Supporting Information for

The unusual structure of Ruminococcin C1 antimicrobial peptide confers clinical properties

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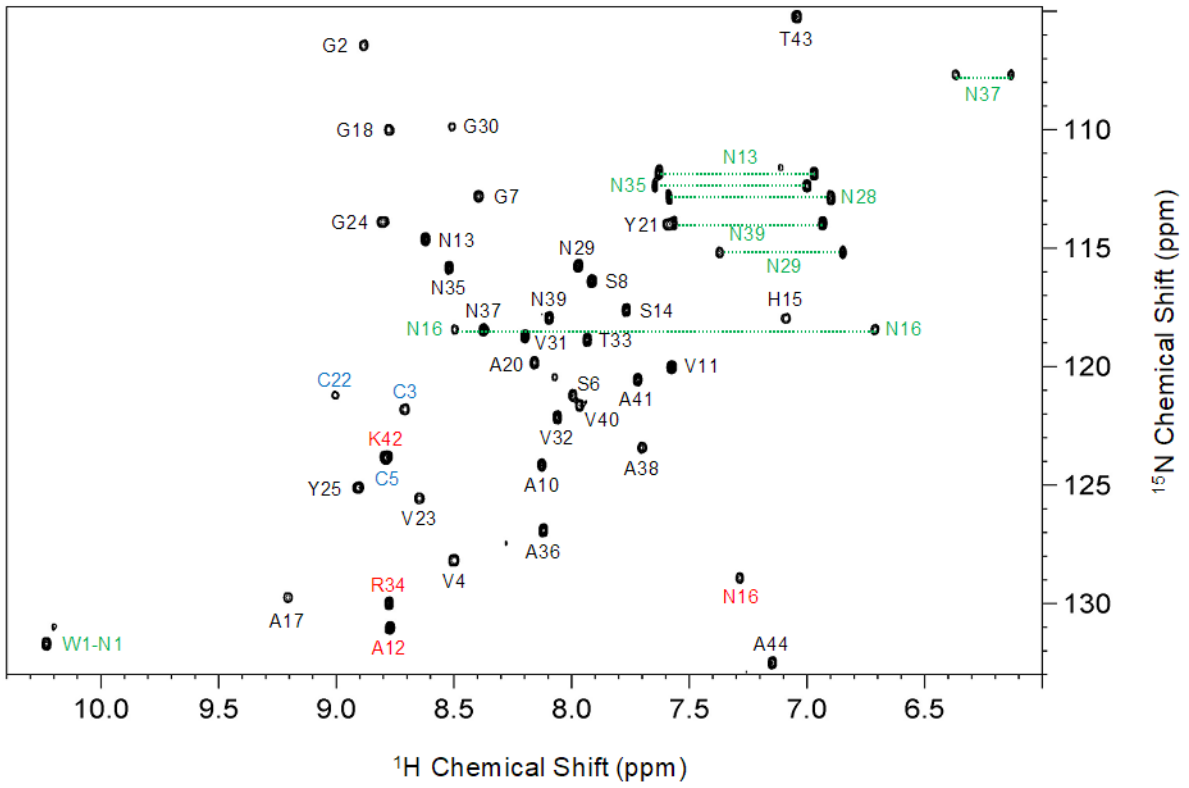


Fig. S1. Two-dimensional [^{1}H, ^{15}N] HSQC spectrum of RumC1. The number and the respective single letter code of amino acids are indicated at each assigned backbone NH cross-peak. Peaks corresponding to asparagine side chain amides are connected with a dotted horizontal line. The tryptophan indole NH group is labeled (W1-N1). Acquisition was done on a Bruker Avance III 600MHz spectrometer equipped with a cryogenically cooled 5 mm TCI probe head. Data was collected with a 0.2 mM sample concentration of ^{13}C and ^{15}N-isotopically enriched RumC1 in 10 mM phosphate buffer, pH 6.8 in 90% H_2O/ 10% D_2O at 27°C.

