Research Article

Journal of PeptideScience

Received: 19 July 2010 Revised: 13 September 2010 Accepted: 20 September 2010 Published online in Wiley Online Library: 19 January 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1316

# Peptides from Royal Jelly: studies on the antimicrobial activity of jelleins, jelleins analogs and synergy with temporins‡

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**Peptides isolated from natural fonts are the object of several studies aimed at finding new molecules possessing antibacterial activity. We focused our studies on peptides originally isolated from the Royal Jelly, the jelleins and on some analogs having a UV reporter at the *N*- or *C*-terminus. We found that jelleins are mainly active against gram-positive bacteria; interestingly, they act in synergy with peptides belonging to the family of temporins such as temporin A and temporin B against *Staphylococcus aureus* A170 and *Listeria monocytogenes*.** 

**Keywords:** antimicrobial; royal jelly; jelleins; temporin

## Introduction

Antimicrobial peptides are natural and evolutionarily conserved weapons possessed by animals and plants to defend themselves against microbes. Owing to the emergence of resistance of several bacterial strains to antibiotics, the interest toward new antimicrobial agents is rapidly growing.

Insects such as moths, flies and bees produce humoral factors in response to pathogens that contribute to defending the host against the invading microorganisms [1,2]. Examples of peptides produced in response to pathogens are cecropins and attacins secreted by *Hyalophora cecropia pupae*, sarcotoxin produced by *Sarcophaga peregrine* and hymenoptaecin, abaecin and defensin by *Apis mellifera* [3–6]. These peptides show broad-spectrum activity against bacteria and yeast. The amphibian skin is also a rich font of antimicrobial peptides such as temporins, japonicin and nigrocin [7]. Temporins isolated from *Rana temporaria* have been studied widely; these are short peptides, 10–14 amino acid long, amidated at the *C*-terminus, mostly bearing a +2 charge. They are active at very low concentrations against gram-positive bacteria, with the exception of temporin L (TL), the only member with a +3 charge, showing strong activities also against gram-negative bacteria. Interestingly, temporins are nontoxic to mammalian cells [8].

A poorly investigated font of antimicrobial peptides is the Royal Jelly (RJ) [9]. The RJ is a nutritive secretion produced by the hypopharyngeal and mandibular glands of worker bees; it is the main nutritional font of the queen honey bee and contains vitamins, carbohydrates, minerals and proteins. Proteins isolated by the RJ include the antimicrobial royalisin and the major royal jelly proteins (MRJPs), to which a nutritional function has been attributed [10,11]. A recently identified family of peptides isolated from the RJ of *A. mellifera* is represented by the jelleins. These are composed of 8–9 amino acids, are amidated at the *C*-terminus [12] and bear a +2 charge. Analysis of the sequence revealed that they may be produced by the processing of MRJP-1. Jelleins share the consensus sequence PFKISIH and differ in the amino acids at the *N*-terminus [Jellein II (RJ II) and Jellein III (RJ III)] and/or at the *C*-terminus. The antimicrobial activity of these peptides has been tested against some gram-positive and some gram-negative bacteria and the yeast *Candida albicans*. Jellein I (RJ I) and Jellein II (RJ II) were active against *Staphylococcus aureus* (ATCC 6535), *Staphylococcus saprophyticus* and B*acillus subtilis* (CCT 2471) among the gram-positive bacteria and *Escherichia coli* (CCT 1371), *Escherichia cloacae* (ACCT 23 355), *Klebsiella pneumoniae* (ACCT 13 883) and *Pseudomonas aeruginosa* (ACCT 27 853) among the gram-negative bacteria [12]. Jellein III (RJ III) showed a less broad spectrum of activity, while Jellein IV was inactive against all the tested microorganisms.

