number of DNA base lesions.

Discussion

Our previous study showed that the most promising non-synthetic compounds effective against spirochaetes and latent forms of *Borrelia* sp. were flavones (baicalein and luteolin) and to some extent also fatty acids (monolaurin and cis-2-decenoic acid), and polyphenols such as rosmarinic acid and micronutrients like ascorbic acid (vitamins C) and cholecalciferol (vitamin D_3), and iodine also showed to be effective (Goc *et al.* 2015).

Table 4 Reciprocal cooperation of phytochemicals and micronutrients against biofilms of *Borrelia burgdorferi* and *Borrelia garinii*

Tested combination	*Borrelia burgdorferi*							*Borrelia garinii*				
	EC_{50}* alone		EC_{50} in combination		FEC			EC_{50} in combination		FEC		
	Agent (μg ml^{-1})		Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FECI	Agent A (μg ml^{-1})	Agent B (μg ml^{-1})	Agent A	Agent B	FECI
Agent A + Agent B	A	B										
Baicalein+Luteolin	750	350	250	87·5	0·33	0·25	0·58	250	87·5	0·33	0·25	0·58
Monolaurin+CDA	750	800	250	200	0·33	0·25	0·58	250	200	0·33	0·25	0·58

EC_{50}, effective concentration causing at least 50% of eradication; FEC, fractional eradication concentration; FECI, fractional eradication concentration index; RA, rosmarinic acid; CDA, cis-2-decenoic acid.
*Published from Goc *et al.* (2015).

In this study, we combined the selected compounds from those we previously tested into 28 different dual combinations and tested them against active and dormant forms of *B. burgdorferi* and *B. garinii*, as predominant pathogenic factors of LD in the United States and Europe. Such an approach is not novel but is based on observations reported by others indicating that it could be more effective in combating all pleomorphic forms of *Borrelia* sp. or other species (Feng *et al.* 2015, 2016b; Qian *et al.* 2015; Siriwong *et al.* 2015; Cai *et al.* 2016). However, to date there is a lack of research data about nonsynthetic reciprocal cooperation of agents with potential pharmacological prospects. Identifying and applying compounds that positively cooperate with each other would allow for lowering the concentrations of individual compounds and retaining their biological efficacy. Moreover, this has additional benefits in alleviating any possible adverse or toxic cellular effects by these agents.

This study revealed that out of the 28 tested dual combinations, baicalein with luteolin and monolaurin with cis-2-decenoic acid showed synergistic cooperation in growth inhibition and in affecting viability of typical motile spirochaetes of both of the two studied *Borrelia* sp. Out of the eight agents tested *in vitro,* only combination of baicalein plus luteolin showed synergistic cooperation in killing knob/round-shaped persistent forms and additive effect in eradication of biofilms formed by the two studied *Borrelia* sp., respectively, as well. This combination was able to eliminate ~90% of active and persistent forms as well as eradicate the mature *Borrelia* biofilms in ~50%. In the case of biofilm, the 90% elimination mark was not reached; however, it is a very demanding requirement even for many antibiotics (Sapi *et al.* 2011). Obtained bactericidal results by utilizing methodology of direct counting and recently developed high throughput screening through spectrofluorometry did not show discrepancies.

Baicalein and luteolin are classified as flavones and belong to the vast group of polyphenols with well-known antiviral, antibacterial and antifungal effects (López-Lázaro 2009; Wenyu *et al.* 2014). Their anti-inflammatory and cell protective properties were also intensively researched worldwide (Woo *et al.* 2006; Cheng *et al.* 2007; Hsieh *et al.* 2007; Nabavi *et al.* 2015). It is worth noting that their cooperation with antibiotics is remarkable as shown by ours and many other research groups (Chang *et al.* 2007; Su *et al.* 2014; Qian *et al.* 2015; Siriwong *et al.* 2015; Cai *et al.* 2016; Goc *et al.* 2016). According to the aforementioned data, baicalein and luteolin have a broad spectrum of antimicrobial activity, but their efficacy is shown to be in relatively high effective concentrations. Thus, more *in vivo* and human studies focusing on their bioavailability and toxicity in higher doses applied long-term are desired, since current studies are limited.