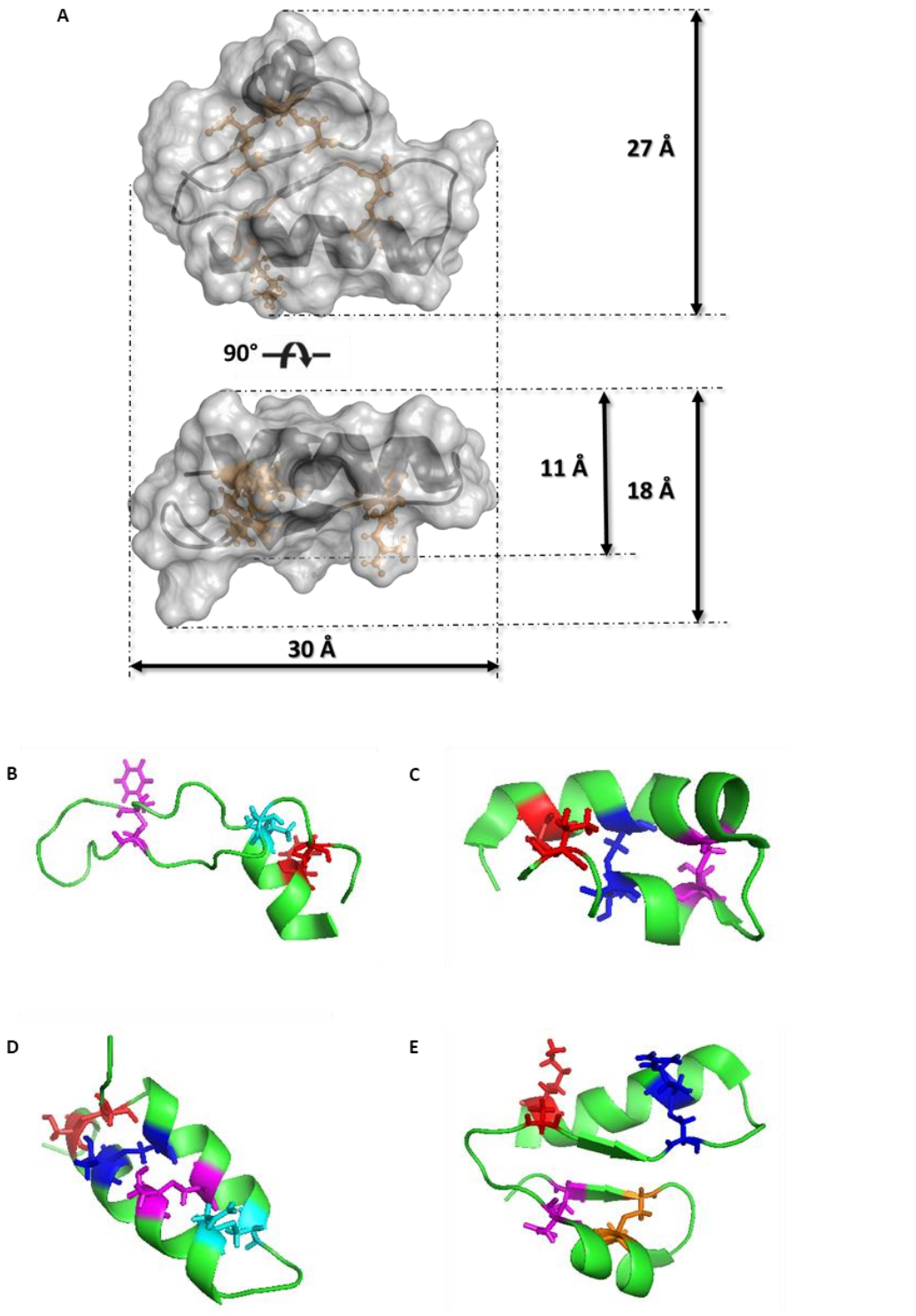


Fig. S2. Dimensions of the NMR structure of RumC1. Comparison of sactipeptides structures. (A) Dimensions of the NMR structure of the DDDD stereoisomer of RumC1. NMR Structures of: **(B)** Subtilosin A, **(C)** Thuricin CD, **(D)** Thurincin H and **(E)** RumC1. Stereoisomers at the α-carbons are LDD, LLD, DDDD and DDDD for subtilosin A, thuricin CD, thurincin H and RumC1, respectively. The Protein Data Bank codes for Subtilosin A, Thuricin CD , Thurincin H and RumC1 are 1PXQ, 2L9X, 2LBZ and 6T33, respectively.

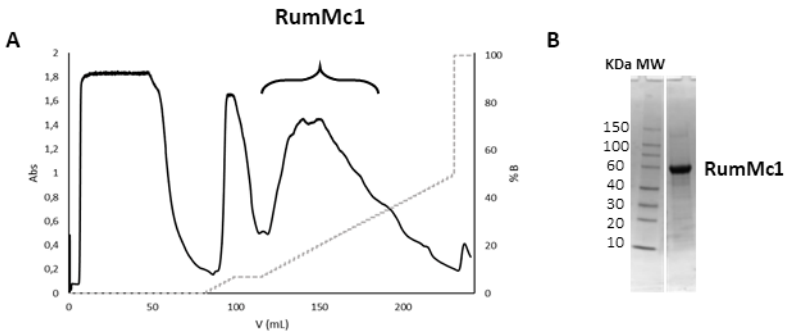
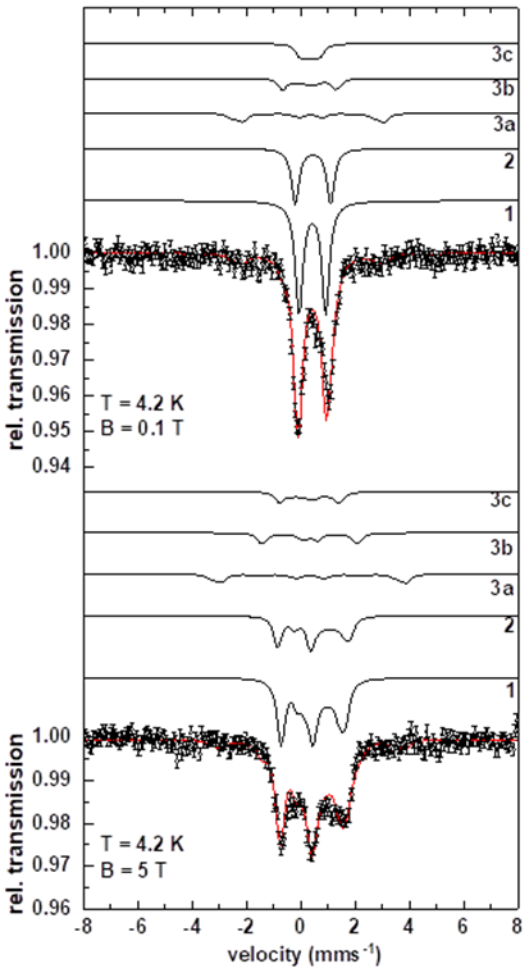