With the aim of determining the structure–activity relationships for jelleins, we studied both the antimicrobial activity and the

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‡ *Special issue devoted to contributions presented at the 12th Naples Worshop on Bioactive Peptides and 2nd Italy-Korea Symposium on Antimicrobial Peptides, 4-7 June 2010, Naples, Italy.*

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**Table 1.** Peptide sequences and mass analysis

| Peptide | Sequence | Calc. mass (Da) | Meas. mass (Da) |
|---|---|---|---|
| RJ I | PFKISIHL | :953.18 | 953.24 |
| RJ II | TPFKISIHL | 1054.30 | 1054.28 |
| RJ III | EPFKISIHL | 1082.32 | 1082.29 |
| RJ IC | PFKISIHLGGY | 1230.46 | 1231.02 |
| RJ IIC | TPFKISIHLGGY | 1331.56 | 1331.90 |
| RJ IIIC | EPFKISIHLGGY | 1359.57 | 1360.10 |
| RJ I N | YGGPFKISIHL | 1230.46 | 1231.02 |
| RJ IIN | YGGTPFKISIHL | 1331.56 | 1331.90 |
| RJ IIIN | YGGEPFKISIHL | 1359.57 | 1360.10 |
| TA | FLPLIGRVLSGIL | 1396.71 | 1396.32 |
| TB | LLPI VGNLLKSLL | 1391.85 | 1391.26 |

secondary structure of RJ I, RJ II, RJ III and some analogs. We also investigated the antimicrobial activity of jelleins combined to temporins.

## Experimental

### Material and Methods

The amino acids used for the peptide synthesis Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(OtBu)-OH, Fmoc-Thr (OtBu)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-Val-OH and the rink amide-(4-methylbenzhydrylamine) (MBHA) and the activators N-Hydroxybenzotriazole (HOBt) and benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were from Novabiochem (Gibbstown, NJ, USA). Acetonitrile (ACN) was from Reidel-deHaën (Seelze, Germany) and dry DMF was from Lab-Scan (Dublin, Ireland). All other reagents were from Fluka (Milan, Italy). LC–MS analyses were performed on a LC-MS equipped with an electrospray single quadrupole souce Finnigan Surveyor MSQ (Thermo-Italy) on a Jupiter 5 µ C18 300 Å, (150 × 4.6 mm) column (Phenomenex-Italy). Purification was carried out on a Jupiter 10 µ Proteo 90 Å (250 × 10 mm) column (Phenomenex-Italy).

### Peptide Synthesis

Peptides were synthesized on solid phase by Fmoc chemistry on the MBHA mmol/g resin by consecutive deprotection, coupling and capping cycles. Deprotection: 30% piperidine in DMF, 5 min (2×). Coupling: 2.5 equivalents of amino acid + 2.49 equivalents of HOBt/HBTU (0.45 M in DMF) + 3.5 equivalents NMM, 40 min. Capping: acetic anhydride/DIPEA/DMF 15/15/70 v/v/v, 5 min. (Refer Table 1 for sequences)

Peptides were cleaved off the resin and deprotected by treatment of the resin with a solution of TFA/TIS/$H_2O$ 95/2.5/2.5 v/v/v, 90 min. TFA was concentrated and peptides were precipitated in cold ethylic ether. Analysis of the crudes was performed by LC–MS using a gradient of ACN (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 30 min. Purification was performed by semipreparative RP-HPLC using a gradient of ACN (0.1% TFA) in water (0.1% TFA) from 5 to 50% in 30 min.

Mass analysis (Da):

Pure peptides were lyophilized, redissolved in $H_2O/CH_3COOH$ and relyophilized prior to use.

### Circular Dichroism

CD spectra were recorded at 25 °C using a 1-cm quartz cell with the J-810 spectropolarimenter (Jasco, Cremella, Italy) using a 260–198 nm measurement range, 100-nm/min scanning speed, 1-nm bandwidth, 4-s response time and 0.5-nm data pitch.

Peptides concentration for CD measurement was 25 µM. CD spectra were registered in 10-mM sodium phosphate buffer at pH 7.4 and in 10-mM sodium phosphate and 20 mM SDS buffer at pH 7.4.

### Bacteria

The study included the following species: *S. aureus* (isolate A170, A172, 007), *Listeria monocytogenes, Salmonella enterica* serovar Paratyphi and *E. coli*. Isolates were obtained from patients hospitalized at the Medical School of the University of Naples. Specimens were analyzed using PCR assay of the genes *sea (S. aureus), MonoA (L. monocytogenes), abe (S. enterica* serovar Paratyphi*) and sat (E. coli)* [13–16].

