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PRODUCTION OF BACTERIOCIN EC2 AND ITS INTERFERENCE IN THE GROWTH OF *SALMONELLA* TYPHI IN A MILK MATRIX

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ABSTRACT

Bacterial interference can occur through various mechanisms, including the production of peroxides, acids, ammonia, bacteriolytic enzymes or bacteriocins. The strain *Escherichia coli* EC2 produces the antimicrobial substance (AMS) EC2, able to inhibit different strains of Gram-negative bacteria isolated from food, as *E. coli* and *Salmonella* sp. The activity of AMS EC2 was lost after treatment with proteolytic enzymes, indicating the presence of an active proteinaceous compound, suggesting that it is a bacteriocin. The substance, renamed bacteriocin EC2, has its better production when the producer strain is grown on Casoy medium, at 37ºC and pH 6.0, without NaCl addition, but it is also able to be produced in milk. When co-cultivated in UHT milk with the producer strain *E. coli* EC2, the growth of the indicator strain *Salmonella* Typhi is totally inhibited within the first 4 hours of incubation, suggesting a potential application of bacteriocin EC2 in the control of *Salmonella* sp. e.g. in foods.

Keywords: *Escherichia coli* EC2, bacteriocin EC2, *Salmonella* Typhi, bacterial interference, milk

INTRODUCTION

Consumers are constantly being warned about possible adverse health effects caused by the presence of chemical additives in foods and as a result, opting for natural and fresher foods without the addition of preservatives. This perception, coupled with the increasing demand for minimally processed foods and longer shelf-lives, have stimulated the interest in researching new and more efficient natural preservatives against spoilage and pathogens as *Salmonella* (**Chen and Hoover, 2003; Vignolo *et al.*, 2012**).

According to the Public Health Agency of Canada, there are about 1.3 billion cases of non-typhoid salmonellosis worldwide each year and the WHO estimates that there are 17 million cases and nearly 500,000 deaths per year caused by typhoid fever. Generally, peaks of the disease occur between summer and autumn in developing countries, and salmonellosis is responsible for about 20% of deaths related to diarrheas caused by bacteria (**PHAC, 2012**). It is believed that the incidence of salmonellosis in Brazil is extremely high, however, underreported, since only typhoid fever is compulsory notification mandatory in our country, thus hampering the data collection. With the exception of *Salmonella* Typhi and *S.* Paratyphi, salmonellas usually generate a self-limiting clinical with spontaneous reversion in 48 hours, and antibiotics in the treatment of gastroenteritis is not recommended, as it prolongs the period of excretion of the agent, characterizing the asymptomatic carrier, and promoting the emergence of multiresistant strains (**Shinohara *et al.*, 2008**).

It has been found that in nature, bacteria interact with each other, trying to establish and control their environment. Some of these interactions , the bacterial interferences - are synergistic, while others are antagonists, since the microorganisms can interfere with the growth of the other to compete for its ecological space having thus an important role in the maintaining of normal flora, preventing invasion by exogenous bacteria (**Brook, 1999; Altenhoefer *et al.*, 2004**). The bacterial interference can operate through various mechanisms such as production of hydrogen peroxide, lactic acid, ammonia, bacteriolytic enzymes or bacteriocins (**Tolinački *et al.*, 2010**).

Bacteriocins are ribosomally synthesized peptides with antimicrobial activity against more or less related bacteria (**Nes & Holo, 2000**).These substances have gained attention due to their powerful spectrum of activity, its stability and its low or even absent toxicity to humans (**Bastos *et al.*, 2009; Budič *et al.*, 2011; Lohans and Vederas, 2012**). Bacteriocins produced by Gram-positive bacteria are the most studied, aiming its use as food biopresservatives, but the antimicrobial substances produced by Gram-negative bacteria can also have this potential application (**Fleming *et al.*, 2010; Fleming *et al.*, 2011**).

