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ORIGINAL PAPER

A central composite rotatable design (CCRD) approach to study the combined effect of antimicrobial agents against bacterial pathogens

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Abstract The combination of antimicrobial agents has been proposed as a therapeutic strategy to control bacterial diseases and to reduce the emergence of antibiotic-resistant strains in clinical environments. In this study, the interaction between the lantibiotic bovicin HC5 with chloramphenicol, gentamicin, nisin, lysostaphin and hydrogen peroxide against *Staphylococcus aureus* O46 was evaluated by MIC assays. The central composite rotatable design (CCRD), a robust and economic statistical design, was used to combine concentration levels of different antimicrobials agents with distinct mechanisms of action and the presence of significant interactions among the antimicrobials was determined by regression analysis. According to the adjusted model, there were no significant interactions between bovicin HC5 and gentamicin, lysostaphin, nisin or hydrogen peroxide. However, bovicin HC5 showed a significant interaction ($P < 0.02$) with chloramphenicol. This is the first study applying the CCRD approach to evaluate the combined effect of antimicrobials against *S. aureus*. Based on our results, this approach is an effective strategy to determine synergistic interactions between antimicrobial agents applied in human and veterinary medicine against bacterial pathogens.

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Introduction

The emergence of resistant bacteria to antibiotics is a leading public health concern (CDC 2013). In recent years, bacteria isolated from hospital-acquired infections often show resistance to at least one of the drugs most commonly used in the clinical treatment (Indrawattana et al. 2013). In this context, the combination of antimicrobial agents with different mechanisms of action has been proposed to reduce the emergence of human and livestock antibiotic-resistant strains and improve their efficacy against microbial pathogens by targeting multiple sites in the cell (Branen and Davidson 2004; Laxminarayan et al. 2006; del Pozo and Patel 2007; Lacasse et al. 2008; Dosler and Mataraci 2013).

Most studies evaluating the combined effect among antimicrobials use a complete factorial design to assess the activity of such inhibitors (Aaron et al. 2002; Nazer et al. 2005; Desbois and Coote 2011). Although these approaches allow the evaluation of several combined treatments, a large number of experimental units are often required to assess the interactions between treatments, with a remarkable increase in workload and experimental costs. Additionally, in many cases, the interpretation of the results does not always allow a clear interpretation of the interactions between antimicrobials being tested (Chou 2006; Tallarida 2011).

In this scenario, the central composite design (CCD) offers a robust regression analysis that can be used to design experiments with a lower number of treatments without losing discrimination power and maintaining its sensitivity to the occurrence of outliers in the experimental design (Chou 2006; Ukaegbu and Chigbu 2014). Among

the different types of CCD, the central composite rotatable design (CCRD) has shown great potential to target the stability region of the design around a central point determined by its properties of rotatability and orthogonality (Hader and Park 1978). The CCRD approach has several applications, especially in studies aiming the optimization of experimental conditions or containing several treatments or variables. Although the CCRD has been applied to optimize the concentrations and exposure times of antimicrobials against the target bacteria (de Oliveira et al. 2012), the use of this approach to study the combined effects of different antimicrobial agents against pathogenic organism has been largely overlooked until now.

In this study we aimed to evaluate if the interaction between an antimicrobial peptide (bovicin HC5) and other antimicrobial agents could improve the inhibitory activity of bacteriocins against the pathogenic bacterium *Staphylococcus aureus*. The use of bacteriocins for therapeutic purposes has been considered an attractive alternative to control bacterial infections and to avoid the development of resistant strains (Hoffmann et al. 2002; Cao et al. 2007; Wu et al. 2007; Field et al. 2008). Bovicin HC5, a lantibiotic produced by *Streptococcus equinus* HC5, is highly effective against several bacterial pathogens, including *S. aureus* (de Carvalho et al. 2007; Pimentel-Filho et al. 2013, 2014). To test our hypothesis, a statistical approach using CCRD was applied to combine concentration levels of each antimicrobial and regression analysis was used to determine the interactions between these factors.

Materials and methods

Microorganisms and culture conditions

Streptococcus bovis HC5 was cultivated under anaerobic conditions, at 39 °C, in basal medium containing, per liter: 0.292 g K_2HPO_4, 0.292 g KH_2PO_4, 0.48 g $(NH_4)_2SO_4$, 0.48 g NaCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.064 g $CaCl_2{\cdot}2H_2O$, 0.6 g cystein hydrochloride, 0.1 g trypticase®, 0.5 g yeast extract, 4 g Na_2CO_3 and 16 g glucose.