Interestingly, the mode of action of both these flavones affects many targets in a plethora of Gram-positive and Gram-negative bacteria, but a main repeatedly reported one is the membrane permeability (Wang and Xie 2010; Yun *et al.* 2012; Siriwong *et al.* 2015; Joung *et al.* 2016). This corroborates our results implying that baicalein and luteolin alone and in combination increases membrane permeability of studied *Borrelia* sp. as well. That could at least partially explain their similar effects on both actively and slow or nonproliferating persistent forms. Moreover, their efficacy towards mature biofilms of *Borrelia* sp. is encouraging and showed to have a possible wilder bacterial spectrum (Cao *et al.* 2008; Shen *et al.* 2014; Chen *et al.* 2016). Furthermore, our results showed that the DNA of studied *Borrelia* sp. is not a major target for conditional stress caused by these two compounds. This was determined by measuring the number of apurinic/apyrimidinic sites (AP) in their DNA that can be generated either spontaneously under physiological conditions

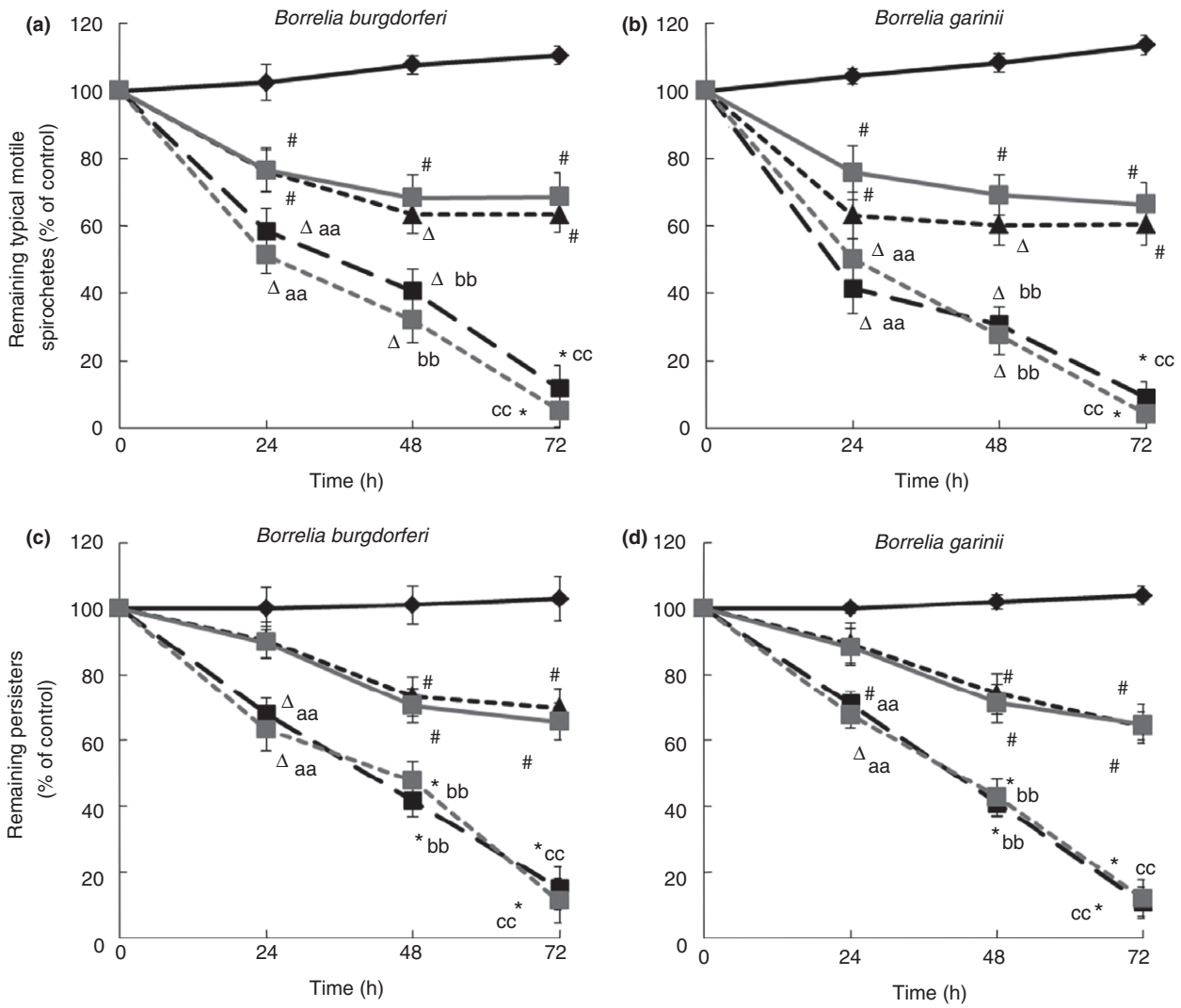

Figure 2 Kinetic evaluation of susceptibility of typical motile spirochaetes (logarithmic phase) (a and b) and persisters (stationary phase) (c and d) of *Borrelia burgdorferi* and *Borrelia garinii* to antibacterial agents determined by LIVE/DEAD BacLight staining. Tested compounds: 62·5 μg ml^{-1} baicalein, 62·5 μg ml^{-1} luteolin, 10 μg ml^{-1} Dap—daptomycin, 10 μg ml^{-1} Dox—doxycycline, 10 μg ml^{-1} Cef—cefoperazone, control (0·2% v/v ethanol) valid for both *Borrelia* sp.; #$P \leq 0{\cdot}05$, $\Delta P \leq 0{\cdot}01$, *$P \leq 0{\cdot}001$ compared with control; $^{a}P \leq 0{\cdot}05$, $^{b}P \leq 0{\cdot}01$, $^{c}P \leq 0{\cdot}001$ compared with flavones; —♦— control, —■— luteolin, -·▲·- baicalein, –■– baicalein+luteolin, ··■·· Dap+Dox+Cef.