### Antibacterial Activity

Bacteria were grown at 37 °C in tryptic soy broth (TSB) (*S. aureus* and *L. monocytogenes*) or in Luria–Bertani (LB) medium (the remaining bacterial species), harvested in exponential phase ($OD_{600}$ nm; 0.6–0.8), centrifuged ($8 \times 10^3$ g for 10 min), washed with saline (0.15 M NaCl), resuspended in Muller Hinton (MH) broth at the concentration of approximately $10^6$ colony-forming units (CFU)/ml and distributed, in triplicate, into 96 well plates (60 µl/well), mixed with increasing concentrations of the antimicrobial peptides dissolved in sterile distilled water (5–400 µg/ml, 40 µl/well) and incubated at 37 °C for 20 h. The minimal peptide concentration at which 100% inhibition of microbial growth was observed is defined as MIC and determined by measuring the absorbance at 600 nm (Biorad microplate reader model 680, Hercules, CA, USA). The bactericidal activity of the peptides was measured by spotting an aliquot (10 µl) of each well on tryptic soy agar (TSA) or LB agar and counting the CFU. In addition, a potential synergism between two different antimicrobial peptides was evaluated by adding combinations of the two peptides to wells containing $10^5$ CFU/well in a final volume of 100 µl. The ranges of peptide dilutions used were 5–400 µg/ml, 40 µl/well. The presence of synergism was established as described [17]. The fractional inhibitory concentration (FIC) index for combination of two peptides was calculated according to the equation: FIC index $= FIC_A + FIC_B = A/MIC_A + B/MIC_B$, where A and B are the MICs of drug A and drug B in the combination, $MIC_A$ and $MIC_B$ are the MICs of drug A and drug B alone, and $FIC_A$ and $FIC_B$ are the FICs of drug A and B. The FIC indices were interpreted as follows: $\leq 0.5$, synergy; $>0.5$, no interaction.

### Electron Microscopy

Bacteria (*S. aureus* or *S. enterica* serovar Paratyphi; $10^6$ CFU/60 µl) were mixed on a microscope slide with the antimicrobial peptide (*S. aureus*: 100 µg RJ I in a total volume of 40 µl; *S. enterica* serovar Paratyphi: 100 µg RJ I in a total volume of 40 µl), incubated overnight in 2.5% gluteraldehyde and then treated for 1 h, in succession, with 20, 40 and 60% ethanol and overnight with 70% ethanol. The slides were finally washed with 90 and 100% ethanol for 1 h, dried at 37 °C for 15 min and observed with the SEM Zeiss microscope (Evo 40, Jena, Germany).



  

### Test of the Hemolytic Activity of the Antimicrobials

RJ1, temporin A (TA) and temporin B (TB) were tested individually for their hemolytic activity using mouse red blood cells. The blood was collected from the tail of the animals, centrifuged ($4 \times 10^2$ g for 3 min) and washed with saline. A 4% suspension of mouse red blood cells was mixed with the peptide combination (50 μg/ml RJ I + 11 μg/ml TB in 100 μl saline; 100 μg/ml RJ I + 6 μg/ml TA in 100-μl saline) and incubated for 1 h at 37 °C. The hemolytic activity was measured according to the formula $(OD_{peptide} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}) \times 100$ where the negative control (0% hemolysis) is represented by erythrocytes suspended in saline and the positive control (100% hemolysis) is represented by the erythrocytes lysed with 1% Triton X100 [18].

## Results and Discussion

### Synthesis and CD Studies

Our studies are focused on RJ I, RJ II and RJ III. Peptides were synthesized on solid phase by Fmoc chemistry, purified by HPLC and characterized by electrospray mass analysis. Sequences of the peptides are reported in Table 1. Analogs of jelleins were designed to have a UV reporter amino acid for the evaluation of their concentration and the successive studies of their secondary structure. We chose Tyr as the reporter, as it shows a strong absorbance at 280 nm. Tyr was separated by the wild-type sequence of the jelleins by two Gly residues to avoid interferences in the CD spectra, which we recorded to check the presence of secondary structure of the peptide. It is known, in fact, that aromatic side chains, similar to those from Tyr, influence CD absorption in the far ultraviolet, where the signals of $\alpha$-helices appear [18]. As we could not predict the influence of these modifications on the structure of the peptides, we synthesized two set of peptides, one having at the *N*-terminus the sequence YGG and the other with the GGY at the *C*-terminus, so that Tyr was always separated by the peptides.

