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Isolation and partial characterization of antimicrobial peptide-enriched fractions from *Parabacteroides distasonis*

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Parabacteroides distasonis **is a part of the indigenous human microbiota and plays a significant role in disease etiopathogenesis. This study aimed to evaluate the efficacy of peptides isolated from *P. distasonis* strains. We focused on the expression, purification, characterization, antimicrobial efficacy through antagonism assays, and in silico studies of antimicrobial peptides produced by 78 *P. distasonis* strains. Methodology: Seventy-eight *P. distasonis* strains isolated from broiler (*Gallus gallus domesticus*) feces were evaluated. Antagonistic activity was assessed using the double-layer diffusion method. The influence of pH, temperature, proteolytic enzymes, and organic solvents on the intracellular peptide fraction (C-50) was analyzed. MIC was determined according to CLSI guidelines with adaptations. The peptide was purified, and its N-terminal amino acid sequence was determined using MALDI-TOF. Sequence identity was confirmed through BLAST-P analysis. Membrane docking was also conducted. Results: Fifty percent of the tested isolates produced antagonistic substances against at least one indicator strain. The C-50 fraction displayed stable antagonistic activity across a wide pH range (5.0–10.0) but was inactivated above 70 °C. MIC and MBC values for *P. distasonis* ATCC 1295 were 1.81 mg/mL (6.25 AU/mL) and 23.24 mg/mL (160 AU/mL), respectively. Ion-exchange chromatography revealed two peaks of activity. The major active peptide showed 100% identity to segments of histone-like H1 from *Bacteroides fragilis*. Exploratory molecular docking suggested plausible interactions. These computational findings are preliminary and require experimental validation. This study reports the discovery and partial biochemical characterization of antimicrobial peptides from *P. distasonis*. The peptides demonstrated extract-level activity and sequence similarity to histone-like proteins. Detailed mechanism of action and safety validation require further investigation.**

Keywords *Parabacteroides distasonis*, Antimicrobial peptides, Expression, Efficacy, Broiler chickens, Antagonism, Molecular docking

The burden of infectious diseases and the emergence of antimicrobial resistance have made disease treatment challenging[1]. The emergence of antimicrobial resistance is driven by a combination of different factors; however, the misuse and prolonged use of antibiotics trigger significant antimicrobial resistance[2]. For example, the use of the sulfonamides antibiotics started in the 1930s and till the 1950s, sulfonamide-resistant strains were circulating[3]. Natural and biological resources are frequently explored to develop new antimicrobials to combat the multidrug-resistant bacteria. Anaerobes have been a source of active antimicrobial substances, including bacteriocins, which are effective even in low concentrations[4]. Continue advancements in multiomics and

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heterologous expression systems increase the importance of anaerobes to be explored as potential reservoirs of bioactive natural products[5]. Additionally, these compounds are less prone to resistance and relatively safe[4].

Bacteria of the *Parabacteroides* genus are Gram-negative obligate anaerobic rods, members of the indigenous microbiota of the oral cavity, and the respiratory, intestinal, and urogenital tracts of humans and other animals[6]. Sakamoto & Benno proposed that this genus be used to reclassify some species of *Bacteroides*[7]. Subsequently, other species were classified in this group, including bacteria isolated from fecal specimens of animals[7]. Among the representative species of this newly created genus, *Parabacteroides distasonis* deserves mention, previously named *Bacteroides distasonis*[2]. This species, together with some *Bacteroides* and other *Parabacteroides*, is recognized as the predominant obligate anaerobe in the gut of humans, and other animals, including chickens, play an essential role in the host[8]. *Parabacteroides* species have also been reported as well-known inducers of antimicrobial peptides such as Reg3β and Reg3γ[10]. Additionally, the lysate of *P. distasonis* is reported to influence the abundance of the genera *Anaerostipes*, *Parabacteroides*, and *Prevotella*, and alter the expression of antimicrobial peptides in the intestinal mucosa[9]. The *P. distasonis* also showed protection against several diseases, including liver fibrosis, obesity, colorectal cancer, inflammatory bowel disease, diabetes, and splenic abscesses[10]. Other anaerobic bacteria that frequently produce antibiotics are *Clostridium* and *Actinomyces*, which are effective against even drug-resistant bacteria[5]. However, the obstacles and challenges of these compounds, such as production scalability and synthetic accessibility, hinder their translational potential[11]. For example, clostrubin antimicrobial, which is a potent anti-VRE, has low production levels and a complex biosynthetic pathway, which are key challenges[5].

The predominance in complex environments has as one of the justifications for the expression by microorganisms of a broad range of competitive abilities, which are important advantages, especially in heavily colonized environments. Antimicrobial peptides play an essential role in bacterial ecological relationships, benefiting the producer strain in competition against other bacteria[12]. Although the synthesis of these substances has been reported for a wide range of Gram-negative and Gram-positive bacteria, few studies involving more detailed characterization of these compounds are available in the literature when produced by obligate anaerobes, such as *Parabacteroides*. Furthermore, little is known about the ecological importance of antimicrobial peptides synthesized by strains of this genus. Knowledge of microorganisms' antimicrobial activities may help elucidate the complex microbial relations in several ecosystems[13–15]. Besides the ecological relevance, new perspectives emerge regarding the use of antimicrobial substances that produce bacteria as probiotics for therapy against bacterial infections or in the food industry for preservation[16]. Thus, considering the importance of the topic and the paucity of studies on antagonistic substances from obligate anaerobic bacteria, the present study aimed at the extraction, purification, and characterization of antimicrobial substances produced by a *P. distasonis* strain.

Herein, we isolate and characterize antimicrobial peptides, explicitly focusing on their antagonistic effects against Gram-negative pathogens. Further expression, purification, and structural characterization of these peptides were performed. Alongside evaluating their antimicrobial activity under various environmental conditions, such as temperature, pH, and enzyme exposure. Additionally, the research investigates the peptides' binding interactions with *P. distasonis* receptors through molecular docking.

Materials and methods

Bacterial strains

This study included 78 *P. distasonis* strains isolated from broiler [Gallus gallus domesticus] feces as producers[17]. The strains were identified according to[18]. Reference strains, along with strains isolated in the present and previous studies, were used as indicators to evaluate hetero-antagonistic (inhibition of strains from another bacterial genus), iso-antagonistic (inhibition of other Parabacteroides strains), and auto-antagonistic activity (self-inhibition by the producer strain). Based on the screening results, *P. distasonis* P15 was selected as the producer strain, and its antagonistic activity was further tested against clinically relevant indicator strains, including *P. distasonis* and Bacteroides spp. Isolated from patients with intra-abdominal infections.