or formed by DNA-damaging inducers (Lindahl and Nyberg 1972; Kubo *et al.* 1992). While it was reported that both baicalein and luteolin inhibit proliferation of the eukaryotic cells, it is also known that AP sites are not skirted by DNA polymerase in bacterial cells, thus DNA lesions can result in blocking DNA replication. Additionally, it was also reported that baicalein and luteolin, respectively, among other targets, affect DNA topoisomerase I and II in *Staphylococcus aureus* (Wang and Xie 2010, Yun *et al.* 2012). Finally, it should be mentioned that the total number of AP sites per 10^5 bp DNA is approximately 10 times higher in the *B. burgdorferi* B31 strain DNA than in untreated *Escherichia coli* DNA, due to higher the number of telomeres present in the genome of *B. burgdorferi*, and that *B. burgdorferi* DNA is not the major target for oxidative damage either (Boylan *et al.* 2008).

In summary, this study documents *in vitro* efficacy of several combinations of phytochemicals and micronutrients against active and latent forms of *B. burgdorferi* and *B. garinii* and identifies the combination of baicalein with luteolin to synergistically and/or additively affect all pleomorphic forms of *Borrelia* sp. There are no published reports about reciprocity between the phytochemicals and micronutrients, which emphasizes the novelty of this study. The intrinsic anti-borreliae activity of this