The reference strain *S. aureus* O46 used in this study was kindly provided by the Institut National de la Recherche Agronomique (INRA)—UMR1253, Science et Technologie du Lait et de l'Oeuf, Rennes, France. *S. aureus* O46 was isolated from a ewe diagnosed with mild mastitis (Le Marechal et al. 2011) and was cultivated overnight under microaerophilic conditions, at 37 °C, in Mueller–Hinton (MH) media.

Preparation of the antimicrobial agents

Bovicin HC5 extracts were prepared and purified by reversed phase cromatography as previously described (Mantovani et al. 2002; Paiva et al. 2012). Purified bovicin HC5 was resuspended in phosphate buffer (PB, 30 μmol l^{-1}, pH 7.0) and stored at −20 °C until use. Nisin stock solution was prepared by dissolving the appropriate amounts of commercial nisin (Sigma, N5764, 2.5 %) in 0.85 % NaCl prepared in 0.2 N HCl (pH 2.0). The other antimicrobials combined with bovicin HC5 were chloramphenicol (Fluka, 23275), gentamicin sulfate (Sigma, G3632) and lysostaphin (Sigma, L0761). These antimicrobials were prepared by dissolving the appropriate amounts of powder from commercial products into sterile deionized water with the exception of hydrogen peroxide (Synth, d 1.130), in which the stock solution was prepared by direct dilution in distilled water. Stock solutions of all antimicrobial agents were stored at −20 °C until use.

Minimum inhibitory concentration (MIC) assays

The MIC assay for different antimicrobials was performed using standard broth microdilution methods (CLSI 2013). Incubations were performed using MH media and the concentration range of each antimicrobial agent was (in μmol l^{-1}): 1.0–125.0 for bovicin HC5, 16.2–130 for chloramphenicol, 0.2–25.0 for gentamicin, 373.1–5970.0 for hydrogen peroxide, 1.0–140 for lysostaphin 0.8–100 for nisin. The MIC values were used to determinate the concentration range of each antimicrobial tested in the combined treatment.

Combined effects of antimicrobial agents

Overnight cultures of *S. aureus* O46 (10^5 CFU ml^{-1}) were inoculated into 96-well plates containing MH media added with combined concentrations of antimicrobials. The concentrations of each antimicrobial agent were determined using the CCRD approach. The maximum concentrations corresponded to ½ MIC values and the minimum concentrations were ten times lower than the MIC. When necessary, distilled water was added to adjust the final volume in each well to 200 μl. The optical densities (OD_{600nm}) were monitored every 30 min for up to 12 h of incubation.

Data analysis

Each antimicrobial concentration used in this study was coded according to the standardization and principles of analysis of the CCRD approach. Levels of bovicin HC5 (factor A) were combined with five levels of each antimicrobial (factor B), generating nine treatments (Table 1). Three technical replicates were used for each treatment, yielding a total of 135 experimental units for each biological replication.

Table 1 Coded treatments used in the central composite rotatable design (CCRD) and combination levels of bovicin HC5 (factor A) and the companion antimicrobial (factor B)

Treatments	A factor	B factor
1	−1	−1
2	1	−1
3	−1	1
4	1	1
5	−1.4142	0
6	1.4142	0
7	0	−1.4142
8	0	1.4142
9	0	0

The final OD_{600nm} values (obtained after 12 h of growth) were subjected to regression analysis to assess the combined effect of the antimicrobials on bacterial growth. The following first-order model considering the interaction of each antimicrobial with bovicin HC5 was used:

$$y_{ij} = \beta_0 + \beta_1 a_i + \beta_2 b_j + \beta_3 (a_i b_j)$$

where, a_i is the level i of the factor A (bovicin HC5), if $-1.4142 \leq a_i \leq 1.4142$ and b_j is the level j of the factor B (each antimicrobial that was combined with bovicin HC5), if $-1.4142 \leq b_j \leq 1.4142$.

Depending on the model, non-significant coefficients ($P > 0.02$) were removed one at a time, based first on the interaction between factors, and subsequently, based on the interaction that had a higher P value according to the Student's t test.

Results

The MIC values for each antimicrobial agent tested against *S. aureus* O46 are summarized in Table 2. The concentration range for each antimicrobial agent was determined using the CCRD approach and tested in the treatments combined with bovicin HC5. The concentration range of each antimicrobial agent was (in μmol l^{-1}): 0.1–1000 for bovicin HC5, 1.61–16.13 for chloramphenicol, 0.01–18 for gentamicin, 37.30–373.05 for hydrogen peroxide, 0.12–1.25 for lysostaphin and 0.07–0.78 for nisin (Table 3). The growth kinetics of *S. aureus* was always influenced by the presence of antimicrobials in the MH media. In general, the lag phases were prolonged and the growth rate and final OD_{600nm} were lower compared to the control treatments (Fig. 1).