Antagonistic assays

Culture preparation

Producer strains were cultivated in brain heart infusion broth (BHI, Difco), enriched with 0.5% yeast extract, 0.1% hemin, and 0.1% menadione (BHI-S). Cultures were incubated for 48 h at 37 °C in an anaerobic chamber (Forma Scientific Company, Marietta, OH, USA) under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2.

Double-layer agar diffusion method

Following incubation, 10 µL of each producer culture was spotted onto brain heart infusion agar (BHIA-S) plates (adjusted to pH 7.2) using a Steer's replicator. The plates were incubated anaerobically at 37 °C for another 48 h. To terminate growth, the producer colonies were exposed to chloroform vapor for 30 min. After residual chloroform had evaporated, 3.5 mL of soft BHIA-S (0.7% agar) inoculated with indicator strains was overlaid. Indicator strains were prepared in broth, and specific inoculum volumes were used according to their physiological classification: 0.2 mL for obligate anaerobes, 0.01 mL for aerobes, facultative anaerobes, and microaerophiles, and 0.05 mL for yeasts. The overlaid plates were incubated for 24 and 48 h at 37 °C under the appropriate atmospheric conditions specific to each indicator organism.

Evaluation of antagonistic activity

Antagonistic activity was assessed by observing clear zones of inhibition surrounding the producer strain spots, indicating suppression of indicator strain growth. All antagonism assays were performed in triplicate.

Detection of interfering factors

A piece of agar [3 mm in diameter] from the inhibition zone was removed aseptically, according to the method described by[19], to exclude the possible presence of bacteriophages. Then, the agar block was homogenized, centrifuged, placed onto the surface of BHIA-S, and overlaid with 3.5 ml of soft BHIA-S containing the indicator strain. After incubation for 48 h under anaerobiosis, the Petri dishes were evaluated for the presence of lytic zones. To exclude a possible inhibition by long-chain fatty acids, tests were conducted on BHIA-S, with and without adding 1% soluble starch. BHI supplemented with or without 0.03% [w/v-1] catalase [Sigma Company; St. Louis, MO, USA] was used to investigate the inhibition by hydrogen peroxide. Finally, antagonistic activity was also determined without the use of chloroform to exclude a possible interference of residual chloroform on the growth of indicator strains. After the growth of the producers, the indicator strains were inoculated with a loop near the spots of the producer strains without previous exposure to chloroform. Cultures were incubated at 37 °C for 48 h, under anaerobiosis, and then examined for the presence of inhibition zones.

Extraction of antimicrobial substances

Culture conditions

The selected producer strain (*P. distasonis* P15) was cultured in 1.2 L of BHI-S broth (pH 8.0) at 37 °C for 48 h in an anaerobic chamber under the same gas composition previously described. The producer strain was cultivated in 1.2 L of BHI-S [Difco], pH 8.0, in an anaerobic chamber at 37 °C for 48 h.

Protein precipitation and fractionation

After incubation, the culture was centrifuged at 12.520 g, at 4 °C, for 30 min, and proteins from the supernatant were precipitated with ammonium sulfate [Vetec, Duque de Caxias, RJ, Brazil] at three increasing concentrations [30, 50, and 80%] under constant slow stirring in an ice bath. After centrifugation at 23.741 g for 30 min, the precipitates were dissolved in 5 mL of 10 mmol/L of Tri-HCl buffer, pH 8,0, and dialyzed [Spectra/Por Membrane 1000 MW; Spectrum Laboratories Inc., Rancho Dominguez, CA, EUA] for 12 h at 4 °C. The extracellular fractions obtained were called S-30, S-50, and S-80.

Cells recovered after centrifugation were washed three times with 10 mmol/L Tri-HCl buffer, pH 8.0, suspended in 15 ml of the same buffer, and sonicated [Branson Sonifier 450; Danbury, CT, USA] for 20 cycles of 40 s at 50 W in an ice bath. Cellular disruption was checked by microscopy. The extract was centrifuged at 29.829 g for 30 min at 4 °C, and the proteins from the supernatant were precipitated with the same concentrations of ammonium sulfate, centrifuged, suspended in Tris–HCl buffer, pH 8.0, and dialyzed as described for the extracellular fractions. The intracellular fractions obtained after dialysis were called C-30, C-50, and C-80.

Test of activity, titration of crude antimicrobial substances, and determination of protein concentration

Antagonistic activity was determined as reported by Apolônio et al.[20], and titration of fractions was performed as previously described, and the result was given in arbitrary units AU/mL[21]. Protein concentration was determined according to Bradford[22], using bovine serum albumin as standard [Sigma, St Louis, MO, USA].

Effect of different treatments on antimicrobial substances activity

The effect of pH, temperature, proteolytic enzymes, and organic solvents on C-50 was evaluated by the presence of antagonistic activity after each treatment. All assays were carried out in duplicate. *P. distasonis* ATCC 1295 was chosen as the indicator strain. To evaluate pH influence, values from 2.0 to 12.0 were tested using the Britton-Robinson universal buffer[23], adjusted to each pH value. C-50 was diluted 1:1 [v: v] in each adjusted buffer and incubated in a water bath at 37 °C for 15 min up to 30 days. C-50 was diluted in a universal buffer, pH 8.0, and buffers without C-50 were employed as controls. For temperature influence, the following values and incubation periods were tested: -80, -20, 4, 25, 37, 50, 70, 100, and 121 °C for 10 min up to 12 months. Each aliquot was diluted 1:1 [v/v-1] in 10 mmol l-1 Tris–HCl buffer, pH 8.0, and then incubated as described above. All the samples were cooled at 4 °C before testing for antagonistic activity. Untreated diluted C-50 aliquots were employed as controls[24]. To test enzyme influence, papain [diluted in phosphoric buffer 50 mmol l-1 pH 5.0], proteinase K [Tris–HCl 20 mmol l-1 pH 7.2], pepsin [citrate buffer 100 mmol l-1 pH 3.0], α-chymotrypsin and trypsin [Tris–HCl 20 mmol l-1 pH 8.0] [Sigma] [at 1 mg.ml-1] were used. A dilution 1:1 [v: v] of C-50 in each enzyme solution was incubated at 37 °C for 15 min up to 1 h. Untreated crude C-50 aliquots, C-50 diluted in each buffer, buffers without C-50, and each enzyme solution were used as controls. For the influence of organic solvents, aliquots of C-50 were diluted 1:1 [v/v-1] in acetone, acetonitrile, butane-1-ol, chloroform, hexane, propane-2-ol, ethanol, or methanol [10%]. After incubation at 25 °C for 30 min to 2 h, antagonistic activity was determined. C-50 was diluted in Tris–HCl 10 mmol/L, pH 8.0, and each organic solvent was employed as a control[25].