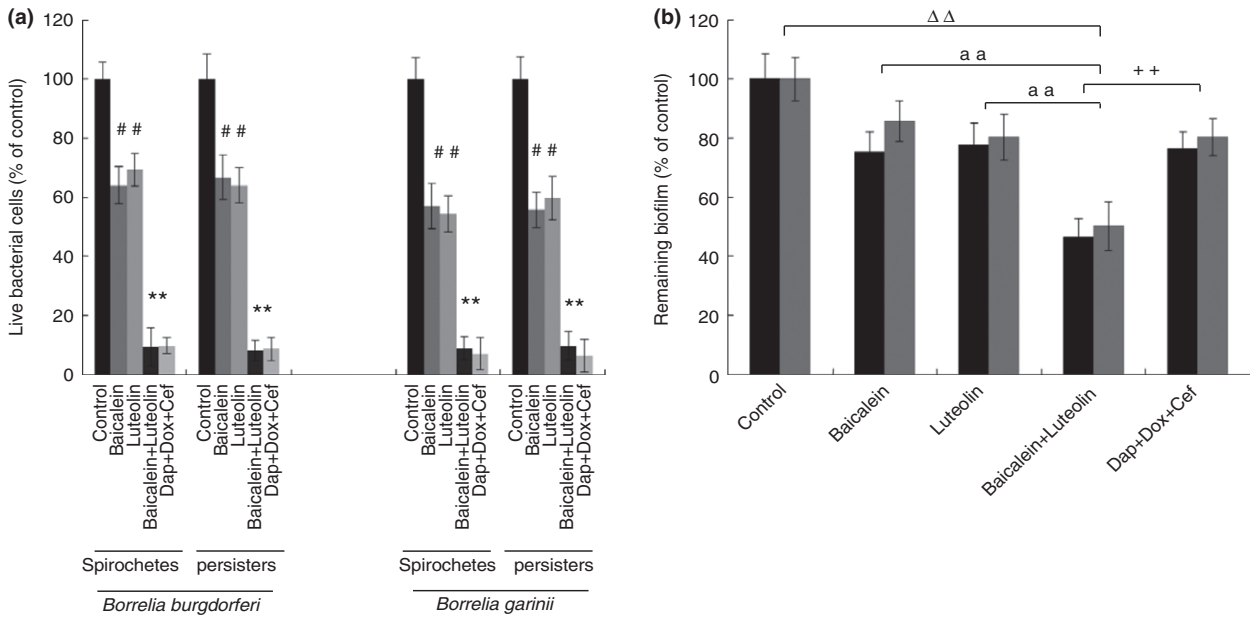

Figure 3 Susceptibility of active and latent forms of *Borrelia burgdorferi* and *Borrelia garinii* to antimicrobial agents. (a) Susceptibility of typical motile spirochaetes and persisters determined by SYBER Green/IP staining and spectrofluorometry after 72 h post-treatment with tested compounds: 62·5 μg ml^{-1} baicalein, 62·5 μg ml^{-1} luteolin, 10 μg ml^{-1} Dap—daptomycin, 10 μg ml^{-1} Dox—doxycycline, 10 μg ml^{-1} Cef—cefoperazone, control (0·2% v/v ethanol) valid for both *Borrelia* sp. (b) Susceptibility of *Borrelia burgdorferi* (■) and *Borrelia garinii* (■) biofilms grown on collagen-coated surface to antibacterial agents after 72 h determined by crystal violet staining. Tested compounds: 250 μg ml^{-1} baicalein, 87·5 μg ml^{-1} luteolin, 10 μg ml^{-1} Dap—daptomycin, 10 μg ml^{-1} Dox—doxycycline, 10 μg ml^{-1} Cef—cefoperazone, control (0·2% v/v ethanol) valid for both *Borrelia* sp.; #$P < 0{\cdot}05$, $\Delta P \leq 0{\cdot}01$, *$P \leq 0{\cdot}001$ compared with control; $^{a}P \leq 0{\cdot}05$ compared with individual flavones; +$P \leq 0{\cdot}05$ compared with Dap+Dox+Cef.

