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RESEARCH, EXTRACTION AND PARTIAL CHARACTERIZATION OF BACTERIOCIN-TYPE ANTAGONIST SUBSTANCE PRODUCED BY *Fusobacterium necrophorum*

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Abstract: Fusobacterium necrophorum is a Gram-negative anaerobic bacillus, from the amphibiotic microbiota of animals, including humans, and is associated with diseases, in different places, such as *Lemierre Syndrome*. Considering the clinical relevance of this microorganism, the *in vitro* production of an antagonist substance by F. necrophorum ATCC 25286 against 14 reference samples that integrate the human oral microbiota was evaluated. The species that exhibited greater sensitivity to the antagonist substance were *Peptostreptococcus anaerobius*, *Porphyromonas gingivalis*, F. *nucleatum Streptococcus mutans and Prevotella intermedia*, the phenomenon being best evidenced in *Brain Heart Infusion* Agar supplemented with yeast extract and glucose at pHs 6.0 and 7.0. It was shown that the antagonistic ability was not related to the production of acids, bacteriophages, hydrogen peroxide or residual chloroform. The protein fractions precipitated by ammonium sulfate that showed activity were extra and intracellular, precipitated at 50 and 80%. It was observed that C50 and C80 maintained their activity at pH values 7.0 to 9.0 and 5.0 to 9.0, respectively. Regarding heat treatment, C50 was more unstable than C80. After treatment with proteases, the active extracts lost activity, confirming the protein nature of the substance. The molecular mass of the substance was estimated between 37 and 50 kDa, according to the "in situ" activity against P. *anaerobius*. This is the first report of antagonistic activity of F. *necrophorum* and the heteroantagonism relationship observed evidences a possible ecological role of the substance in the oral ecosystem, possibly favoring indigenous bacteria with pathogenic potential, such as F. *necrophorum*, which is why this phenomenon is considered related to virulence.

Keywords: anaerobes, *Fusobacterium necrophorum*, antagonist substance,

bacteriocin-like substance.

INTRODUCTION

Fusobacterium necrophorum is a Gram-negative obligate anaerobic pleomorphic rod considered to be an important opportunistic pathogen in both human and veterinary medicine [3; 18; 24]. On the basis of morphological, biochemical, biological properties and differences in DNA level in 16S rRNA and DNA repeats two subspecies of *F. necrophorum* can be differentiated, *F. necrophorum* subsp. *necrophorum* considered to be mainly an animal pathogen, and *F. necrophorum* subsp. *funduliforme* more frequently associated with infections in humans [3; 6; 18; 26; 29].

Although overall *F. necrophorum* seems to be responsible for a low number of human infections its relevance must not be neglected. The bacterium shows a clearly established association with the ethiopathogenesis of a systemic disease known as necrobacillosis, postanginal sepsis, or Lemierre's syndrome a life-threatening septicaemic illness. The bacterium has also been implicated as an agent of community-acquired sore throat being responsible for as much as 10% of pharyngitis cases among adolescents and young adults [1; 6; 18; 24]. In addition to a possible association of the pathogen with cases of colorectal cancers [29].

Several virulence abilities that help the microorganism to evade from host defense mechanisms and contribute for tissue damage including lipopolysaccharide, hemolysin, hemaglutinin, capsule, adhesins, platelet aggregation factor, dermonecrotic toxin, several extra acellular enzymes, and mainly leukotoxin have already been described [16; 19]. However, we are not aware of any report on the production of antagonistic substances such as bacteriocin by *F. necrophorum*.

Bacteriocins are defined as bactericidal or bacteriostatic proteinaceous substances that play an ecological role in regulating bacterial populations either by preventing or enabling the establishment of a microorganism in an already existing microbial community. Whatever roles bacteriocins play their expression seems to be influenced by biotic and abiotic environmental factors. Bacteriocins synthesized by Gram negative bacteria are called microcins and colicins with molecular weights of up to 10 kDa and 25 to 80 kDa, respectively [7; 23].

Taking into consideration, the relevance of *F. necrophorum* as a cause of animal and human life-threatening diseases, the importance of bacterial antagonistic substances as putative virulence factors, and the lack of studies evaluating the production of this kind of substances by the bacterium we carried out this investigation aiming to search for bacteriocin-like antagonist substances production by *F. necrophorum* and to partially characterize the substance.