Table 2 Minimum inhibitory concentration (MIC) of the antimicrobial agents used against *S. aureus* O46

Antimicrobial agent	MIC (μmol l^{-1})
Bovicin HC5	2.00
Chloramphenicol	32.27
Gentamicin	0.38
Hydrogen peroxide	746.10
Lysostaphin	2.18
Nisin	1.56

MIC values were determined in MH media by standard broth microdilutions test (37 °C), under aerobic conditions

The regression analysis of the first-order interactions observed in our study allowed the evaluation of the combined effects between bovicin HC5 and other antimicrobials. The estimated equations, used for the interpretation of the combined effects of the antimicrobials on microbial growth were composed only by significant terms and are described in Table 4, as well as their respective coefficients of determination (R^2). The mean value of R^2 for the estimated equations was 0.744. When bovicin HC5 was combined with different antimicrobial agents, positive interactions in inhibitory activity were observed only between bovicin HC5 and chloramphenicol ($P < 0.02$), an antibiotic that inhibit peptide bond formation by 70S bacterial ribosomes. However, our results did not show interaction ($P > 0.02$) between bovicin HC5 and antimicrobial agents that target other structural or biochemical cellular components (e.g. 30S ribosomal subunits, peptidoglycan, lipid II, membrane lipids and macromolecules). Therefore, the decrease in bacterial growth shown in Fig. 1b–e is explained mainly by the inhibitory activity of bovicin HC5 against *S. aureus.*

Discussion

Staphylococcus aureus is an opportunistic pathogen frequently isolated from chronic infections (Indrawattana et al. 2013). Diseases caused by *S. aureus* are often difficult to treat and cure, and the raise of methicillin-resistant *S. aureus* strains (MRSA) emphasizes the need for more effective therapeutic strategies to control *S. aureus* infections (Römling and Balsalobre 2012; Otto 2013). Therefore, the combination of antimicrobials with distinct mechanisms of action could enhance their efficacy and therapeutic effects, reducing the time or the concentration required during clinical treatment (Dosler and Mataraci 2013).

In this study, the utilization of the CCRD approach to define the combined effects of different antimicrobial agents resulted in a regression analysis with significant interaction for the combination of bovicin HC5 and chloramphenicol ($P < 0.02$). This synergistic effect could be

Table 3 Code levels determined by CCRD and the corresponding concentration of the antimicrobial agents used in this study

CCRD code levels	Factor A (μmol l^{-1})	Factor B (μmol l^{-1})				
	Bovicin HC5	Chloramphenicol	Gentamicin	Hydrogen peroxide	Lysostaphin	Nisin
−1.4142	0.100	1.614	0.188	37.305	0.126	0.078
−1.0000	0.232	3.740	0.044	86.473	0.292	0.181
0.0000	0.550	8.875	0.103	205.178	0.692	0.429
1.0000	0.868	14.010	0.163	323.882	1.092	0.677
1.4142	1.000	16.137	1.880	373.050	1.258	0.780

Concentrations of factors A and B (μmol l^{-1}) were determined by CCRD and the maximum concentration of each antimicrobial corresponded to the ½ MIC values shown in Table 2

partially related with the mechanisms of action of these antimicrobial molecules. Chloramphenicol is a broad-spectrum antibiotic that diffuses through the cytoplasmic membrane and specifically prevents protein chain elongation in the 50S ribosomal subunit by reversibly inhibiting the transfer of amino acids to the growing peptide (Lin et al. 1997). Bovicin HC5 binds with high affinity to lipid II ($Ka = 3.4 \times 10^6$ mol l^{-1}) and is able to form a pre-pore-like structure (Paiva et al. 2011, 2012). Our previous results suggested that bovicin HC5 is too short to permeabilize lipid bilayers composed of phospholipids with C18 or longer acyl chains. Nonetheless, the antibacterial activity of bovicin HC5 is often more persistent than the activity of other lantibiotics that also target lipid II (e.g. nisin) and potassium efflux if often observed only if the bacteriocin is allowed to interact with sensitive cells for longer periods of time (Mantovani and Russell 2008; Paiva et al. 2011, 2012).