The strain of *Escherichia coli* EC2, studied by our group, produce an antimicrobial substance initially named AMS EC2, which has antagonistic activity against different strains of Gram-negative bacteria isolated from food, including *E. coli* and *Salmonella* sp., many of them, resistant to antibiotics (**Fleming *et al*, 2010**). In this work, we verified the proteinaceous nature of the antimicrobial substance EC2 in order to characterize it as a typical bacteriocin, the best conditions to its production and, given the importance of *Salmonella* as a foodborne pathogens, the ability of bacterial interference from the producer strain *E. coli* EC2 on a strain of *Salmonella enterica* subsp. *enterica* serotype Typhi, using milk as a food matrix.

MATERIAL AND METHODS

Strains and milk samples

Strain *E. coli* EC2, producer of AMS EC2, was isolated from cheese as described by **Fleming *et al.* (2010)**. Non-bacteriogenic strains *Salmonella enterica* subsp. *enterica* serotype Typhi ATCC19214 and *E. coli* ATCC25922 were used as indicators. As food matrix, integral UHT milk was used.

Investigation of the proteinaceous nature of AMS EC2

The effects of the proteolytic enzymes pronase, proteinase K and trypsin (Sigma-Aldrich, São Paulo, Brazil) on antimicrobial substance activity were determined according to **Giambiagi-deMarval *et al.* (1990)**. The enzymes (1mg/ml) were prepared in 0.05 M-Tris pH 8.0, 0.01 M-$CaCl_2$ and 40 □l were applied around the producer colonies after chloroform treatment. The plates were incubated at 37ºC for a further 4 h and sprayed with the indicator strain. After the treatment with the enzymes, the absence of inhibition zones when the indicator strains were used indicates the proteinaceous nature of the antimicrobial substance. Antimicrobial substances were also treated with 0.2 N NaOH to discard that the inhibition exhibited was due to organic acids produced by the producer strain during its metabolism.

Plasmid isolation

This method was performed according to **Takahashi and Nadano (1984)**. Five milliliters of the cultures were harvested by centrifugation at room temperature at 10.000 x g for 5 min. Either 200 µl of buffer A (40 mM Tris-acetic acid - 2 mM disodium EDTA, pH 8.0) was added to the cell pellet. Then, 400 µl of lyse solution (4% sodium dodecyl sulfate (SDS) -100 mM Tris - 0,4 N NaOH) was added to the cell suspension. The tube was gently inverted 5 to 10 times and allowed to stand at room temperature for 5 min. For neutralization, 300 µl of cold buffer B (3 M sodium acetate-acetic acid, pH 5.5) was added and gently mixed by inversion 10 to 20 times. After being maintained at 4°C for 5 min, the tube was centrifuged at room temperature and then again maintained at 4°C for 10 min. Salt-precipitated material was centrifuged at 4°C, and the supernatant was transferred to another tube by decantation (ca. 700 µl). An equal volume of chloroform was added and emulsified by inversion 5 to 10 times, followed by centrifugation at 4°C to break the emulsion. Aqueous phase, (ca. 500 µl) was carefully transferred to another tube. Cold ethanol (1 ml) was added and inverted 5 to 10 times. The tube was maintained at 0°C for 5 min. The precipitate was collected by centrifugation at 4°C, and the supernatant was removed by decantation. The pellet was dissolved in 100 µl of buffer C (10 mM Tris-acetic acid and 2 mM disodium EDTA, pH 8.0). The plasmid DNA solution was subjected to electrophoresis in 0.7% (w/v) agarose gel at 100 V.

Optimization of AMS EC2 production

Tests were performed as described by **Fleming *et al.* (2011)**. Different culture media, pH ranges and NaCl concentrations were evaluated to the production of AMS EC2. In all tests, the production was evaluated by the diameter of the inhibition zones against the indicator strain *Escherichia coli* ATCC25922. The following solid media were used: BHI (Brain heart infusion, Himedia, Brazil), CAS (casaminoacids 1.5% [w/v], Merck, Germany), Casoy (Himedia, Brazil), HI (heart infusion, Himedia, Brazil), MH (Müller-Hinton, Himedia, Brazil), NA (Nutrient agar, Himedia, Brazil) and TSA (Trypticase soy agar, Oxoid, England). The effect of the growth temperature on bacteriocin production was evaluated by incubating Caosy medium plates at 25°C, 37°C and 42°C for 18 h, after spotting the producer strains. The influence of the initial pH was determined by adjusting Casoy medium with HCl or NaOH, to achieve pH values of 4.0, 5.0, 6.0, 6.0, 7.0, 8.0, and 9.0 before spotting the producer strains. The plates were incubated at 37°C for 18 h. The influence of salt was determined by growing the producer strains on Casoy medium plates with NaCl concentrations of 0, 1, 2 and 3% (w/v). The plates were incubated for 18h at 37°C.