Determination of minimal inhibitory concentration [MIC] and minimal bactericidal concentration [MBC]

MIC was determined in triplicate according to CLSI [2012] guidelines, with adaptations for anaerobic growth conditions (Mueller–Hinton broth; inoculum ~ 10^6 CFU/mL; incubation for 48 h instead of 16–20 h due to slower growth of anaerobes). Aliquots of 2 µl of *P. distasonis* ATCC 1295 culture previously adjusted with 0.85% saline to Mac Farland 0.5 range were added to each well of a 96-well microplate, corresponding to a final concentration of 106 colony forming units [CFU] ml-1.Then, 0.1 ml volume of different concentrations of the C-50 and 98 µl of BHI-S were added. A mixture of 198 µl of BHI-S with 2 µl of indicator culture and 98 µl of BHI-S with 100 µl of extract solution and 2 µl of saline solution was used as positive and negative control, respectively. After 48 h incubation, turbidity was observed in the wells. MIC was expressed in AU/mL, and MBC was recorded as the lowest concentration with no growth on agar plates. For the determination of MBC, the content of each well was

plated onto BHIA-S and checked for growth after 48 h of incubation at 37 °C. MIC was determined as the lowest concentration of bacteriocin that resulted in the absence of turbidity in the well. MBC was recorded as the lowest concentration of bacteriocin that resulted in the absence of growth of *P. distasonis* ATCC 1295 on BHIA-S plates.

Chromatographic purification and mass analysis of active fractions

C-50 was submitted to ion exchange chromatography on a fast performance liquid chromatography system [FPLC] using a mono-Q HR 5 / 50 GL Tricorn column [Pharmacia, Uppsala, Sweden] equilibrated with Tris–HCl buffer 20 mmol l^{-1}, pH 8.0, and eluted with the same buffer using a gradient of 1 mol l^{-1} NaCl, at a flow rate of 1 ml min^{-1}. Aliquots were lyophilized and suspended in the same buffer before testing for antagonistic activity as described above. Active fractions were further purified by gel filtration chromatography on an FPLC system equipped with a Superose 12 HR 10/30 column [Pharmacia] equilibrated with 20 mmol l^{-1} Tris–HCl buffer, pH 8.0, added with 200 mmol l^{-1} NaCl and eluted with the same buffer at a rate of 30 ml h^{-1}. Aliquots were processed as described for ion exchange. Each active fraction was submitted to reversed-phase chromatography on a high-performance liquid chromatography system [HPLC] equipped with a C18 column [300 × 4.6 mm] [Pharmacia]. The mobile phases A and B were 0.05% trifluoroacetic acid [TFA] and 100% aqueous acetonitrile solution containing 0.05% TFA, respectively. Aliquots were treated as described. Active fractions obtained from the previous steps were submitted to a high-performance liquid chromatography system with an LC18 column [SupercosilTM, 460 • 250 mm] [Pharmacia]. Finally, the mass of active fractions was determined using an AutoflexTM III MALDI-TOF/TOF mass spectrometer [Bruker Daltonics, Billerica, MA, USA]. Elution profiles of both FPLC and HPLC analysis were monitored at 280 nm.

Structural analyses

Determination of N-terminal amino acid sequences

MS/MS determined the protein sequence, operated in an Ultraflex III MALDI-TOF spectrometer [Bruker Daltonics, Billerica, MA], and was conducted using an in-source decay (ISD) protocol[26]. 1,5-diaminonaphthalene (DAN) was used as a matrix, prepared at 20 mg/ml in H_2O/acetonitrile (1/1, v/v) containing 0.3% TFA. The instrument was operated in the reflectron positive mode, and the accelerating voltages were as follows: 20.00 kV to ion source 1, 17.65 kV to ion source 2, and 7.00 kV to the lens; the voltages at reflectors 1 and 2 were 21.10 and 10.85 kV, respectively. The amino acid sequences of peptides were determined manually using the Flex Analysis software to assign the c ion series whenever possible. It is important to point out that the peptide in the fractions was sufficiently pure to allow unequivocal ion assignments. The amino acid sequences of peptides that could be detected were identified by comparison with the deposited sequences on the Basic Local Alignment Search Tool program (BLAST-P- P).

Results

Antagonism

Fifty percent of the tested isolates of *P. distasonis* produced antagonistic substances against at least one of the indicator strains. Iso-antagonism was the most frequently observed, followed by hetero-antagonism (9 out of 20) and auto-antagonism (2 out of 20). Amongst indicator strains, activity was observed only against Gram-negative bacteria, mainly for phylogenetically related groups, including other *Bacteroides* and *Parabacteroides* isolated from poultry cecal and reference strains, but also acting on bacteria like *Fusobacterium necrophorum* and *Aggregatibacter actinomycetemcomitans*. *Bacteroides vulgatus, B. thetaiotaomicron*, and *P. distasonis* were the most sensitive indicators (Table 1/ Fig. S1).

Results based on triplicate assays; reproducible across independent experiments.

P. distasonis P15 was the producer strain that most often expressed antagonism, and for these characteristics, was selected for the subsequent stages of the study. As *P. distasonis* ATCC 1295 showed the more significant and clearer growth inhibition zones, this strain was used as an indicator strain for these stages (Fig S2). When *P. distasonis* P15 was evaluated using *Campylobacter*, *Salmonella Typhimurium*, important bacteria for the health of the chickens, and clinical strains of *P. distasonis* and *Bacteroides* as indicators, antagonism was observed only against the phylogenetically related *Parabacteroides* and *Bacteroides*.

Interfering factors

Tests for the presence of bacteriophages in the inhibition zone showed no lytic activity. The possibility of inhibition by long-chain fatty acids was also discarded because of the lack of interference upon adding soluble starch to the medium. The addition of catalase to the culture medium did not inhibit the expression of antagonism. Finally, the experiments carried out without using chloroform gave the same results as those that included exposure to chloroform to kill the producer strain (Fig S3).

Antagonistic activity of extracted proteins

Only extracts C-30 (50 ± X AU/mL; 10.92 ± X mg protein ml-1, n = 3), C-50 (200 ± X AU/mL; 29.06 ± X mg ml-1, n = 3), and S-50 (400 ± X AU/mL; 42.76 ± X mg ml-1, n = 3) obtained from the P15 producer strain presented antagonistic activity. S-50 showed a higher titration of antagonist activity and higher protein concentration but was more unstable than C-50, losing its activity in a week. Thus, C-50 was selected for subsequent stages of the study (Fig S4).