Fig. S3. Anaerobic purification of RumMc1. (A) FPLC chromatogram of overexpressed RumMc1 on a nickel-charge IMAC column. %B is the % of a 50 mM HEPES, pH 7,5, 300 mM NaCl, 500 mM imidazole buffer solution. **(B)** SDS-PAGE analysis of purified RumMc1

A



B

	1	2	3a	3b	3c
δ (mm/s)	0.42 (±0.02)	0.44 (±0.02)	0.33 (±0.03)	0.33 (±0.03)	0.33 (±0.03)
ΔE_Q (mm/s)	0.98 (±0.02)	1.30 (±0.02)	0.49 (±0.03)	0.49 (±0.03)	0.49 (±0.03)
Γ (mm/s)	0.35 (±0.02)	0.33 (±0.02)	0.33 (±0.03)	0.33 (±0.03)	0.33 (±0.03)
η	1 (-0.4)	1 (-0.4)	0 (+0.5)	0 (+0.5)	0 (+0.5)
$A_{xyz}/\mu_N g_N$ (T)	(0/0/0)	(0/0/0)	(36/28/32)[1]	(11/11/11)[1]	(2.5/2.5/2.5)[1]
Area (%)	50	25	8.33	8.33	8.33

Fig. S4. Field dependent spectra of RumMc1. (A) Mössbauer spectra of RumMc1 with the simulation (red solid line) representing the sum of the subcomponents **1**, **2** and **3** (black lines). Component **1** and **2** are simulated in a ratio of 2:1 and represent two diamagnetic $[4Fe4S]^{2+}$ clusters. Component **3** is divided into three subcomponents **3a**, **3b** and **3c** that represent three Fe^{3+} high spin ions antiferromagnetically coupled to a total spin of S = 1/2 as present in a $[3Fe4S]^{1+}$ cluster. **(B)** Mössbauer parameters obtained from the simulation of the field dependent spectra of RumMc1. [1] Values for $A_{xyz}/\mu_N g_N$ were taken from B. H. Huynh *et al.* (1).

Fig. S5. Alignment of selected radical-SAM enzymes with RumMc1. Alignment of anSME (anaerobic Sulfatase Maturating Enzyme), AlbA, and RumMc1. Conserved residues are marked "*" and cysteine residues are highlighted in green.