Production of AMS EC2 on milk agar

This method was performed as described by Giambiagi-deMarval *et al*. (1990). The producer cells were grown in Casoy broth (Himedia, Brazil) for 24 h. Five microliters of culture were spotted onto Casoy (positive control) and Milk agar (UHT milk with 1% w/v agar) plates. After 18 h at 37°C, the bacteria were killed by exposure to chloroform vapor and the plates were sprayed with 3 ml of Casoy soft agar added by 0.3 mL of the culture of indicator strain previously grown. The plates were further incubated for 18 h at 37°C and the inhibition zones were measured.

Bacterial interference experiments

The producer strain *E. coli* EC2 and the indicator strain *Salmonella* Typhi were grown overnight on Casoy agar. Isolated colonies from the cultures were separately inoculated on saline solution 0.85% (w/v) until achieve the turbidity of a 0.5 from McFarland standard (~$1{,}5\times10^8$ cfu/mL). The suspensions were diluted 300 times and then, 100µL of each suspension were transferred concomitantly (interference experiments) to Erlenmeyers flasks containing 50 ml of UHT milk. The suspensions were also inoculated separately, as control. Immediately after homogenization, aliquots of the co-culture and the controls were diluted and plated onto EMB (Eosin-methylene blue, Himedia, Brazil) and Hektoen enteric agar (Himedia, Brazil), to quantify both, producer and indicator strains. After periods of 2h, 4h and 24h of incubation to 37ºC, the quantification was repeated.

Statistical analysis

The statistical analyses were performed using the GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego, California, USA). For all significance tests, p values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Although bacteriocins produced by Gram-negative bacteria are not as studied as bacteriocins produced by Gram-positive bacteria, different applications have been suggested for these substances. According to **Gillor *et al.* (2005)**, administration of colicins and microcins may reduce levels of enteric pathogens in animals, and prevent the acquisition of pathogenic strains. The potent activity exhibited by these bacteriocins, added by the narrow spectrum of bacterial targets, makes them attractive tools for food preservation applications (**Duquesne *et al*., 2007**). Other examples include their potential use antitumor agents and also to control diarrheal disease caused by enteropathogenic bacteria (**Lagos *et al*., 2009**).

The strain *Escherichia coli* EC2 (formerly strain *E. coli* 02) was isolated from a cheese sample supplied by a commercial establishment in Rio de Janeiro, Brazil. This strain produces the antimicrobial substance (AMS) EC2, which has antagonistic activity against different strains of Gram-negative bacteria isolated from food, including *E. coli* and *Salmonella* sp., many of them, antibiotic resistant (**Fleming *et al*., 2010**).

To optimize conditions for production of AMS EC2, seven growth media were compared (Table 1). The media HI, Müller-Hinton and NA did not allow the production of AMS EC2. No significant difference ($p > 0.05$) was detected in the inhibition zones obtained in media as BHI, TSA, CAS and Casoy. However, visibly, strain EC2 produced the largest and clearest zones of inhibition in Casoy medium. This medium was therefore used in subsequent experiments.

Table 1 Effect of the growth conditions on the production of AMS EC2

Growth Conditions	Inhibition zones (mm)
Growth medium	
AN	-
BHI	10.7 ± 1.5
CAS	12.0 ± 1.4
Casoy	13.0 ± 1.4
HI	-
MH	-
TSA	11.0 ± 1.3
pH	
4.0	-
5.0	-
6.0	14.4 ± 0.9
7.0	10.7 ± 0.5
8.0	-
9.0	-
NaCl concentration	
None	13.0 ± 1.4
1.0%	10.2 ± 0.4
2.0%	11.8 ± 0.4
3.0%	-

Legend: The numbers represent the means and standard deviations of the diameters of inhibition zones (in mm) from three independent experiments; -, absence of bacteriocin production. *Escherichia coli* ATCC25922 was used as indicator strain. The diameter of the producer growth ranged from 0.3 and 0.4 mm.