Effect of different treatments on C-50

The antagonistic activity of the C-50 fraction was stable in a wide pH range (5.0 to 10.0) but was lost by heating at temperatures higher than 70 °C for 20 min. This activity was preserved during long-term storage at -20 °C and

Indicator microorganism	Source	Activity
Gram-positive bacteria		
Staphylococcus aureus	ATCC 33,591	–
Streptococcus epidermidis	ATCC 12,228	–
Peptostreptococcus anaerobius	ATCC 27,337	–
Enterococcus faecalis	ATCC 19,435	–
Propionibacterium acnes	ATCC 6919	–
Streptococcus mutans	ATCC 25,175	–
Bifidobacterium breve	ATCC 15,700	–
Bifidobacterium longum	ATCC 15,707	–
Actinomyces israelii	ATCC 12,102	–
Streptococcus sanguis	ATCC 10,557	–
Streptococcus uberis	ATCC 9927	–
Gram-negative bacteria		
Escherichia coli	ATCC 25,922	–
Pseudomonas aeruginosa	ATCC 27,853	–
Fusobacterium necrophorum	ATCC 25,286	+ +
Aggregatibacter actinomycetemcomitans	FDC Y4	+ +
Eikenella corrodens	ATCC 23,834	–
Porphyromonas gingivalis	FD 381	+ +
Prevotella intermedia	ATCC 25,611	+ +
Prevotella nigrescens	ATCC 33,569	+ +
Bacteroides fragilis	ATCC 25,285	+ +
Bacteroides vulgatus	ATCC 8482	+ +
Bacteroides thetaiotaomicron	ATCC 29,741	+ +
P. distasonis	ATCC 1295	+ +
P. distasonis [n = 78]	[Garcia et al.,17]	+ +
Bacteroides spp. [n = 93] *B. fragilis, B. vulgatus, B. ovatus* and *B. stercoris*	[Garcia et al., 17]	+ +
Yeast		
Candida albicans	ATCC 18,804	–
Cryptococcus gatii	ATCC 24,065	–
Cryptococcus neoformans	ATCC 62,066	–
Tested only for *P. distasonis* P15		
Campylobacter jejuni	ATCC 33,291	–
Campylobacter coli	ATCC 33,559	–
Salmonella Typhimurium	ATCC 14,028	–
P. distasonis [n = 10]	[Santos et al.[54]]	+
Bacteroides spp. [n = 24] *B. fragilis, B. vulgatus, B. uniformis, B. ovatus, B. caccae, B. caplillosus* and *B. thetaiotaomicron*	[Santos et al.[54]]	+

Table 1. Indicator strains used and results of antagonist activity for all the *P. distasonis* [n = 78] tested, and only for *P. distasonis* P15. *– no inhibitory activity; + inhibitory activity; First + : inhibitory activity from strains of *P. distasonis*; Second + : inhibitory activity from *P. distasonis* P15.

-80 °C (> 12 months). The activity of the fraction was completely lost following treatment with papain, trypsin, and α-chymotrypsin, but preserved after treatment with all the organic solvents tested (Table 2).

Values represent mean ± standard deviation (SD) from triplicate experiments.

Determination of MIC and MBC

The MIC of the C-50 extract against *P. distasonis* ATCC 1295 was 1.81 ± 0.05 mg·mL^{-1} (6.25 AU/mL; 95% CI: 1.72–1.90), and the MBC was 23.24 ± 0.11 mg·mL^{-1} (160 AU/mL; 95% CI: 23.0–23.5) after 48 h in Mueller–Hinton broth. The resulting MBC/MIC ratio was 12.8, consistent with a bacteriostatic effect.

Antimicrobial substance purification

After ion-exchange chromatography, antagonistic activity was retained in compounds corresponding to two peaks, eluted at nearly 0% and 20% NaCl concentration (Fig. 1). A summary linking the purification steps with the corresponding HPLC peaks, exact masses, peptide identification, and biological assays is presented in Table S1.

Fractions corresponding to both peaks were submitted to gel filtration chromatography, and the resulting active fractions were submitted to reversed-phase HPLC. The antagonistic activity was found in only three

Treatment	Conditions/time	Activity [AU/mL]
Temperature		
Control	–	100
120 °C & 100 °C	10 min	0
70 °C	10 min	*
50 °C	4 h	100
37 °C	30 days	*
25 °C	30 days	100
4 °C	50 days	100
– 20 °C & – 80 °C	12 months	100
pH		
Control	–	200
2, 3 & 4	6 days	100
5 & 6	30 days	100
7, 8, 9 & 10	30 days	200
11 & 12	0 min	0
Enzymes (1 mg·mL^{-1}, 37 °C)		
Control	–	100
Trypsin	15 min	0
α-Chymotrypsin	15 min	0
Papain	15 min	0
Pepsin	1 h	*
Proteinase K	1 h	*
Organic solvents (10%, 25 °C, 2 h)		
Control	–	100
Acetone	2 h	100
Acetonitrile	2 h	100
Isopropanol	2 h	100
Butanol	2 h	100
Ethanol	2 h	100
Hexane	2 h	100
Methanol	2 h	100

Table 2. Effects of different treatments on C-50 antagonistic activity. *, activity only in the undiluted extract.

fractions, eluted at 60–65% acetonitrile (Fig. 2), and originated from fractions corresponding to the first peak of ion-exchange chromatography (Fig. 1). The active fractions were pooled and submitted to mass spectrometer analysis, giving rise to the three separated ions with mean mass values of 6,426.3, 7,397.2, and 9,007.3 Da (Fig. 3). The ion with molecular mass 7, 397.2 is largely predominant, allowing its sequencing by in-source decay MALDI mass spectrometry, a technique in which ion dissociation occurs before ion extraction, thus not allowing ion selection.

Determination of partial amino acid sequences

Since the mass spectrum (Fig. 3) shows a predominant ion, it was submitted to the top-down in-source decay (ISD) (Fig. 4), which furnished a partial sequence, DANAQIENGNKAAGTRARKASLEIEKAMKEFRKVSLEE.

The sequence demonstrated 100% identity and query coverage with four segments of histone-like H1 from *Bacteroides fragilis* deposited in NCBI (accession number: WP_049141787.1, WP_069187673.1, WP_002562150.1, WP_005780999.1). Top-down ISD yielded a partial histone-like motif (38 aa) consistent with the major intact species (~ 7.4 kDa; 7397.2 Da) detected by MALDI. Because ISD provides fragment-level evidence (c-series), the full proteoform remains unresolved.