A

	RumC1	RumC1-44	RumC1-44-LS
% maturation	90%	< 1%	< 10%

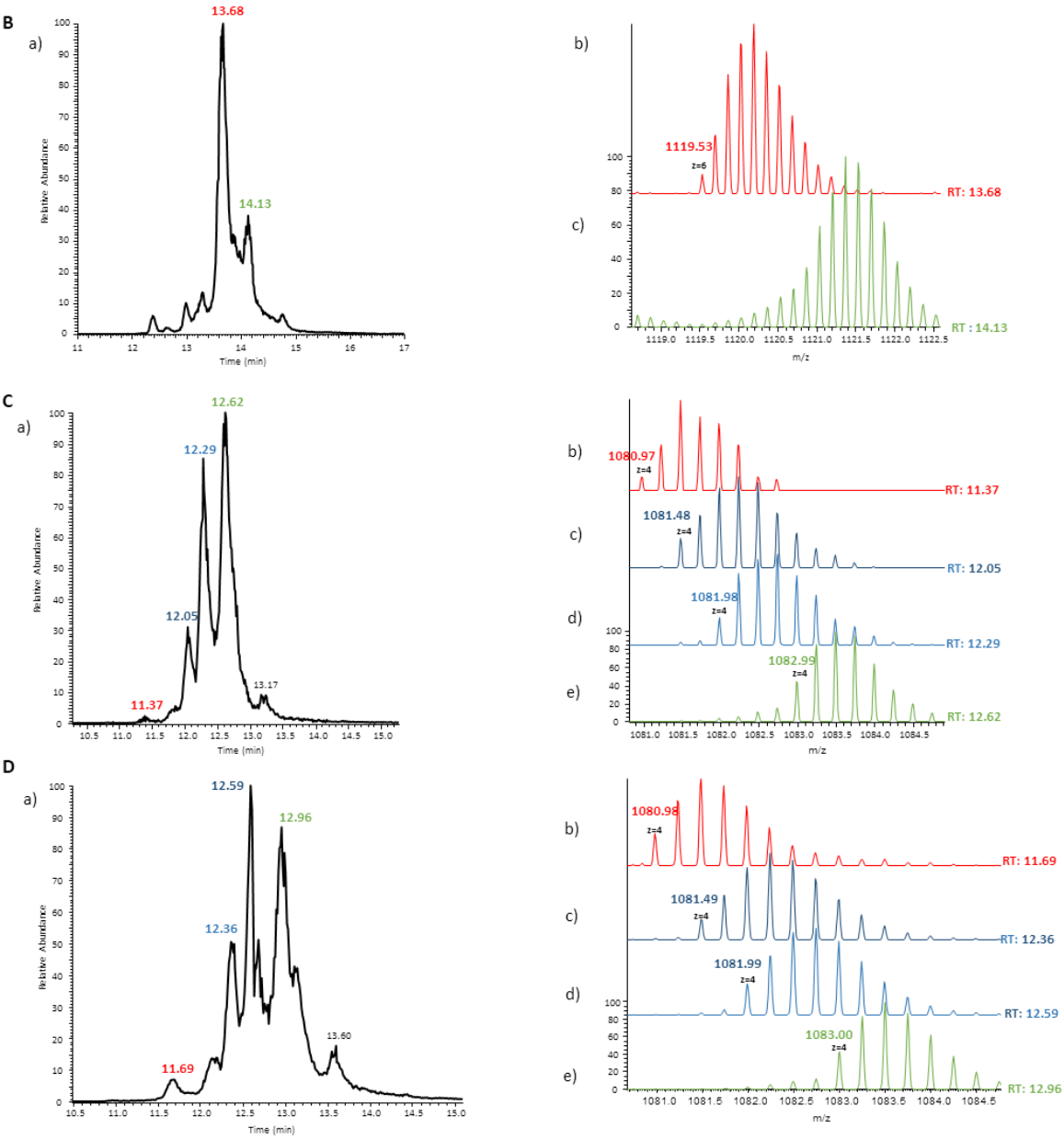


Fig. S6. LC-MS analyses of leader peptide-dependent maturation of RumC1. (A) Percentage of the full maturation for the different constructions. **(B)** Maturation of RumC1: (a) LC-MS trace of *in vitro* matured RumC1. Zoom-in spectra at RT = 13.68 (b) and RT = 14.13 (c) for m/z corresponding to RumC1. (b) A major ion $[M+6H]^{6+}$ = 1119.5 Da corresponds to the fully maturated form containing 4 thioether bonds. (c) A mixture of unmaturated forms containing no, one or two disulfide bonds are observed. **(C)** Maturation of RumC1-44: (a) LC-MS trace of *in vitro* matured RumC1-44. Zoom-in spectra at RT = 11.37 (b), RT = 12.05 (c), RT = 12.29 (d), and RT = 12.62 (e) for m/z corresponding to RumC1-44. (b) A major ion $[M+4H]^{4+}$ = 1081 Da corresponds to the fully maturated form containing 4 thioether bonds. (c) A major ion $[M+4H]^{4+}$ = 1081.5 Da corresponds to a species containing 3 thioether bridges. (d) A major ion $[M+4H]^{4+}$ = 1082 Da corresponds to a species with 2 thioether bridges. (e) A major ion $[M+4H]^{4+}$ = 1083 Da corresponds to the non maturated form of RumC1-44. **(D)** Maturation of RumC1-44-LS: (a) LC-MS trace of *in vitro* matured RumC1-44-LS. Zoom-in spectra at RT = 11.69 (b), RT = 12.36 (c), RT = 12.59 (d), and RT = 12.96 (e) for m/z corresponding to RumC1-44-LS. (b) A major ion $[M+4H]^{4+}$ = 1081 Da corresponds to the fully maturated form containing 4 thioether bonds. (c) A major ion $[M+4H]^{4+}$ = 1081.5 Da corresponds to a species containing 3 thioether bridges. (d) A major ion $[M+4H]^{4+}$ = 1082 Da corresponds to a species with 2 thioether bridges. (e) A major ion $[M+4H]^{4+}$ = 1083 Da corresponds to the non maturated form of RumC1-44-LS.