Regarding temperature, AMS EC2 was produced at the three temperatures tested, with a slightly higher production at 37ºC. The growth in Casoy medium with initial pH 6.0 and 7.0 did not affect the antimicrobial substance production. However, under pH 4.0, 5.0, 8.0 and 9.0, there was no production of AMS EC2 (Table 1). This type of behavior has been observed in some bacteriocins, where the optimal temperature and pH for cell growth did not correspond to the requirements for maximum production of these substances (**Møretrø *et al*., 2000, Fleming *et al*, 2011**). The ability to produce AMS EC2 under the presence of NaCl was also evaluated. The production was unaffected by addition of concentrations of 1% and 2%, but was inactivated when the producer strain was grown with 3% NaCl (Table 1).

The Figure 1 illustrates the inhibition of *Salmonella* Typhi by AMS EC2 when the producer *E. coli* strain is grown under the best conditions of production (Casoy medium, 37ºC, pH 6.0, without NaCl addition). These conditions were also employed to check the sensitivity to proteolytic enzymes and to obtain the active supernatant from EC2 culture to bacterial interference in milk.

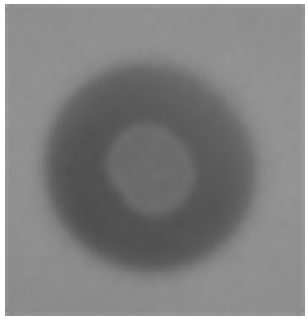

Figure 1 Agar-spot assay on Casoy medium demonstrating the antimicrobial activity exhibited by *E. coli* EC2 against the indicator strains *Salmonella* Typhi, as seen by a clear zone of inhibition around producer strain growth (central spot).

The confirmation of the suspicion that the AMS EC2 is a bacteriocin was performed by testing for susceptibility to proteolytic enzymes (Figure 2). The AMS EC2 was sensitive to all the three enzymes tested (trypsin, protease, and pronase) characterizing its proteinaceous nature. Furthermore, it also proved to be resistant to NaOH, discarding the possibility that inhibition of indicator is being made by acids released by the producer strain. Similar results were found with the antimicrobial substance produced by the strains *Klebsiella ozaenae* K, which was sensitive to the same three proteolytic enzymes used, and with the substance produced by the *Raoultella terrigena* L, that was sensitive to two enzymes tested, suggesting that these substances are also bacteriocins (**Fleming *et al.*, 2010**).

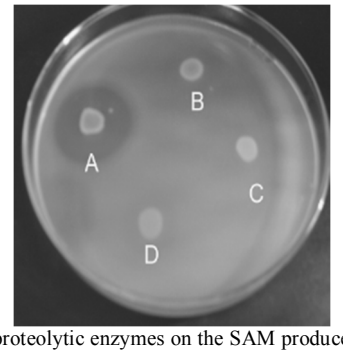

Figure 2 Action of proteolytic enzymes on the SAM produced by the strain EC2. Spots from the producer strain were done by application of 10 □l of the bacterial culture onto surface of the plate. Block letter A corresponds to control, without treatments. B, C and D correspond to the treatment with proteinase K, pronase XXIII, trypsin and NaOH, respectively. The indicator strain used in these experiments was *Salmonella* Typhi.

Additionally, the location of genetic elements involved in the bacteriocin EC2 production was also investigated. Plasmid DNA isolation revealed that strain EC2 has no plasmids (**Figure 3**), suggesting that genes responsible for the bacteriocin production are chromosomal. Although most of the bacteriocins produced by Gram-negative strains are encoded by plasmids well studied bacteriocins, as the microcin E497 produced by *K. pneumoniae,* are chromosomal encoded (**Cascales *et al.*, 2007; Duquesne *et al.*, 2007**).