Analysis of the secondary structure of the peptides was carried out by CD (Figure 1) in phosphate buffer and SDS, which is commonly employed as a model for the negatively charged bacterial membrane. Interestingly, peptides bearing three extra amino acids at the *C*- or *N*-terminus show different secondary structure. RJ III, the *N*-terminal-modified RJ IIIC and the *C*-terminal-modified RJ IIIN were hard to dissolve in phosphate buffer, and did not show clear conformational preference in SDS. In phosphate buffer, unmodified RJ I and RJ II exhibit similar behavior; CD spectra show a deep minimum at 200 nm and a shallow minimum at 224 nm; in SDS a minimum at 207 nm and a shoulder at 229 nm appear. It seems that in phosphate buffer the peptides do not assume a defined conformation, while in SDS they seem to approach a helix-like conformation. Ratio between the dichroic intensities at 229 and 207 nm indicates that the peptides assume the conformation of a $3_{10}$ helix [19]. *C*-terminal-modified RJ IC and RJ IIC in phosphate buffer precipitate or aggregate, while in SDS they show a minimum at 220 nm and a maximum around 200 nm, indicative of a $\beta$-sheet conformation.

The *N*-terminal-modified peptides RJ IN and RJ IIN do not assume a preferred conformation in phosphate buffer, where again likely aggregation occurs, while in the presence of SDS they show one minimum at 206 nm and a shoulder around 216 nm, which indicates the trend to assume the conformation of a $3_{10}$ helix.

Comparison of CD data suggests that even a subtle modification of the primary structure of peptides influences their secondary structure. Interestingly, when the reporter tail YGG is at the *C*-terminus the peptide assumes a beta sheet conformation in SDS, while the unmodified peptide is an $\alpha$-helix in the same conditions.

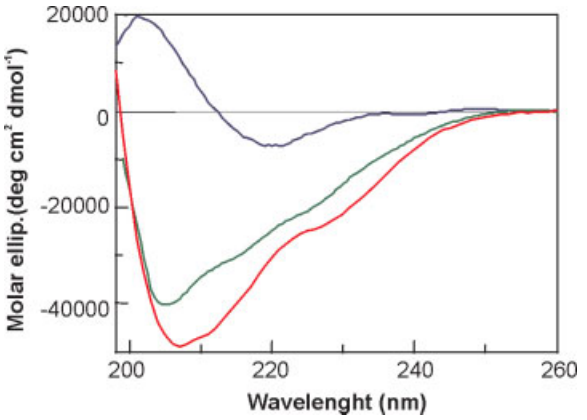


**Figure 1.** CD spectra of RJ II (brown), RJ IIN (green) and RJ IIC (blue) 25 μM in phosphate buffer 10 mM, SDS 20 mM, pH 7.4.

### Antimicrobial Activity

Data obtained by the analysis of the secondary structure of the peptides stimulated us to explore the antimicrobial activity of all modified and natural peptides to verify whether any structure–activity relationship may be brought to light. Analysis of the antimicrobial activity was carried out on gram-positive and gram-negative bacteria. The peptide RJ I showed total inhibition of bacterial growth for *S. aureus* A170, *L. monocytogenes* and *S. typhimurium* only at very high concentrations (≥200 μg/ml). At these concentrations, RJ I also exhibited bactericidal activity against these three bacterial species, as demonstrated by lack of growth on agar (data not shown). RJ II and RJ III instead were inactive even at 200 μg/ml. Studies by scanning electron microscopy showed the ability of the antimicrobial peptide RJ I to perforate the bacterial membrane of either *S. aureus* 170 or *S. typhimurium* (Figures 2 and 3). Interestingly, the antimicrobial activity of RJ I was potentiated when the jellein was administered together with TA and TB. These antimicrobial peptides, secreted by the granular glands of the European red frog *R. temporaria*, are mainly active against gram-positive bacteria at low concentrations (10–20 μg/ml) [20]. Antimicrobial activity of TA and TB against gram-negative bacteria is observed only at high concentrations (>100 μg/ml) [17]. Different combinations of RJ I with TA and TB were tested on *S. aureus* and *L. monocytogenes*, the bacteria against which RJ I showed some antimicrobial activity. When RJ I at 50 μg/ml was administered with 11 μg/ml of TB, we observed a 90% inhibition of bacterial growth on *S. aureus* A170; the same doses of the two peptides alone caused the inhibition of the bacterial growth of 21% for RJ I and 36% for TB (Figure 4). Experiments on *L. monocytogenes* showed a 99% inhibition of the bacterial growth when 100 μg/ml of RJ I was administered with 6 μg/ml of TA, while the same doses of the two peptides had no effect on bacterial growth (Figure 5). The FIC indices of 0.3 for RJ I + TB and of 0.4 for RJ I + TA against *S. aureus* A170 and *L. monocytogenes* respectively confirm the synergistic effect between the peptides (an FIC index of ≤0.5 indicates synergy) [17]. Synergism has been observed between different peptides produced by the same organism; examples are represented by