3. Discussion

Histones are among the most conserved and abundant proteins that offer several biological properties, including gene regulation, chromatin structure, and antimicrobial potential of histone-derived peptides, which make them fascinating proteins for biotechnological and therapeutic applications[27]. In recent times, histone-like proteins have shown excellent antimicrobial potential against pathogens by disrupting bacterial membranes or interfering with nucleic acids. Gram-negative bacteria, members of the indigenous microbiota of humans and other animals as well as agents of infectious disease affecting primarily humans, have been the target of investigations conducted by our research group[28–30], including the description of new antagonistic substances. Antagonistic substances produced by obligate anaerobic bacteria such as *Bacteroides* and *Parabacteroides* are still poorly studied. Antagonistic substance production by *P. distasonis* (previously *Bacteroides distasonis*) strains was reported[31], evaluating strains isolated from the human oral cavity and intestinal tract. Besides that study, we

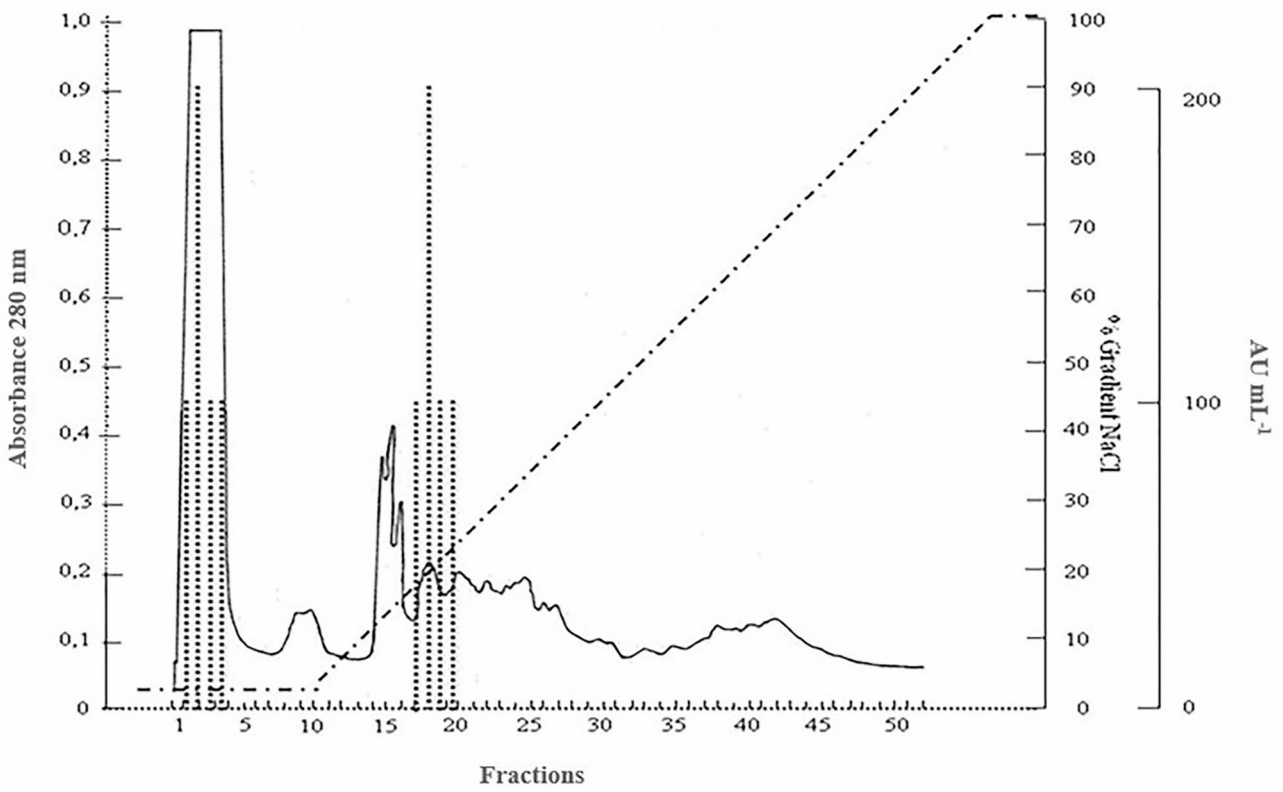

Fig. 1. Mono-Q ion exchange, fast performance liquid chromatogram [FPLC] of *Parabacteroides distasonis* P15 antagonistic substance. Linear gradient 0–100% of NaCl 1 mol l^{-1} aqueous solution at a flow rate of 1 ml min^{-1} and room temperature (__ Absorbance 280 nm; ….., AU/mL ^{1} and __. , Gradient NaCl).

are unaware of any other investigation on antimicrobial substances produced by *P. distasonis*, and consequently, this is the first description of the characterization and purification of an antagonistic substance produced by *P. distasonis*.

Antagonistic bacteria are an important part of microbial communities that survive by inhibiting the growth of or killing their neighboring bacteria[32]. Antagonistic bacteria have been a source of new antimicrobials that are considered a breakthrough in medicine. The antagonism is carried out by several bacterial phyla with other bacteria that produce synergistic efficacy and lethality[33]. Antagonism may disrupt microbial ecosystems, resulting in instability. However, these competitive interactions may also increase microbial diversity and stability, because antagonism forces bacteria to adapt and specialize in different areas (spatial structuring), allowing many species to coexist rather than one dominating the ecosystem[33].

The strains tested here showed mainly a high frequency of iso-antagonism [50%] against members of the poultry ceca. A study reported the presence of Bacteroides and Parabacteroides species isolates from human intestinal microbiota, among which 55.2% strains produced antagonistic substances. The iso-antagonistic effect was observed in *B. fragilis* (40.9%), *B. vulgatus* (50%), and *B. uniformis* (14.2%)[34]. The finding supports the idea that antimicrobial substances may play an important role in the relationship between bacterial groups in complex and diverse ecosystems[35].

Recent investigations showed that *Parabacteroides* and *Bacteroides* are among the most predominant genera in the intestinal tract of birds[36], which could be explained by their ability to produce antimicrobial substances, conferring an ecological advantage. This phenomenon could influence the microbial populations and be related to the balance of the indigenous microbiota and host health[37].

Antimicrobial peptides, such as bacteriocins, synthesized by Gram-negative bacteria, have a narrower action spectrum when compared with Gram-positive bacteria and usually inhibit only strains of the same species or phylogenetically related species[38]. The present study confirmed such observation, with samples of *P. distasonis* able to inhibit bacteria from the *Bacteroides*, *Prevotella*, and *Porphyromonas* genera. However, antagonistic activity against taxonomically more distant bacteria, such as *Fusobacterium necrophorum* and *Aggregatibacter actinomycetemcomitans,* was also observed.

Among the producer strains tested, *P. distasonis* P15 showed action against both reference and clinical indicators, and its inhibition zone was clear and visible. Practically all samples of bacteria isolated from patients with intra-abdominal infection demonstrated susceptibility to the antagonistic substance produced by P15. This suggests a potential practical application in medicine in the future, mainly as an alternative to infections caused by resistant bacteria.