MATERIALS AND METHODS

BACTERIAL STRAINS

The reference strain *F. necrophorum* ATCC 25286 was tested as producer of antagonistic substance(s). Additionally to the test strain the following strains were also used as indicators: *Aggregatibacter actinomycetemcomitans* FDC Y4, *Actinomyces israelii* ATCC 12102, *Eubacterium lentum* ATCC 25559, *Fusobacterium nucleatum* ATCC 10953, *Peptostreptococcus anaerobius* ATCC 27337, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Cutibacterium* (*Propionibacterium) acnes* ATCC 6916, *Staphylococcus aureus* ATCC 33951, *Streptococcus mutans* IM/UFRJ, *Streptococcus sanguinis (S. sanguis)* ATCC 10557, *Streptococcus sobrinus* ATCC 27351, and *Streptococcus uberis* ATCC 9927. Stock

cultures were maintained in Brucella Broth (BBL, Sparks, MD, USA) supplemented with 10% (v/v) glycerol at -86ºC. All samples are part of the library of the Laboratory of Oral and Anaerobic Microbiology.

EVALUATION OF ANTAGONISTIC ACTIVITY

Antagonistic activity expression was evaluated by the overlay method [4; 8]. The test strain was cultivated in Brain Heart Infusion (Difco, Sparks, MD, USA) supplemented with 0.5% hemin, 0.1% menadione, 0.5% yeast extract, and 0.05% L-cysteine, pH 7.2 (BHI) at 37ºC for 48 h in an anaerobic chamber (Forma Scientific Inc., Marietta, OH, USA) containing 85% N_2, 5% CO_2, and 10% H_2. The culture was spotted onto the surface of the following media (Difco): Brain Heart Infusion Agar (BHIA), Brucella Agar (BA), and Tryptic Soy Agar (TSA) all of them supplemented with 0.5% hemin, 0.1% menadione, and 0.075% L-cystine added or not with 0.5% yeast extract (YE), 0.5% glucose (GLU), or both and adjusted at pH 6.0, 7.0, 8.0, and 9.0. After incubation at 37ºC for 48 h under anaerobic conditions, the strain was killed by exposure to chloroform vapour for 30 min. Residual chloroform was allowed to evaporate and the test cultures were overlaid with 3.5 ml of melted Brain Heart Infusion Soft Agar (0.7%) supplemented with 0.5% hemin, 0.1% menadione, and 0.5% yeast extract, pH 7.0 (BHISA), which had been inoculated with 0.2 ml of a 24 h BHI culture of each indicator strain. Plates were then incubated for an additional 24-48 h period at 37ºC under appropriate atmospheric conditions according to the physiology of the indicator strain and evaluated for the presence of inhibition zones. Considering all possible culture media, supplementations, and pH values combinations forty-eight distinct conditions were employed for the evaluation of antagonistic activity expression.

DETECTION OF INTERFERING FACTORS

All assays performed to evaluate possible interfering factors employed the following culture conditions. Producer and indicator strains were cultivated in BHI for 24 h at 37ºC under anaerobic atmosphere. Agar cultures were done in BHIA, pH 7.0 and incubated for 24 h at 37ºC in anaerobiosis unless otherwise specified. *P. anaerobius* ATCC 27337 and *F. nucleatum* ATCC 10953 were employed as indicator strains.

The flip-streak method was used to eliminate bacteriophage activity as the cause for antagonism [11]. Briefly, an overnight liquid culture of *F. necrophorum* ATCC 25286 was streaked along the middle of a 2 to 3 mm-thick agar plate. The plates were incubated for 48 h after which the agar was flipped over using a forceps and an overnight culture of *P. anaerobius* or *F. nucleatum* was swabbed over the reverse side of the agar. Following incubation evidence of inhibition of the indicator strain was recorded.

The occurrence of inhibition caused by long-chain fatty acids synthesized by the test strain was evaluated by using agar plates added or not with 1% (w/v) soluble starch. To search for interference of acidity resulting from bacterial metabolism the pH of the surface of the culture medium inside and outside the inhibition zones was measured with the aid of a microelectrode (Microelectrode Inc., Foster City, CA, USA) [30].