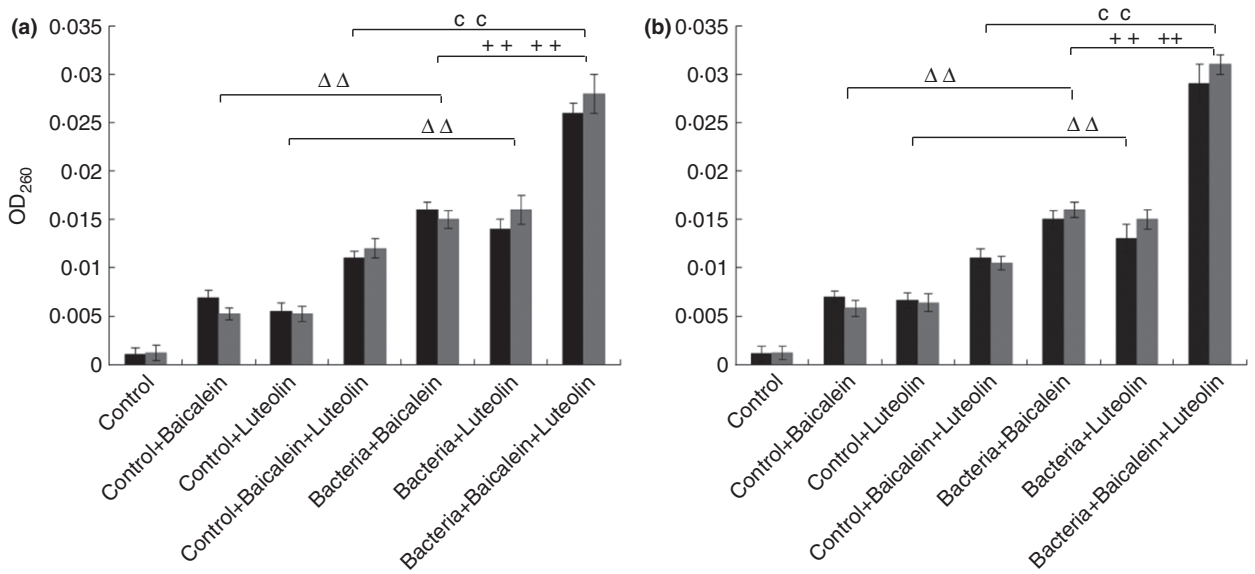

Figure 4 The presence of 260-nm absorbing materials in supernatants of typical motile spirochaetes (logarithmic phase) (a) and persisters (stationary phase) (b) of *Borrelia burgdorferi* (■) and *Borrelia garinii* (■) after 72 h treated with appropriate antibacterial agents. Tested compounds: 62·5 μg ml^{-1} of baicalein, 62·5 μg ml^{-1} of luteolin, control (0·2% v/v ethanol) valid for both *Borrelia* sp.; $\Delta P \leq 0{\cdot}01$, *$P \leq 0{\cdot}001$ control+flavone compared with bacteria+flavone; $^{c}P \leq 0{\cdot}001$ control+flavone+flavone compared with bacteria+flavone+flavone; +$P \leq 0{\cdot}01$, bacteria+flavone compared with bacteria+flavone+flavone.

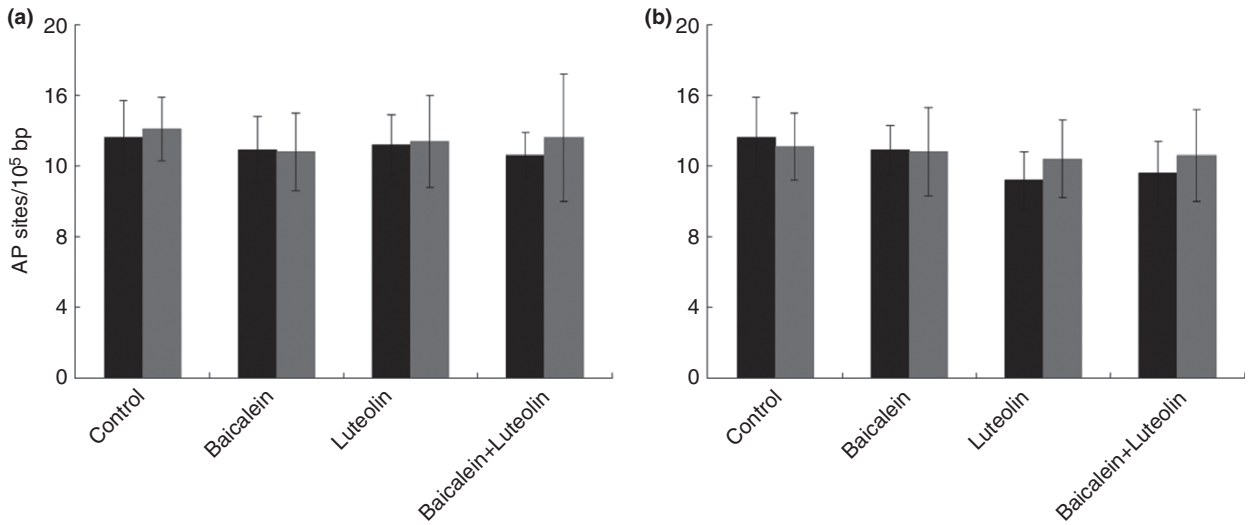

Figure 5 Effect of antibacterial agents on DNA damage of typical motile spirochaetes (logarithmic phase) (a) and persisters (stationary phase) (b) of *Borrelia burgdorferi* (■) and *Borrelia garinii* (■) determined as the number of aldehyde reactive probe (DNA base lesions) per 10^5 bd DNA using standard curve. Tested compounds: 62·5 μg ml^{-1} of baicalein, 62·5 μg ml^{-1} of luteolin, control (0·2% v/v ethanol) valid for both *Borrelia* sp.

combination supports the hypothesis that it might represent a valuable addition to combat *Borrelia* sp. This finding may improve prospects for developing LD approaches with improved efficacy and scope; however, further *in vivo* and human studies are needed to support this conclusion.

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Conflict of Interest

No conflict of interest to declare.

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