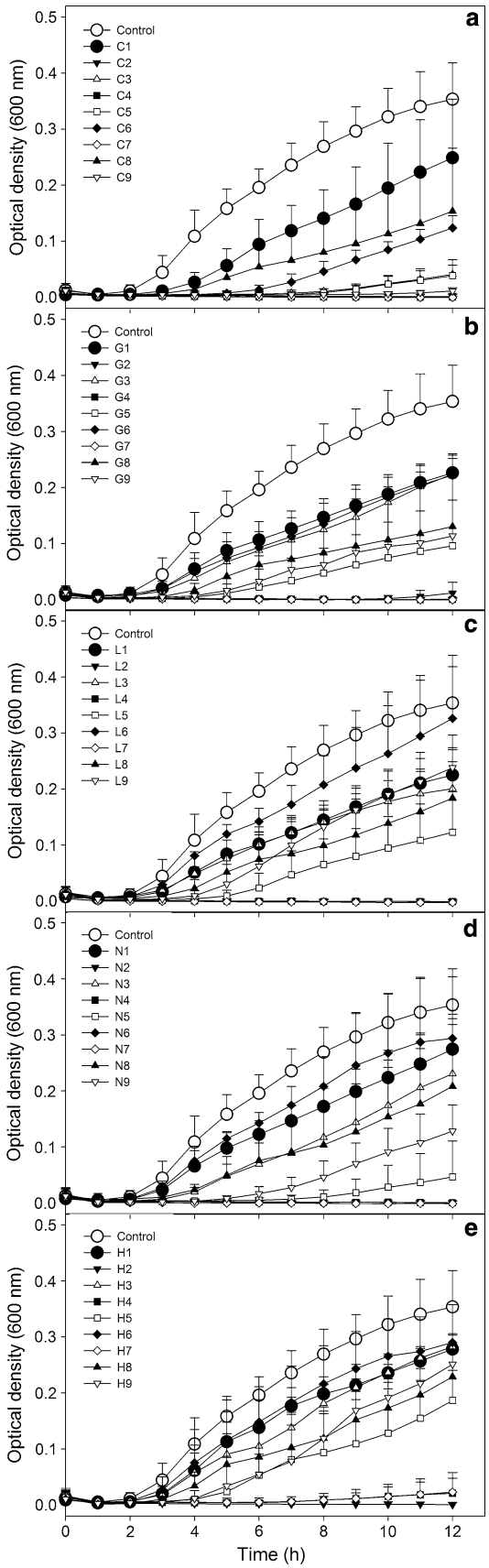
Based on these results, molecules acting against the cell membrane could facilitate other molecules to access the cell interior and reach its primary target (Park et al. 2004). This could be the case for the interaction between bovicin HC5 and chloramphenicol, in which, the latter could inhibit anabolic reactions in the cytoplasm and prevent a stress response in *S. aureus* cells. Additionally, bovicin HC5 is capable to cause efflux of cations that are relevant to maintain cell homeostasis (e.g. potassium). Therefore, a decrease in intracellular ATP pools and an increase in respiratory activity might be expected in these combined treatments (Mantovani and Russell 2008).

The regression analysis of the first-order interactions observed in our study also allowed the evaluation of the combined effects between bovicin HC5 and other antimicrobial agents. According to the adjusted models, there was no significant interaction between bovicin HC5 and gentamicin, hydrogen peroxide, lysostaphin or nisin, indicating that these antimicrobials neither act synergistically with bovicin HC5 nor interfere ($P > 0.02$) with the inhibitory activity of the peptide against *S. aureus*. The absence of synergism and the antagonism between antimicrobial agents has been previously described. When the antimicrobial activity of nisin and ramoplanin were evaluated against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) strains, an antagonistic effect was observed if the antimicrobials were added simultaneously to BHI media (Brumfitt et al. 2002).

In the case of gentamicin, lysostaphin and nisin, which showed much lower MIC values compared to chloramphenicol, the interaction with bovicin HC5 was probably not required to improve the antimicrobial activity of these molecules used alone. Gentamicin is an aminoglycoside that irreversibly binds to ribosomal proteins and 16S rRNA found in the 30S ribosomal subunit, inhibiting the initiation complex required for protein synthesis in the cell (Yoshizawa et al. 1998). The MIC values for gentamicin were approximately 85-fold lower than those observed for *S. aureus* cultures treated with chloramphenicol, indicating that this antibiotic can reach its target in the cytoplasm successfully without other "auxiliary" compounds.