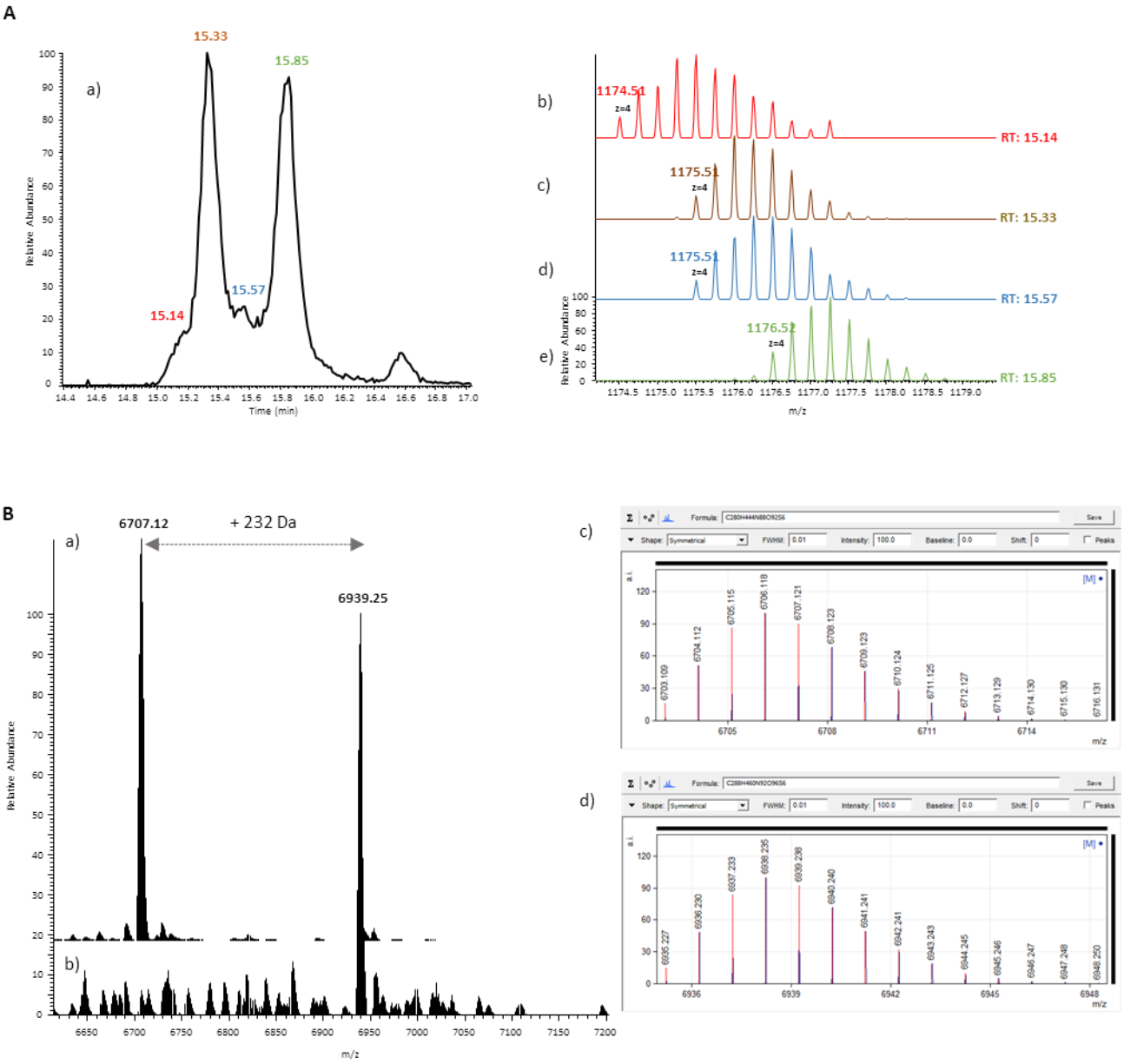


Fig. S7. Analysis of *in vivo* maturation of RumC1-44 and RumC1-Ala18/Ala19 variant. (A) LC-MS analysis of *in vivo* maturated RumC1-44. (a) LC-MS trace of *in vivo* matured RumC1-44. Zoom-in spectra at RT = 15.14 (b), RT = 15.33 (c), RT = 15.57 (d) and RT = 15.85 (e) for m/z corresponding to RumC1-44. (b) A major ion $[M+4H]^{4+}$ = 1174.5 Da corresponds to the fully maturated form containing 4 thioether bonds. (c) and (d) A major ion $[M+4H]^{4+}$ = 1175.5 Da corresponds to species with respectively 2 disulfide bridges and 2 thioether bridges, according to their corresponding MS/MS spectra. (e) A major ion $[M+4H]^{4+}$ = 1176.5 Da corresponds to the non maturated form of RumC1-44. **(B)** MS Analysis of *in vivo* maturated RumC1-Ala18/Ala19. (a) The deconvoluted mass obtained for the main species is 6707.1 and corresponds to a species containing 2 disulfide bridges. (b) After DTT reduction and iodoacetamide alkylation, the mass is shifted to 6939.2 corresponding to RumC1-Ala18/Ala19 alkylated 4 times (mass increased by 232 Da corresponding to 4 alkylations and 2 disulfide bridge reductions). (c) and (d) Simulated MS profiles of RumC1-Ala18/Ala19 with disulfide bonds and fully alkylated cysteines, respectively.