Agar medium added or not with 0.03% (w/v) catalase (Sigma Company; St. Louis, MO, USA) was employed to evaluate the interference of hydrogen peroxide [10]. The occurrence of inhibition due to residual chloroform was investigated by performing antagonistic activity assays without using the substance. After growth of the test strain,

the indicators were inoculated with a loop near the spots of the producer strain without touching them [17].

PROTEIN EXTRACTION FROM INTRACELLULAR AND EXTRACELLULAR FRACTIONS OF *F. necrophorum* ATCC 25286

F. necrophorum ATCC 25286 was grown in 800 ml of BHI at 37°C under anaerobic conditions until early stationary phase (24 h). The culture was centrifuged at 6,000 x *g* for 30 min and cell-free supernatant was precipitated with ammonium sulfate at 30, 50, and 80% saturation levels under constant slow stirring at 4°C for 30 min in order to obtain extracellular fractions S-30, S-50, and S-80, respectively. The protein precipitate was collected by centrifugation at 12,000 x *g* for 30 min, and resuspended (1/10 of the original volume) in 10 mM Tris-HCl, pH 7.2. The crude extract was then dialysed twice (Spectra/Por® 1; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against the same buffer during 24 h at 4°C under agitation on a magnetic stirrer.

The cells pelleted by centrifugation as described above were washed three times with same buffer, suspended in 20 ml and sonicated (Branson Sonifier 450; Danbury, CT, USA) for 5 min (10 cycles of 30 sec) at 50 W in an ice bath. Cellular disruption was checked by microscopy. The extract was centrifuged at 12,000 x *g* for 60 min at 4°C and the proteins from the supernatant were precipitated with ammonium sulfate, centrifuged, suspended in Tris-HCl, and dialyzed as described above. Intracellular fractions obtained were named C-30, C-50, and C-80. Extracellular and intracellular crude extracts were filter-sterilized (Millex-GP, 0.22 µm, PES; Millipore, Carrigtwohill Co., Cork, Ireland) and tested for antagonistic activity [27].

ANTAGONISTIC ACTIVITY ASSAYS

Aliquots of 0.2 ml of a 24 h BHI culture of *P. anaerobius* ATCC 27337 and *F. nucleatum* ATCC 10953 were transferred to 3.5 ml of BHISA and the inoculums were poured over BHIA plates. Aliquots of 20 µl from each intracellular and extracellular fraction were dropped over the surface of the medium. Evidence of activity was provided by the presence of zones of growth inhibition of the indicator strain after 24 h of incubation at 37°C under anaerobic atmosphere. Active fractions were aliquoted and maintained at -80°C [14].

DETERMINATION OF PROTEIN CONCENTRATION AND SEMI-QUANTITATIVE BIOASSAY OF THE INHIBITORY ACTIVITY OF INTRACELLULAR EXTRACTS C-50 AND C-80

Protein concentration of active crude extracts was estimated according to [5].

Titration of antagonistic activity was performed. Briefly, C-50 and C-80 active extracts were titrated through serial dilutions in 0.01 M Tris-HCl, pH 7.2 sterile and tested for antagonistic activity. The presence or absence of inhibition zones was recorded. Titer expressed as arbitrary units of bacteriocin per milliliter (AU/ml) was defined as the reciprocal of the highest dilution that resulted in inhibition of the indicator lawn [14; 31].

EFFECT OF PHYSICOCHEMICAL FACTORS ON ANTAGONISTIC ACTIVITY

The effect of proteolytic enzymes on antagonistic activity was determined by using α-chymotrypsin, papain, proteinase K, and trypsin (Sigma) diluted (1.0 mg ml^{-1}) in 0.1M Tris-HCl/0.15 M NaCl, pH 7.4. Enzyme solutions were filter-sterilized (Millex-GP, 0.22 µm, PES), aliquots of C-50 and C-80 were

two-fold diluted in each enzyme solution, and mixtures were incubated at 37ºC for 2 h. Remaining antagonistic activity after enzyme treatment was determined as described previously. Diluted C-50 and C-80 (0.01 M Tris-HCl, pH 7.2) and enzyme solutions were used as controls [28].