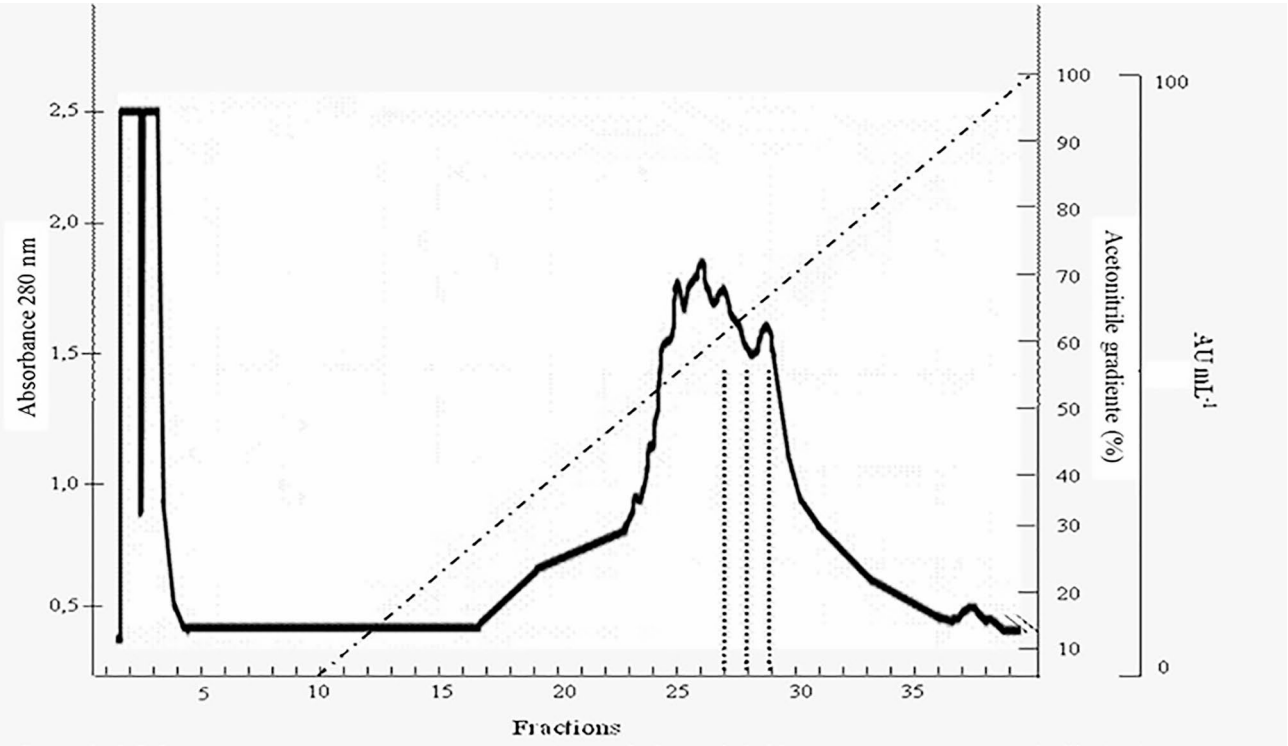

Fig. 2. C18 –Reversed-phase high-performance liquid chromatogram [HPLC] of *Parabacteroides distasonis* P15 antagonistic substance. Linear gradient 0–100% of B solution (80% aqueous acetonitrile solution containing 0.1% trifluoroacetic acid) at a flow rate of 1 ml min^{-1} and temperature of 35ºC (_ Absorbance 280 nm; ….., AU/Ml and __. , Gradient Acetonitrile).

Additionally, among the bacteria used as indicators, *P. distasonis* ATCC 1295 showed the most significant susceptibility and the sharper inhibition zones. For this reason, *P. distasonis* P15 and *P. distasonis* ATCC 1295 were used for the subsequent steps of this study as producer and indicator strains, respectively. The antagonistic activity detected for *P. distasonis* P15 was not due to bacteriophages, fatty acids, hydrogen peroxide, or chloroform, as demonstrated by the experiments with interfering factors. These results suggest that an antimicrobial peptide is responsible for the observed inhibitory effects.

The stability of the C-50 extract under different conditions, including temperature, pH, exposure to digestive enzymes, and organic solvents, was evaluated (Table 2). These results provide insight into the potential behavior of the antimicrobial peptide(s) in the gut environment. For instance, the peptide retains activity at physiological temperatures and neutral pH, but is sensitive to high temperatures and certain proteases, indicating that its activity may be modulated by digestive conditions. Overall, these findings highlight the ecological relevance of the antimicrobial substances produced by *P. distasonis* and suggest that they could remain active under specific conditions in the gastrointestinal tract.

Three active fractions, two intracellular [C-30 and C-50] and one extracellular [S-50], were obtained during the purification process. While S-50 presented greater antagonistic activity, this fraction was not selected for the next stages of the study due to its instability, which probably resulted from the presence of extracellular proteolytic enzymes. For this reason, the C50 fraction was selected for the purification steps of the investigation, as in other studies on antagonistic substances of our group[39]. Antimicrobial activity is maintained in a wide range of pH values but is lost at high temperatures, and these characteristics have been reported in other studies on inhibitory compounds produced by Gram-negative anaerobes[40]. As reported above, the proteinaceous nature of the antimicrobial substance[s] suggested by the high-temperature sensitivity was confirmed by the complete activity loss after treatment with some peptidases. Together, these data suggest the production of an antimicrobial peptide by *P. distasonis* P15. The MBC value of intracellular C50 extract against *P. distasonis* ATCC 1295 was 12.8 times higher than its MIC value. According to the literature, a compound is considered a bactericide if the MBC value is less than four times the MIC[41]. Therefore, these results indicated a bacteriostatic activity of C50 against the indicator tested. Here, it is important to emphasize that the results obtained in our study and discussed so far refer to the gross intracellular extract of *P. distasonis* P15 precipitated at a 50% ammonium sulfate concentration, implying that several substances can be responsible for its antibacterial activity.

Several protocols and chromatographic methods have been proposed for the analytical purification of antimicrobial peptides. Chromatographic methods, such as ion exchange or size exclusion, are usually applied after an initial concentration step by salt precipitation[42] or acid extraction. Such a sequential strategy was used in the present study with salt precipitation, followed by ion exchange, gel filtration, and reversed-phase chromatography protocols. The elution pattern in the ion exchange step suggested the presence of two different