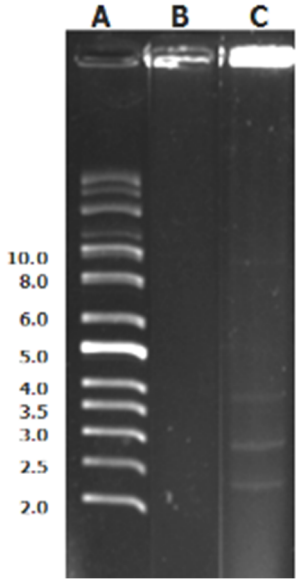

Figure 3 Electrophoresis in agarose gel (0.7%, w/v) from the plasmid DNA. A, supercoiled DNA ladder (Invitrogen); B, *E. coli* EC2 (plasmid free); C, *Pseudomonas aeruginosa* 8.3 (control of DNA extraction). Numbers at left represent size in kb.

The first step to evaluate the bacterial interference between *E. coli* EC2 and *Salmonella* Typhi strains in the food matrix consisted on verifying the production of bacteriocin EC2 on milk agar, which was initially verified by diffusion on solid medium, using milk agar. The results showed that the activity of the bacteriocin against both tested indicators is not influenced by the milk, even though this is a compound extremely rich in carbohydrates and proteins. The inhibition zones on milk agar presented no significant difference in comparison with the control agar Casoy under the best conditions for the EC2 production (Table 2). Some studies related that the production of bacteriocin on food matrix can be affected by the food components (**Stergiou *et al.*, 2006; Galvéz *et al.*, 2007**). At least in relation to milk, bacteriocin EC2 seems to be stable.

Table 2 Comparison of the production of bacteriocin EC2 in Casoy agar and Milk agar

Indicator strains	Inhibition zones (in mm)	
	Casoy agar	Milk agar
E. coli ATCC25922	14.0 ± 0.5	14.0 ± 0.5
S. Typhi ATCC19214	10.5 ± 1.0	9.0 ± 1.0

Legend: The diameter of the producer growth ranged from 0.3 and 0.4 mm.

The bacterial interference experiments using milk as the liquid matrix showed that the presence of EC2 strain was able to inhibit the growth of the indicator *Salmonella*, and this was observed within the first four hours of incubation (Table 3).

Table 3 Bacterial interference between *E. coli* EC2 e *Salmonella* Typhi in milk.

Incubation time (h)	Positive control: *Salmonella* Typhi (cfu/ml)	Bacterial interference	
		E. coli EC2 (cfu/ml)	*Salmonella* Typhi (cfu/ml)
0	1.2×10^2	1.0×10^2	1.0×10^2
2	1.0×10^2	1.0×10^2	1.5×10^2
4	5.0×10^2	2.0×10^2	< 1.0
24	1.4×10^9	1.9×10^9	< 1.0

Legend: cfu, colony-forming units.

The same experiment was conducted with a non-bacteriocinogenic strain of *E. coli* ATCC25922 (data not shown) and no reduction or inhibition of *Salmonella* cells compared to control was observed. An active preparation containing 1 600 AU/ml of the bacteriocin EC2 was able to be obtained from the supernatant of an *E. coli* EC2 culture. This preparation was diluted and used on milk. It resulted in the *Salmonella* inhibition after 2 and 4 hours of incubation. However, after 24 h, the quantification of *Salmonella* on milk was similar to the control (data not shown), suggesting that a larger number of AU/ml of bacteriocin is needed. This amount is achieved when the strain EC2 is grown in milk concurrently with the *Salmonella* indicator strain. New experiments employing higher amounts of bacteriocin EC2 aiming its purification is already being performed.

CONCLUSION

Although initial, our results are encouraging, since the bacteriocin EC2 can be produced in milk and resulted in the complete inhibition of the *Salmonella* Typhi indicator strain. A classic example of bacterial interference is the modulation of the gastrointestinal microbiota by the non-pathogenic strain of *E. coli*, which is introduced into the microbial host organism to compete and remove pathogens such as *Salmonella*. This probiotic is marketed in many countries under the name of Mutaflor® and is composed by the strain *E. coli* Nissle 1917 (**Dezfulian *et al.*, 2008; Schierack *et al.*, 2011; Inovera Bioscience, 2012**). This strain also produces an antimicrobial substance, further reinforcing the real potential application of bacteriocins produced by Gram-negative bacteria and encouraging studies with bacteriocin EC2.

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