Thermostability was evaluated by incubating two-fold diluted aliquots of C-50 and C-80 in 0.01 M Tris-HCl, pH 7.2 at 37, 45, 65, 80, 100 and 121ºC for 15, 30, 60, and 90 min, 2, 3, 4, 5, 6, and 7 h. After treatment, temperature was set at 4ºC and each aliquot was tested for antagonistic activity. Untreated diluted aliquots of C-50 and C-80 were used as controls [28].

The effect of pH was examined by employing the following buffers: citrate 0.01 M, pH 3.0, 4.0, 5.0, and 6.0 and 0.01 M Tris-HCl, pH 7.0, 8.0, and 9.0. C-50 and C-80 were two-fold diluted in each filter-sterilized (Millex-GP, 0.22 µm, PES) buffer and incubated at 37ºC for 2 h. Antagonistic activity expression was evaluated. C-50 and C-80 aliquots two-fold diluted in 0.01 M Tris-HCl, pH 7.2 and buffers without extracts were employed as controls [28].

TRICINE-SDS-PAGE AND *in situ* ASSAY

Fractions C-50 and C-80 were submitted, in duplicate, to Electrophoresis in Gel of Polyacrylamide Sodium Tricine Duodecyl Sulfate (TRICINE-SDS-PAGE, 16.5%) [25]. The gel was cut into two vertical halves and one of them was thoroughly washed (six periods of 30 min each) with sterile Milli-Q® water (Millipore, Molsheim, France) under agitation. After that the gel was employed for detection of antagonistic activity by overlaying it with 30 ml of BHISA inoculated with 0.85 ml of a 24 h BHI *P. anaerobius* culture. After incubation at 37ºC overnight in anaerobic atmosphere the presence of growth inhibition zones around protein bands was evaluated. The other half of the gel was stained with silver nitrate (CENNABRAS, Guarulhos, SP, Brazil) and the molecular mass of the active antagonistic substance was estimated by employing a 10 to 250 kDa standard (Precision Plus Protein™ Dual Color Standard; Bio-Rad Laboratories Inc., Hercules, CA, USA).

EVALUATION OF POPULATION HETEROGENEITY

The producing sample, grown in broth, for 24 h, in anaerobiosis, at 37°C, was submitted to serial dilution (10^{-1} to 10^{-8}). Each dilution was plated and incubated under the same conditions for 48 h. After growth, the Colony Forming Units (CFUs) were counted on the plates that showed bacterial counts in the range of 30 to 300 colonies. These plates were submitted to the antagonist activity test. The reading was carried out by counting the number of bacteriocin-producing CFUs, considering the presence of an inhibition halo in the revealing sample, followed by the estimate, by percentage of producing individuals [28].

STATISTICAL ANALYSIS

Data were analyzed for statistical differences by employing χ^2 test with Yates correction or Fisher's exact test when applicable (Epi Info™ version 3.5.1; CDC, Atlanta, GA, USA). The level of significance was set at $p < 0.05$.

RESULTS

EVALUATION OF ANTAGONISTIC ACTIVITY

A total of 241 (35.9%) out of the 672 performed assays for evaluating the expression of antagonism by *F. necrophorum* ATCC 25286 yielded positive results (TAB. 1). The test strain exhibited heteroantagonistic

activity against a wide pannel of indicator strains. Autoantagonism was not detected.

In regard to heteroantagonism when the analysis considered each indicator strain individually *P. anaerobius* showed to be the most sensitive indicator ($p < 10^{-7}$, OR = 11.01, 95% CI = 4.76-29.56 when compared to the sum of the results obtained for all other indicator strains). Antagonistic activity was detected in 41 (85.4%) out of 48 assays that employed this strain. High numbers of positive tests were found for *P. anaerobius*, *P. gingivalis, F. nucleatum*, *S. mutans* and *P. intermedia* (TAB.1). *S. sobrinus* was not inhibited in any tested condition.

Data regarding the influence of medium composition on antagonistic activity expression are depicted in TAB. 1. BHIA showed to be the most adequate medium for heteroantagonism expression (50.0% positive tests) when the analysis considered the three non-supplemented culture media. Statistical difference was not detected between BA and TSA ($p = 3.3 \times 10^{-1}$). Supplemented culture media showed to be more adequate for heteroantagonism detection ($p = 8.2 \times 10^{-5}$, OR = 2.28, 95% CI = 1.50-3.51). With greater emphasis on the supplemented BHIA medium compared to the supplemented BA and TSA.