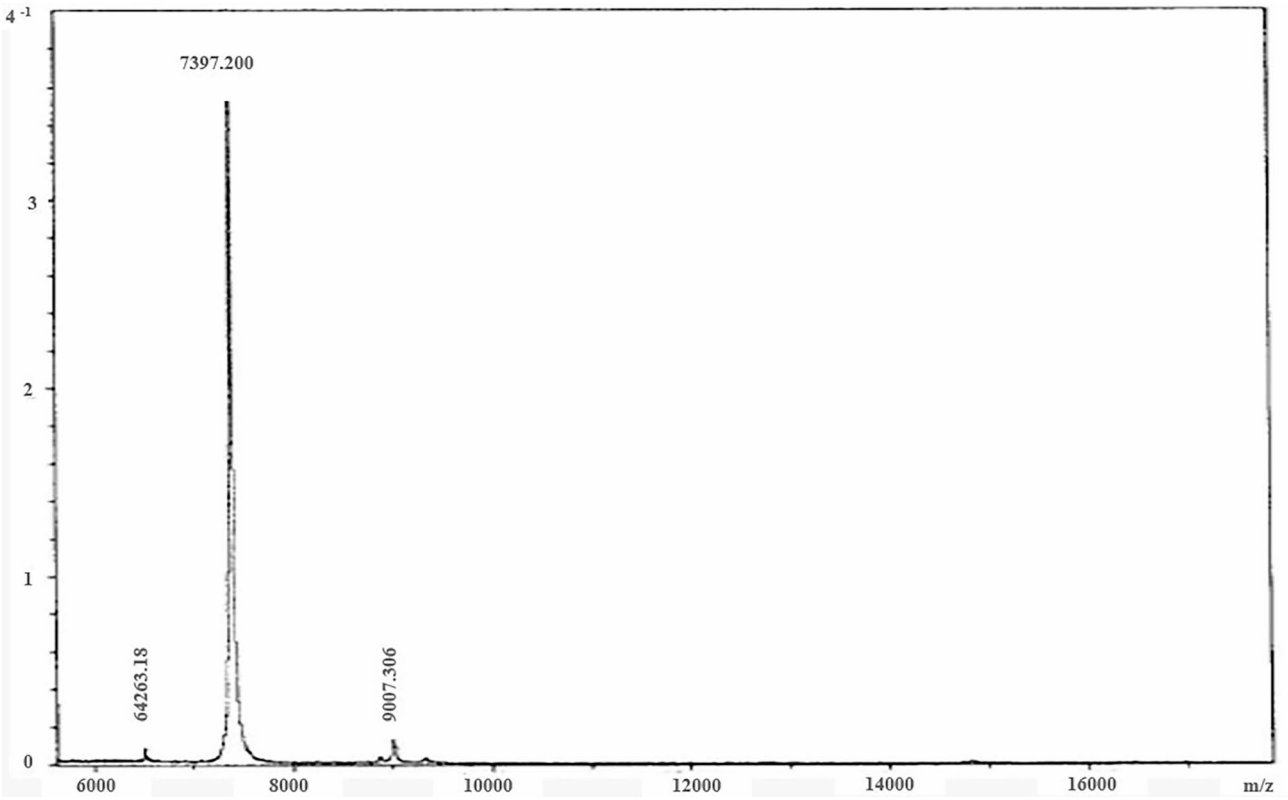

Fig. 3. Mass spectrum of ions corresponding to the reversed-phase pooled fractions with antagonistic activity. Values corresponding to mean molecular masses [m/z]. The spectrum was obtained linearly in an Ultraflex III MALDI mass spectrometer (Bruker Daltonics).

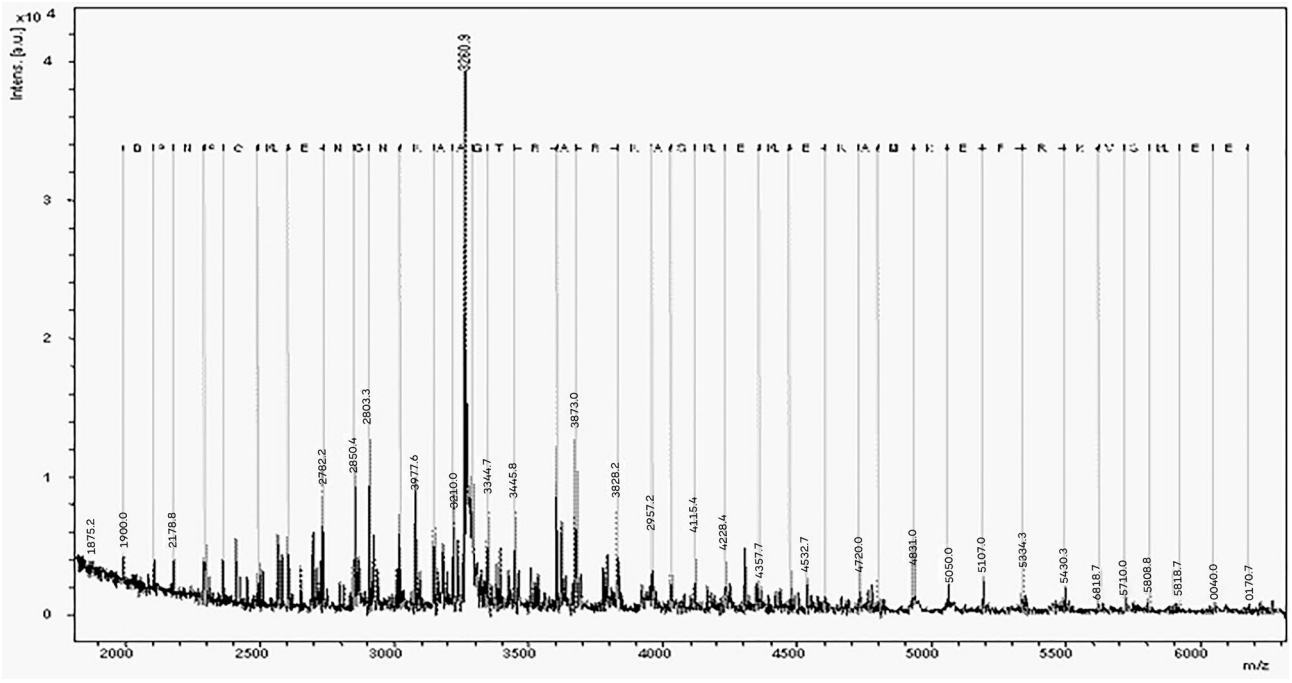

Fig. 4. In-source decay (ISD) MALDI mass spectrum acquired on an Ultraflex III mass spectrometer (Bruker Daltonics), showing signal intensity as a function of mass-to-charge ratio (m/z). The amino acid sequence displayed corresponds to ISD c-series fragment ions assigned to a species with a deconvoluted (neutral) mass of 7,397.2 Da. This sequence represents a partial motif rather than the complete proteoform.

ionic molecules with antagonistic activity, one of a cationic or neutral nature and the other of an anionic nature, demonstrating the presence of more than one antimicrobial substance (Fig. 1). However, antimicrobial activity until the last step of purification was observed only in cationic or neutral substances, possibly because the antagonistic substance was present in low concentration, insufficient to be detected by the test performed.