  

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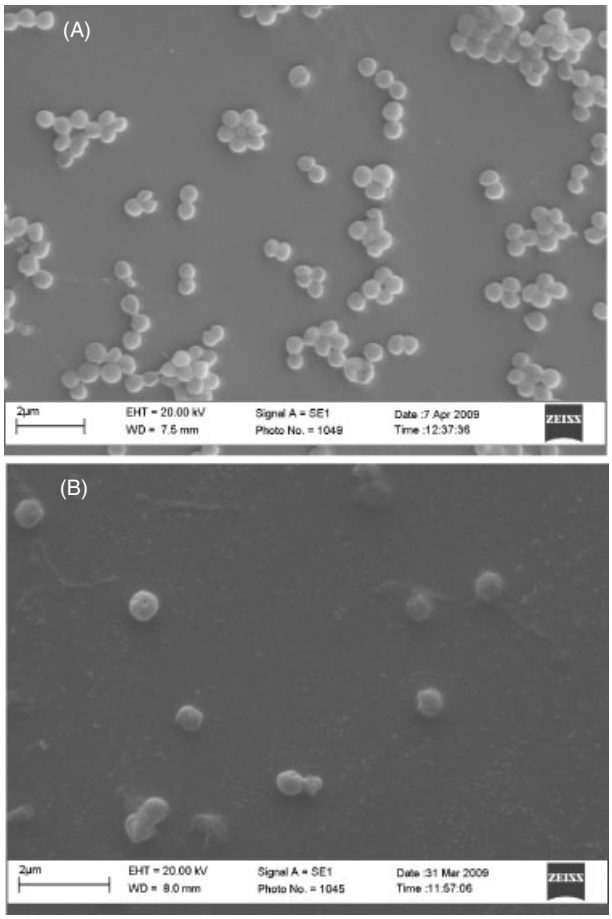