The overall results concerning the effects of supplementation for all culture media demonstrated that the addition of yeast extract had no effect on the frequency of antagonistic activity detection ($p = 6.4 \times 10^{-2}$). Glucose favored the expression of antagonism ($p = 2.4 \times 10^{-4}$, OR = 2.51, 95% CI = 1.51-4.18) and the use of both supplements together also enhanced the number of positive assays ($p = 2.4 \times 10^{-5}$, OR = 2.85, 95% CI = 1.72-4.75) (TAB. 1).

The effect of culture pH on production of antimicrobial substances was investigated (TAB. 2). The optimal pH was 6.0 and 7.0 followed by pH 8.0 and 9.0. In general, higher pH values lead to lower inhibition frequencies for all indicator strains considering the sum of results obtained for all culture media.

DETECTION OF INTERFERING FACTORS

Data obtained ruled out the possibility that inhibition was due to bacteriophages, low pH values resulting from the production of organic acids, long-chain fatty acids, hydrogen peroxide, and residual chloroform.

PROTEIN EXTRACTION AND ASSAY OF ANTAGONIST ACTIVITY OF FRACTIONS OF *F. necrophorum* ATCC 25286

Extracellular fractions S-50, S-80 and intracellular C-50, C-80; originated from *F. necrophorum* ATCC 25286 and obtained by protein precipitation with ammonium sulfate; were active against *P. anaerobius* and *F. nucleatum*. Since the C-50 and C-80 fractions showed the best results.

DETERMINATION OF PROTEIN CONCENTRATION AND SEMI-QUANTITATIVE BIOASSAY OF THE INHIBITORY ACTIVITY OF INTRACELLULAR EXTRACTS C-50 AND C-80

The protein concentration of the C-50 and C-80 extracts were, respectively, 5.53 mg/ml and 6.90 mg/ml. The C-50 extract showed antagonist titers of 12800 (AU/ml) for *F. nucleatum* and 800 (AU/ml) for *P. anaerobius*. The C-80 extract, on the other hand, showed an antagonistic title of 800 (AU/ml) for *F. nucleatum* and 12800 (AU/ml) for *P. anaerobius* (TAB. 3).

EFFECT OF PHYSICOCHEMICAL FACTORS ON ANTAGONISTIC

Culture media[1]	Number of positive antagonistic activity assays[2]												
	Fn	Aa	Ai	El	Pan	Pg	Pi	Ca	Sa	Sm	Ss	Su	Total
BHIA	3	2	1	2	4	3	4	2	1	3	0	1	26
BHIA + YE	3	3	2	2	3	4	2	2	1	2	1	1	26
BHIA + GLU	4	2	2	2	4	4	4	3	3	4	0	0	32
BHIA + YE + GLU	4	1	3	3	4	4	3	3	3	3	0	0	31
Sub-total	14	8	8	9	15	15	13	10	8	12	1	2	115
BA	0	0	0	0	4	3	0	0	0	2	0	0	9
BA + YE	3	0	1	1	4	3	2	0	0	2	2	0	18
BA + GLU	4	0	3	2	4	3	3	3	2	3	0	0	27
BA + YE + GLU	4	3	2	2	4	1	2	2	2	3	0	0	25
Sub-total	11	3	6	5	16	10	7	5	4	10	2	0	79
TSA	1	0	0	0	1	1	0	0	0	1	0	0	4
TSA + YE	1	1	1	0	1	2	2	1	1	1	0	0	11
TSA + GLU	1	0	0	0	4	2	1	2	0	2	0	0	12
TSA + YE + GLU	1	2	0	1	4	3	3	1	2	3	0	0	20
Sub-total	4	3	1	1	10	8	6	4	3	7	0	0	47
Total	29	14	15	15	41	33	26	19	15	29	3	2	241

[1], all media added with 0.5% hemin, 0.1% menadione, and 0.075% L-cystine and tested at pH 6.0, 7.0, 8.0, and 9.0; [2], *F. necrophorum* and *Streptococcus sobrinus* were not inhibit in any tested condition; BHIA, Brain Heart Infusion Agar; BA, Brucella Agar; TSA, Tryptic Soy Agar; YE, 0.5% yeast extract; GLU, 0.5% glucose; Fn, *Fusobacterium nucleatum*; Aa, *Aggregatibacter actinomycetemcomitans*; Ai, *Actinomyces israelii*; El, *Eubacterium lentum*; Pa, *Peptostreptococcus anaerobius*; Pg, *Porphyromonas gingivalis*; Pi, *Prevotella intermédia*; Ca, *Cutibacterium* (*Propionibacterium) acnes*; Sa, *Staphylococcus aureus*; Sm, *Streptococcus mutans*; Ss, *Streptococcus sanguinis (S. sanguis)*; Su, *Streptococcus uberis*.