The results generated by the different purification steps suggest that the molecular mass of the antimicrobial substance is lower than ten kDa, and this was confirmed by mass spectrometry of the final active fractions obtained from reversed-phase chromatography, showing values of 7,397.2, 6,423.3, and 9,007.3 Da. Sequencing by ISD was possible (Fig. 3), which furnished the partial sequence as DANAQIENGNKAAGTRARKASLEIEKAMKEFRKVSLEE, identical to a segment of histone-like H1 from *Bacteroides.* Nevertheless, we could not find a Bacteroides histone with a molecular mass corresponding to 7,397.2 Da, suggesting a different isoform. Histone H1 proteins are arises from proteolytic processing of a larger histone-like protein[43]. Such processing events have been reported in Protozoa and support the plausibility that *P. distasonis* produces histone-derived antimicrobial fragments.

Peptides derived from histones, particularly histone H1, have been shown to possess significant antimicrobial properties. Various studies have reported on histone H1-derived peptides and their ability to disrupt bacterial membranes, leading to cell death. Park et al.[44] demonstrated that fragments of histone H1 from human neutrophils exhibited significant antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, suggesting a role in innate immune defense. Additionally, histone H1-derived peptides from rainbow trout (*Oncorhynchus mykiss*) gill mucus displayed broad-spectrum antimicrobial activity, providing crucial protection against various pathogens by permeabilizing their cell membranes[45]. The mechanism of their antimicrobial action is still not very clear. Their cationic character enables them to bind negatively charged plasma membranes, and there are even reports about their abilities to penetrate the plasma membrane and interfere with DNA structure and replication. Peptides showed binding with two main active membrane proteins, putative lipoprotein[46] and hypothetical protein, the main virulence factors[47].

Although very few studies reported the antimicrobial mechanism of *P. distasonis* P15 producing an antimicrobial peptide, histone-like proteins are membrane-active antimicrobials[48]. The membrane permeabilization ability of histone-like peptides increases through a proline to alanine mutation, which increases the antimicrobial potential. The antimicrobial peptides paired with histones form pores in the membrane[49]. It has been reported that the activity of histone-derived antimicrobial peptides is influenced by arginine composition. The structural modifications of histone-derived antimicrobial peptides by increasing the arginine content result in high antimicrobial potential[50]. Additionally, a histone-like peptide such as BF2 also interacts with other intracellular targets, such as DNA, leading to cell death of microorganisms. Jodoin et al. reported the first histone-like antimicrobial agent with potential antibiofilm properties[51]. This study lacks the in vitro peptide-membrane interaction and in vivo antimicrobial and toxicity tests of the peptide, which will be done in the future.

Ma et al. performed the identification of antimicrobial peptides from the human gut microbiome using deep learning. Based on their occurrence among the most potent candidates, 11 c_AMPs were prioritized for further consideration. Notably, c_AMP67, c_AMP69, and c_AMP1043 demonstrated broad-spectrum activity by consistently ranking highly across multiple strains, whereas peptides such as c_AMP2041 exhibited strong but more strain-specific effects, underscoring the diversity of antimicrobial potential[52]. Two AMPs such as HG2 and HG4 identified from a rumen microbiome metagenomic dataset having sequence MKKLLLILFCLALALAGCKKAP VLGLALIVGGALLIKKKQAKS respectively[53]. Both AMPs having similarity an ABC transporter substrate-binding protein and a hypothetical protein from *Megasphaera elsdenii* respectively. HG2 was found more potent having MIC range of 16–32 µg/ml, while HG4's MIC had 32–64 µg/ml. Their antimicrobial mechanism was related to membrane permeabilisation and decrease the intracellular ATP concentration.

Exploratory docking (Table S2/ Figure S5/S6) suggested plausible peptide–protein contacts. Because scores are software-specific (not experimental free energies) and no negative-control docking was performed, we do not infer mechanism or selectivity.

Conclusions

Several tested isolates of *P. distasonis* produced antagonistic substances against at least one of the indicator strains. Tests for the presence of bacteriophages in the inhibition zone showed no lytic activity. The extracts C-30 (50 AU/mL; 10.92 mg protein ml^{-1}), C-50 (200 AU ml^{-1}; 29.06 mg ml^{-1}) and S-50 (400 AU/mL; 42.76 mg ml^{-1}) obtained from P15 producer strain presented antagonistic activity and the antagonistic activity of the C-50 fraction was stable in a wide pH range (5.0 to 10.0) but was lost by heating at temperatures higher than 70 °C for 20 min. The sequence demonstrated 100% identity and query coverage with four segments of histone-like H1 from *Bacteroides fragilis.* The antimicrobial peptide showed interaction with two target proteins of *P. distasonis*, named 4FVS and 4JHY.Docking is exploratory and requires experimental confirmation; no mechanistic, selectivity or safety claims are drawn. However, future studies will benefit from in vitro assays related to membrane integrity testing and protein interaction studies for validation. The findings underscore the potential of antimicrobial peptides produced by gut microbiota and open new avenues for the development of alternative antibacterial agents. It highlights the intricate interactions within the microbiota and their potential exploitation in enhancing human health. It's recommended to test peptides in vivo in large-scale experiments to assess their antimicrobial efficacy, toxicity, and histological analysis to fully evaluate their potential.

Data availability

The structures analysed during the current study are available in the Protein Data Bank (PDB) repository with a persistent web link and accession number (4FVS, 4JHY). https://www.rcsb.org/structure/4FVS https://www.r

csb.org/structure/4JHY The Mono-Q ion-exchange FPLC of P. distasonis P15 antagonistic substance has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17139301 MIC, MBC, and bactericidal index of the C-50 extract against Parabacteroides distasonis ATCC 1295 has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17305477. Reversed-phase HPLC of P. distasonis P15 antagonistic substance has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17139546. Antagonistic activity exhibited by P. distasonis isolates has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17139591. Antagonistic activity exhibited by P. distasonis P15 using P. distasonis ATCC 1295 has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17139727. Tests for the investigation of interference factors have been deposited in the Zenodo re-pository and is accessible at https://doi.org/10.5281/zenodo.17139772 Antago nistic activity of extracts obtained from Parabacteroides distasonis P15 has been deposited in the Zenodo repos-itory and is accessible at https://doi.org/10.5281/zenodo.17139845 Peptide-protein interaction have been depos ited in the Zenodo repository and is accessi-ble at https://doi.org/10.5281/zenodo.17139904. Mass spectrum of ions corresponding to the reversed-phase pooled fractions with antago-nistic activity has been deposited in the Zenodo repository and is accessible at https://doi.org/10.5281/zenodo.17139982. In-source decay (ISD) MALDI mass spectrum obtained for the ion 7,397.2 Da has been deposited in the Zenodo repository and is accessible https://doi.org/10.5281/zenodo.17140025.

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Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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