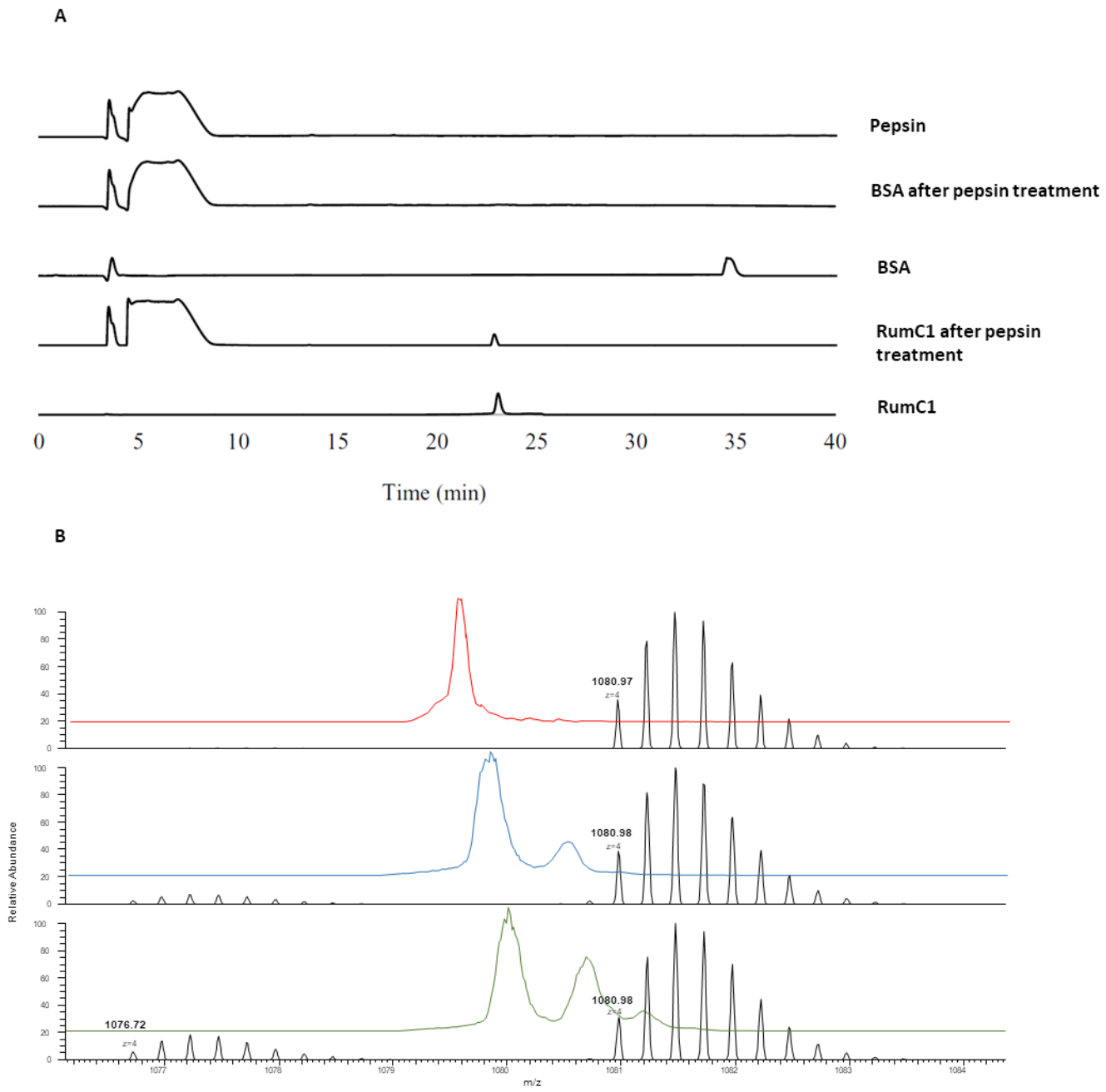


Fig. S8. Chromatographic and spectroscopic analyses to evaluate RumC1 integrity. (A) Chromatogram of RP-C18-HPLC of RumC1, or BSA used as control, treated with pepsin and in conditions simulating the stomach (pH 2,5 37°C 2h). **(B)** LC-MS profiles and MS spectra of RumC1 (top, red) submitted to pepsin (middle, blue) or pancreatic conditions (bottom, green). No change on RumC1 was observed after digestion except the presence of a light amino loss species due to heating (-NH3).

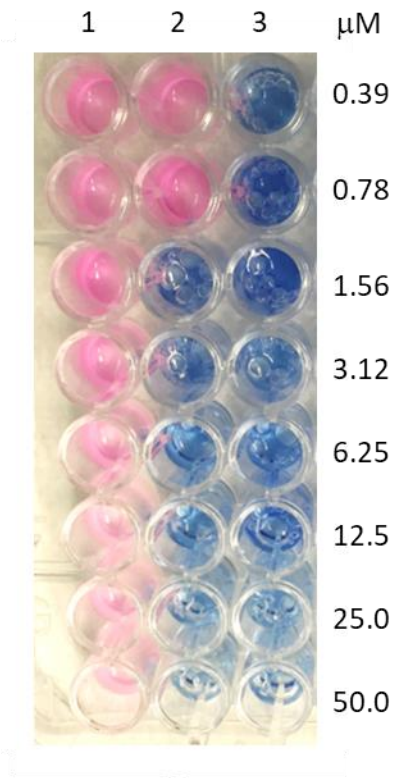


Fig. S9. **Evaluation of RumC1 activity on simulated intestinal epithelium infected by *Bacillus cereus*.** After 24h of incubation, suspension of *B. cereus* grown on Caco-2 and T84 cells were transferred to new 96 well plates free of eukaryotic cells. Resazurin was added to determine the viability of the bacterial cells. Briefly, resazurin is a blue dye that is reduced in pink resorufin in the presence of metabolically active cells. An example of untreated *B. cereus* grown on Caco-2 cells is shown in column 1, whereas the column 2 corresponds to *B. cereus* grown on Caco-2 cells with increasing concentrations of RumC1 and column 3 represents uninfected and untreated Caco-2 cells.

Table S1. Comparison of statistics generated by the 16 stereoisomers of RumC1. Assigned NOEs represent total number of off-diagonal NOE assignments used by CYANA to perform the structure calculation.