Table 1. Influence of culture media on antagonistic activity of *Fusobacterium necrophorum* ATCC 25286

Culture media pH[1]	Number of positive antagonistic activity assays[2]												
	Fn	Aa	Ai	El	Pan	Pg	Pi	Ca	Sa	Sm	Ss	Su	Total
6.0	10	6	6	7	12	10	9	9	8	11	2	1	91
7.0	7	3	6	5	10	8	9	7	4	9	1	0	69
8.0	7	2	2	2	10	9	6	3	3	7	0	0	51
9.0	5	3	1	1	9	6	2	0	0	2	0	1	30
Total	29	14	15	15	41	33	26	19	15	29	3	2	241

[1], Brain Heart Infusion Agar, Brucella Agar, and Tryptic Soy Agar added with 0.5% hemin, 0.1% menadione, and 0.075% L-cystine and supplemented or not with 0.5% yeast extract, 0.5% glucose, and both supplements; [2], *F. necrophorum* and *Streptococcus sobrinus* were not inhibit in any tested condition; Fn, *Fusobacterium nucleatum*; Aa, *Aggregatibacter actinomycetemcomitans*; Ai, *Actinomyces israelii*; El, *Eubacterium lentum*; Pa, *Peptostreptococcus anaerobius*; Pg, *Porphyromonas gingivalis*; Pi, *Prevotella intermédia*; Ca, *Cutibacterium* (*Propionibacterium) acnes*; Sa, *Staphylococcus aureus*; Sm, *Streptococcus mutans*; Ss, *Streptococcus sanguinis (S. sanguis)*; Su, *Streptococcus uberis*.

Table 2. Influence of culture media pH on antagonistic activity of *Fusobacterium necrophorum* ATCC 25286.

ACTIVITY

Differences between these two active extracts regarding proteases, temperature and stability to pH were observed in Table 3.

TRICINE-SDS-PAGE AND *in situ* ASSAY

The stained gel showed good sensitivity to the color of silver, but it was not possible to detect which of the bands refer to substances of the bacteriocins-like contained in the C-50 (2) and C-80 (3) extracts, due to the numerous protein bands visualized (FIG. 1A).

The *in situ* activity assay of C-80 (3) against *P. anaerobius* showed a clear inhibition zone in the region corresponding to bands ranging from 37 to 50 kDa (FIG. 1B).

EVALUATION OF POPULATION HETEROGENEITY

The evaluation of population heterogeneity showed that 99% of the clones of *F. necrophorum* ATCC 25286 are producers of bacteriocin-like substance against the revealing samples *P. anaerobius* and *F. nucleatum* (Fig 2).

DISCUSSION

Despite the increasing knowledge regarding the role of *F. necrophorum* as an agent of human diseases several aspects concerning the relationship between the bacterium and its host still have to be learned. The expression of a number of pathogenicity factors by *F. necrophorum* have already been described but efforts must be made aiming to elucidate in a more comprehensive way the whole set of virulence abilities exhibited by the organism [1].

Among bacterial virulence properties bacteriocins must be mentioned. These antagonistic substances influence microbial relationships, because it aid in competition for nutrients and conquest of a habitat. In addition to their ecological relevance, it has received increasing attention due to their practical application [9; 15].

Considering the genus *Fusobacterium* the elaboration of proteinaceous antagonistic substances has been reported for only two species: *F. nucleatum* [17; 22] and *F. mortiferum* [20].

For evaluating the expression of antagonism by *F. necrophorum* the reference strain *F. necrophorum* ATCC 25286 was tested against a wide pannel of indicator strains. This approach would additionally allow us to determine the activity spectrum of the antagonistic substance(s) possibly expressed and to characterize it(ies) in terms of self-antagonism: when the protein substance produced operate against the producing strain; Isoantagonism: when the produced protein substance act against another isolate of the same species; heteroantagonism: when the produced protein substance work against other species phylogenetically related to the producing species [27].