Nisin is also a lantibiotic that target lipid II and in our assays the MIC values for nisin and bovicin HC5 were comparable to both bacteriocins (1.56 and 2.0 μmol l^{-1}, respectively). The interactions between nisin and bovicin HC5 was not synergistic at the concentrations tested, and the regression analysis parameters further indicated that nisin did not reduce the inhibitory activity of bovicin HC5. These results could be explained by the greater affinity of bovicin HC5 for lipid II compared to nisin (Paiva et al. 2011, 2012).

In the case of lysostaphin, a glycylglycine endopeptidase that can hydrolyse the crosslink bridges in the peptidoglycan of staphylococci, it was initially expected that its mechanism of action could facilitate the activity of molecules that target the cell membrane, such as bovicin HC5. Previous work demonstrated a synergistic antibacterial activity between cell wall hydrolases (e.g. lysozyme, muramidases) and nisin and the authors postulated that these enzymes could enable the antimicrobial peptide to



◀**Fig. 1** Growth of *S. aureus* O46 in presence of bovicin HC5 combined with different antimicrobials. *Panels a* through *e* represent each antimicrobial agent combined with bovicin HC5: *a* chloramphenicol, *b* gentamicin, *c* lysostaphin, *d* nisin and *e* hydrogen peroxide. Control treatments without antimicrobials (*open circles*) are also shown in each *panel*. Levels *1* through *9* represent the coded levels of each antimicrobial agent determined by the CCRD approach. Results are the mean OD_{600nm} obtained from three independent experiments performed in triplicate. *Bars* represent the standard deviation of the mean

cross the cell wall and reach the cell membrane (Prado-Acosta et al. 2010). However, such synergistic effects were not observed in the present study when *S. aureus* was grown in the presence of bovicin HC5 and lysostaphin. These differences might be due to the smaller size of bovicin HC5 (23 amino acids) compared to nisin (34 amino acids), facilitating the diffusion through the cell envelope to reach its target site (cytoplasm membrane).

Hydrogen peroxide causes chemical oxidation of cellular components, including lipids and proteins (Cabiscol et al. 2010). Because these effects generally induces an oxidative stress response, the cell often respond with several changes in gene expression and can become adapted to the stimulus if the concentration of H_2O_2 is not lethal. Our results suggested that hydrogen peroxide does not affect the activity of bovicin HC5 ($P < 0.02$) and even 0.550 μmol l^{-1} of bovicin HC5 could cause complete inhibition of *S. aureus* growth when the bacteriocin was combined with the lowest dose of H_2O_2 (Fig. 1).

Although chloramphenicol was the antimicrobial agent showing greater interaction with bovicin HC5 against *S. aureus*, it should be noted that the clinical use of chloramphenicol is restricted due to its effect inhibiting mitochondrial protein synthesis and associated collateral effects, such as plastic anemia (Barnhill et al. 2012). Nonetheless, studies reporting the use of chloramphenicol in clinical practice and its ability to enhance the inhibitory activity of other antimicrobials or to control microbial pathogens are not rare (Laporte et al. 1998; Zuberbuhler et al. 2014; Maaland et al. 2015; Kiruthika et al. 2015).

Based on our results, it was possible to discriminate the significant interactions between bovicin HC5 and other antimicrobials with distinct mechanisms of action using the CCRD approach to determine the concentration levels and the interactions between the factors being evaluated. The CCRD approach could potentially be used to study the effect of combined antimicrobial agents with greater discriminatory efficiency. Our results shown that even at concentrations below MIC values, bovicin HC5 combined with other antimicrobial agents could prevent the growth of *S. aureus* cultures, and this effect persisted even if the incubation time was as long as 48 h. These results emphasize the potential of bovicin HC5 to inhibit *S. aureus*

Table 4 Effect of antimicrobial agents combined with bovicin HC5 on the growth of *S. aureus* O46

Antimicrobial agent[a]	Equation	R^2
Chloramphenicol	$y = 0.0648 - 0.0388A - 0.0349B + 0.0251AB$	0.746
Gentamicin	$y = 0.1095 - 0.0649A$	0.757
Hydrogen peroxide	$y = 0.1672 - 0.0796A$	0.806
Lysostaphin	$y = 0.1383 - 0.0766A$	0.709
Nisin	$y = 0.1264 - 0.0795A$	0.703

Equations were obtained from linear regressions performed to determine the effect of combined antimicrobials on the growth of *S. aureus* O46

[a] Antimicrobial agent (B) that was combined with bovicin HC5 (A) according to the CCRD code levels specified in Table 3

growth and prevent bacterial infections in vivo and highlight opportunities for improving therapeutic strategies by combining effective antimicrobial agents against microbial pathogens.

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Conflict of interest The authors declare that there are no conflicts of interest.

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