Table entry	Isomers	Thioether bond violations	Assigned NOEs	RMSD (Angstroms)	CYANA average target function value
1	LLLL	3	374	2.5 ± 0.4	0.7
2	LLLD	3	385	1.9 ± 0.8	2.0
3	LLDL	2	400	1.3 ± 0.5	1.3
4	LLDD	2	371	2.7 ± 0.4	3.8
5	LDLL	3	399	1.4 ± 0.5	1.2
6	LDLD	4	404	1.2 ± 0.3	1.5
7	LDDL	3	398	1.1 ± 0.3	0.7
8	LDDD	1	399	1.4 ± 0.2	0.6
9	DLLL	3	405	1.6 ± 0.3	1.1
10	DLLD	4	430	1.5 ± 0.2	0.7
11	DLDL	8	406	2.3 ± 0.4	2.9
12	DLDD	6	377	2.5 ± 0.6	5.3
13	DDLL	3	405	1.5 ± 0.5	1.3
14	DDLD	2	404	1.3 ± 0.2	0.7
15	DDDL	1	394	1.2 ± 0.2	0.3
16	DDDD	0	417	0.9 ± 0.2	0.1

Table S2. Structure calculation statistics for the DDDD stereoisomer of RumC1. Statistics for structure calculation refers to all the twenty structures.

Final NMR restraints in the DDDD structure calculation		
Short-range ($\|i-j\| = 1$)	317	
Medium-range ($1<\|i-j\| < 5$)	99	
Long-range ($\|i-j\| \geq 5$)	80	
Total nOe distance restraints	496	
Hydrogen bonds	50	
Thioether Bridge distance restraints	8	
Dihedral angle restraints	46	
Total restraints	600	13.6 restraints/residu
Residual violations		
CYANA target function	0.36 +/- 1.08	
NOE upper distance constrain violation		
Number > 0.1 Å in at least 1 structure	7	
Dihedral angle constrain violations		
Number > 0.1°	0	
Van der Waals violations		
Number > 0.1 Å	0	
Average structural RMSD to the mean coordinates (Å)		
All backbone atoms	0.81 +/- 0.50	
All heavy atoms	1.19 +/- 0.54	
Ramachandran statistics, % of all residues		
Most favored regions	84.1	
Additional allowed regions	15.4	
Generously allowed regions	0.4	
Disallowed regions	0	

SI Materials and Methods

Heterologous expression and purification of (^{13}C, ^{15}N)-labelled mature RumC1. A synthetic plasmid containing the *E. coli* codon-optimized gene of *R. gnavus* E1 encoding RumMc1 (pET-15b-*rumMc1*, ampicillin-resistant) was obtained from Genscript. Plasmids pET-15b-*rumMc1*, pETM-40-*rumC1* and psuf (chloramphenicol-resistant) containing *sufABCDSE* genes were used to transform competent *E. coli* BL21 (DE3) cells for expression. The resulting strain was grown in 3 L of M9 medium containing kan (50 μg/mL), amp (100 μg/mL), chl (34 μg/mL), vitamin B1 (0.5 μg/mL), $MgSO_4$ (1 mM), $FeCl_3$ (50 μM) and glucose (4 mg/mL) at 37 °C. At an optical density (OD_{600}) of 0.25, cells were harvested by centrifugation (4,000 rpm for 20 min at 4°C). The cells were resuspended in 1 L of labeled minimal medium (Na_2HPO_4 6 g/L, KH_2PO_4 3 g/L, $^{15}NH_4Cl$ 1 g/L) containing kan (50 μg/mL), amp (100 μg/mL), chl (34 μg/mL), vitamin B1 (0.5 μg/mL), $MgSO_4$ (1 mM), $FeCl_3$ (50 μM) and labeled glucose-^{13}C (4 mg/mL). The culture was grown at 25°C to an optical density (OD_{600}) of 0.8. $FeCl_3$ (100 μM) and L-cysteine (300 μM) were then added and the culture was induced with 1 mM IPTG. The cells were grown for 15h under stirring and then were harvested by centrifugation (4,000 rpm for 20 min at 4°C). Labelled MBP-RumC1 was purified as described for the MBP-RumC1 (2). (^{13}C, ^{15}N)-labelled mature RumC1 was obtained after cleavage of the MBP tag by using TEV and purified as previously reported (2). Leader peptide cleavage was performed with trypsin as described by Chiumento *et al.*, (2). Briefly, labelled RumC1was treated with TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)–treated trypsin (Sigma-Aldrich) for 1 hour at 37°C at a molar ratio of 200:1 (RumC1:trypsin). Then RumC1 was purified using RP-C18-HPLC with the following gradient: 10 min at 22% followed by 12 min from 22 to 38% of 90% ACN and 0.1% TFA, on a preparative column (250 mm by 21.2 mm; Phenomenex, Jupiter, 15 μm, 300 Å).

NMR spectroscopy of RumC1. The NMR sample used for sequential assignment of ^{13}C-^{15}N-labeled RumC1 was approximately 0.2 mM in 10 mM phosphate buffer, 90% H_2O/10% D_2O at pH 6.8. All NMR data were collected at 27°C using a Bruker Avance III 600 MHz NMR spectrometer equipped with a TCI 5 mm cryoprobe. The following datasets were performed; 2D: [^{15}N,^{1}H] HSQC and [^{13}C,^{1}H] HSQC; 3D: [^{1}H,^{15}N,^{13}C] HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO, HN(CA)CO, ^{13}C-TOCSY-HSQC (spin lock of 80 ms) and (H)CCH-TOCSY. Backbone resonances were assigned from triple resonance spectra and were extended to give side chain assignments using (H)CCH-TOCSY and ^{13}C-TOCSY-HSQC. ^{1}H assignments for aromatic side chains and asparagines side chain amides were made using 2D [^{1}H, ^{1}H] TOCSY and NOESY (mixing time of

150 ms) spectra. Spectra were processed with Topspin 3.5 and analyzed with CcpNmr Analysis software (3). All of the peak lists and the complete ^{1}H, ^{13}C and ^{15}N backbone and side chain chemical shift assignments have been deposited into the Biological Magnetic Resonance Databank (http://www.bmrb.wisc.edu) under ascension code 50027.