F. necrophorum exhibited a wide spectrum of antagonistic activity. In fact taking into account data generated by each antagonistic activity assay nearly all indicator strains were inhibited by the bacterium, with emphasis on greater sensitivity for *P. anaerobius*, *P. gingivalis, F. nucleatum, S. mutans* and *P. intermedia*. The strong heteroantagonism observed against *F. nucleatum* was already expected, since this is the microorganism most phylogenetically related to *F. necrophorum*. The absence of autoantagonism expression is in agreement with previous studies.

In general literature data demonstrate that bacteriocins produced by Gram negative bacteria exhibit activity mainly against closely related species and that bacteriocin producing strains usually express a specific immune mechanism that protects themseles against their own bacteriocin [23].

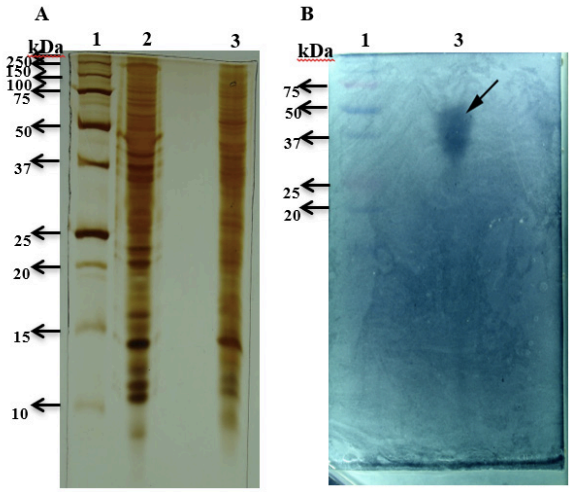

Figure 1. Tricine-SDS-PAGE and *in situ* assay of C-50 and C-80, intracellular extracts from *Fusobacterium necrophorum* ATCC 25286. A, Tricine-SDS-PAGE stained with silver nitrate; B, *In situ* antagonistic activity of C-80 extract from *F. necrophorum* ATCC 25286 against *Peptostreptococcus anaerobius* ATCC 27337 as revealed by Tricina-SDS-PAGE. 1, Standard of molecular mass (Precision Plus Protein Pertained Standards, Dual Color, 10 the 225 kDa); 2, C-50 extract; 3, extract C-80.

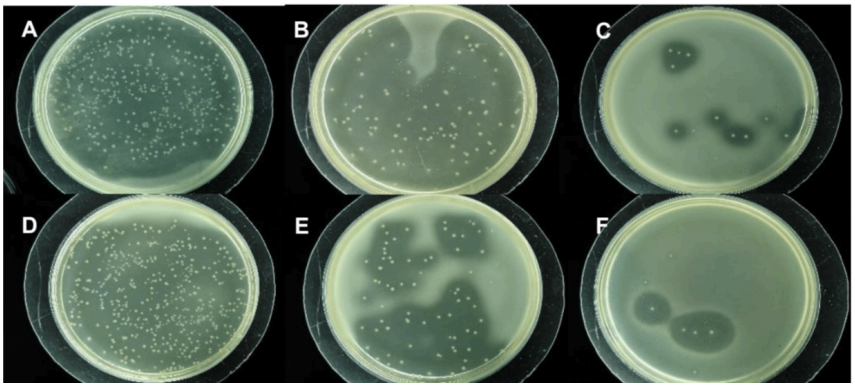

Figure 2. Percentage of subpopulations producing bacteriocin-like substances against *P. anaerobius* (dilutions: A 10^{-3}; B, 10^{-4}; C, 10^{-5}) and *F. nucleatum* (dilutions: D, 10^{-3}; E, 10^{-4}; F 10^{-5}).