**Figure 2.** Electron microscopy images of (A) untreated *S. aureus* and (B) *S. aureus* treated with RJI.

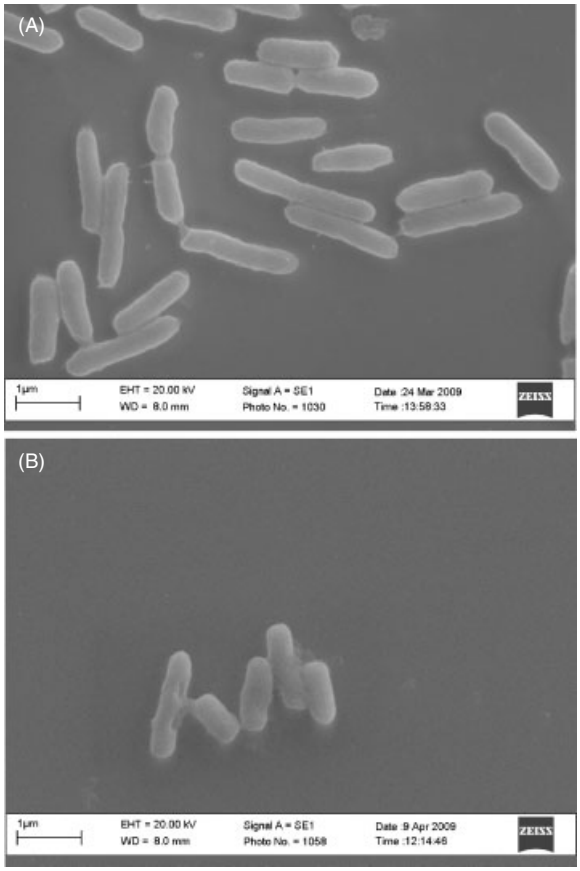


**Figure 3.** Electron microscopy images of (A) untreated *S. paratyphy* and (B) *S. paratyphy* treated with RJI.

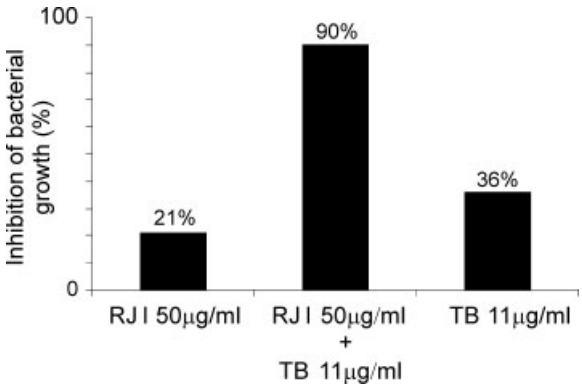


**Figure 4.** Synergistic effect of RJI and TB on *S. aureus*.

different isomers of dermaseptins, cathelicidins and defensins, hepcidin and moronecidin and temporins [17,21,22]. In the case of TA, TB and TL, it was suggested that TL inhibits aggregation of TA and TB, helping them to cross the outer membrane and reach the cytoplasmatic membrane [21]. For catelicidins and defensin, it was hypothesized that the peptides synergistically work augmenting the membrane permeabilization of the target cell. In any case, the synergic effect seems to be mediated by the increased ability to destabilize the cell membrane and this may also be the case for RJ I and temporins.

Very low activity was detected against the same bacteria for all the *N*-terminal-modified peptides. Analysis of the antimicrobial activity of the *C*-terminal-modified jelleins revealed that RJ IC was active at 100 µg/ml against *S. aureus* 170, while RJ IIC and RJ IIIC at the same concentration showed an even poorer activity. Other strains of *S. aureus* such as *S. aureus* 172 and *S. aureus* 7 were also employed in these experiments, but with unsatisfying results. Low-to-null activity was detected against *L. monocytogenes* at concentrations up to 100 µg/ml. Surprisingly, RJ IC was active against *E. coli* at 30 µg/ml and against *S. paratyphyi B* at 80 µg/ml, while RJ IIC and RJ IIIC did not show any activity.

### Test of the Hemolytic Activity of the Antimicrobials

For future therapeutic use of these antimicrobial peptides, they were tested both individually and in combination with each other for their hemolytic activity using mouse red blood cells [23].

The antimicrobial peptides were not hemolytic both individually (RJ I 11%, TB 0%, TA 4%) and in synergy (RJ I + TB 8.5% and RJ I + TA 12.5%).

## Conclusions

Data reported indicate that the amino acid sequence contained in RJ I is necessary for the antimicrobial activity. Modifications at the *N*-terminal end cause a reduction of the activity; in fact, RJ II, RJ III and all the *N*-terminal-modified peptides are less active as compared to RJ I. Furthermore, the secondary structure of the



  

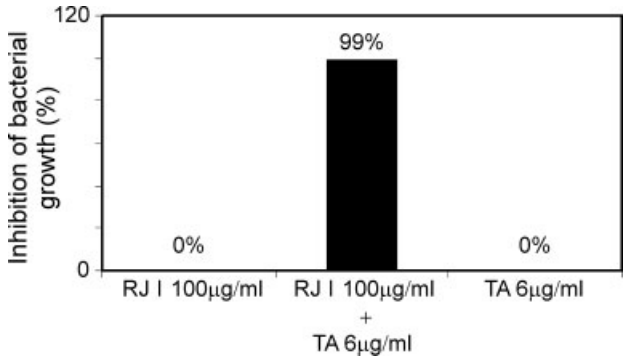


**Figure 5.** Synergistic effect of RJ I and TA on *L. monocytogenes*.

peptides is strongly influenced by the amino acid at the *C* and *N* terminal end: addition of three amino acids at the *C*-terminal induces the formation of a beta sheet in SDS. The antimicrobial activity reported for RJ I in combination with peptides of the temporin family suggests a new approach for the development of antimicrobial agents, based on the use of peptides produced in nature by different organisms.

### Acknowledgement

The work of RC was supported by grant IZS ME 07/09 RC.

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