Extract	**Titer of antagonistic activity (AU/ml)**		**Proteases**				**Thermostability (time)**					**Protein concentration (mg/ml)**	**pH stability**
	Fn	Pan	α-chymotrypsin	Papain	Proteinase K	Trypsin	121°C	80°C	65°C	45°C	37°C		
C50	12800	800	++	+	+	++	15 min	30 min	30 min	30 min	5 h	5.53	7.0-9.0
C80	800	12800	+	+	+	++	15 min	30 min	30 min	2 h	7 h	6.90	5.0-9.0

C-50 and C-80, intracellular fractions precipitated with ammonium sulfate at 30, 50, and 80% saturation levels, respectively; Fn, *Fusobacterium nucleatum*; Pan, *Peptostreptococcus anaerobius*; ++, total inactivation; +, partial inactivation.

Table 3. Partial purification and characterization of antagonistic substance(s) produced by *Fusobacterium necrophorum* ATCC 25286.

Assuming that there are evidences demonstrating that bacteriocin expression is affected by culture conditions we evaluated the influence of composition and pH of culture media on *F. necrophorum* antagonistic activity expression. Data indicating that the number of positive tests was significantly higher when BHIA was employed. Because it is a rich medium, BHIA provides an increase in the population density of bacteria. Bacteriocins are generally produced in higher concentrations when the producing bacterium is under stress conditions, such as high population density [27].

Besides higher amounts of bacteriocin expression some other possible explanations may be formulated. *F. necrophorum* may be able to produce more than one bacteriocin with diverse activity spectrum and the substances may be differentially synthesized under the influence of culture media composition. Besides, it is plausible to hypothesize that any component of each culture media could somehow interfere with antagonism expression both contributing or impairing bacteriocin activity or even affecting the susceptibility of some indicator strains.

In regard to the use of glucose (energy) and yeast extract (nitrogen) to supplement culture media tested overall only glucose had a positive effect on antagonism detection. The positive effect of glucose addition on antagonism expression has already been detected especially for lactic acid bacteria [13]. In relation to *F. necrophorum,* Wahren *et al.* (1971), reports the presents proteases on its cellular wall, whose activity is inversely related to glucose concentration. This could be another explanation for a positive effect of glucose addition inhibiting a proteolytic action on antagonistic substance of proteic nature such as bacteriocins.

The antagonistic action of the substance produced by *F. necrophorum* was higher in a culture medium with pHs 6.0 and 7.0, decreasing with increasing alkalinization until reaching pH 9.0. The pH of the culture medium is important not only for the growth of bacteriocin-producing bacteria, but also for the synthesis and stability of this substance. Some bacteriocins are only produced under controlled pH conditions, while for others this parameter seems not to be essential [11].

The test to verify the presence of interference factors confirmed that the generated antagonistic action did not come from bacteriophages, low pH values resulting from the production of organic acids, long chain fatty acids, hydrogen peroxide and residual chloroform.

Cellular extraction of the antagonist substance(s) was obtained from the extracellular (S) and intracellular (C) media of *F. necrophorum* by adding 30-50% (50) and 50-80% (80) w/v of ammonium sulfate. The best results of the antagonist action against *F. nucleatum* and *P. anaerobius* were found in the intracellular extracts C-50 and C80, which also showed thermostability of the substances. Other authors also reported more satisfactory results in the antagonistic action of the intracellular extract compared to the extracellular extract. This represents an important step in obtaining a greater amount of the molecule for study and other characterization [2; 21; 27].

The protein natures of the substances present in the C-50 and C-80 extracts were confirmed by protein dosage and the total or partial inactivation of the antagonistic action when submitted to the test with proteolytic enzymes. This corroborates the term bacteriocins, which are protein molecules with bactericidal or bacteriostatic action [28]. The thermostability too is a feature present among bacteriocins [27]. Which corroborates the data obtained in the present study.

The *in situ* activity assay of C-80 against *P.*

anaerobius showed the presence the antagonist substance with molecular weight between 37 to 50 kDa. Bacteriocins produced by Gram negative bacteria with molecular weight between 25 to 80 kDa are called colicins. The results obtained support the hypothesis that the substance under study is a colicin-type bacteriocin.

Data in the literature show the increase of the frequency of resistant bacterium samples to drugs. This has stimulated the search of substitute methods for the treatment of infections. Bacteriocin can constitute an alternative solution in the development of new medicines.

Concluding, the present study seems to be the first report of the detection and partial characterization of bacteriocin-like substances produced by *F. necrophorum*, an important microorganism in human and veterinary medicine. The complete purification and sequencing of the antagonistic substances are currently carried out in our laboratory.

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