Structure Calculations. For the structure calculations, a 2D [^{1}H, ^{1}H] NOESY was acquired with a mixing time of 150 ms, using a 2 mM unlabeled RumC1 sample in 10 mM phosphate buffer, 90% H_2O/ 10% D_2O, pH 6.8 at 27°C, performed on the Bruker Avance III 600 MHz spectrometer. The structures of the 16 stereoisomers were calculated with CYANA 2.1 (4), using NOE restraints measured from the 2D [^{1}H, ^{1}H] NOESY, 3D [^{1}H, ^{15}N, ^{1}H] NOESY, 3D [^{1}H, ^{13}C, ^{1}H] NOESY experiments and angle restraints obtained from the TALOS+ server (5). The NOEs were calibrated within CYANA according to their intensities. The same nOe peaks list and angle restraints were used for the structure calculations of each stereoisomer, following the same procedure as previously described by John Vederas and co-workers (6–8). Twenty lowest target function value conformations were generated for each of the 16 stereoisomers. Coordinates of the twenty conformations of DDDD stereoisomer of RumC1 have been deposited into the Protein Data Bank under ascension code 6T33.

Expression of RumMc1. We obtained a commercially supplied codon-optimized synthetic plasmid of *Ruminococcus gnavus* RumMc1 (pET-28a–*rumMc1*, kanamycin-resistant) from Genscript. Vectors pET-28a-*rumMc1* and pDB1282 (ampicillin-resistant), which carries the *isc* operon required for proper assembly of the Fe-S clusters in RumMc1, were subsequently co-transformed into chemically-competent BL21 (DE3) *E. coli* cells. A 500 mL sterile culture erlenmeyer containing 100 mL Luria Bertani medium (LB) supplemented with kan (50 μg/mL), amp (100 μg/mL) was inoculated with a single colony of BL21 (DE3) cells carrying pET-28a-*rumMc1* and pDB1282. The 100 mL culture was grown overnight at 37 °C, 200 rpm and used to inoculate 10 liters M9 minimal medium on a fermenter. The minimal medium was prepared by supplementing M9 Minimal Salts (Sigma) with a final concentration of 20 mM glucose, 2 mM MgSO4, 50 μg/mL kan, 100 μg/mL amp) and 50 μM $FeCl_3$. The fermenter culture was grown at 37 °C, 200 rpm to an OD_{600} nm ~ 0.3-04 and then supplemented with a final concentration of 50 μM $FeCl_3$, 300 μM L-Cys, and 13.3 mM L-arabinose. At an (OD_{600}) of 1.2, the culture was cooled at 24°C, supplemented with a final concentration of 1 mM IPTG and grown for 15h under stirring. The cells were harvested by centrifugation (4,000 rpm for 20 min at 4°C).

Purification of RumMc1. The purification protocol was carried out under strictly anaerobic conditions. The cell pellet was suspended in 40 mL of buffer A (50 mM HEPES, pH 7.5, 300 mM NaCl), sonicated and lysate clarified by centrifugation at 40,000 rpm at 4 °C for 40 min. The supernatant was collected and passed over nickel-charge IMAC column (HisTrap^{TM} HP 5mL GE Healthcare). Columns were washed with 4 volumes of buffer A. RumMc1 was eluted from nickel-charge IMAC columns with a gradient of 0-50% of buffer B (50 mM HEPES, pH 7.5, 300 mM NaCl, 500 mM imidazole). Fractions containing RumMc1were pooled and concentrated in a 30,000 MWCO filter in an Amicon® Ultra centrifugal filter devices. To eliminate imidazole and salts, sample was passed on NAP^{TM} Column, NAP-25 equilibrated in buffer C (HEPES 50mM, NaCl 100 mM pH 7.5). Anaerobic UV-visible spectra were recorded on an Uvikon XL100 spectrophotometer (Bio-Tek instruments) connected by optical fibers to the cuvette holder in the anaerobic chamber. The protein concentration was estimated by UV-visible spectroscopy by using an extinction coefficient at 280 nm of 73266 $M^{-1}.cm^{-1}$. Iron content of RumMc1 samples were measured according to the Fish method (ref). The calibration curve was obtained by measuring iron standards with iron content form 2 to 30 nmol.

Site directed mutagenesis of *rumC1* and heterologous expression of RumC1 variant. Site directed mutagenesis of the *MBP-rumC1* construct was performed to produce the Ala18/Ala19 variant of RumC1. Mutagenesis was done by following the instructions from the Q5 Site-Directed Mutagenesis Kit (New England BioLabs®). The NEBaseChanger tool was used to generate the 2 primer sequences (5'-CCATAACGCGGCTGCGGCGTACTGCG, 5'-CTGTTCGCAACCGCGGTG) and annealing temperatures. Template plasmids were digested using DpnI and were transformed into competent Top10 cells. The mutant plasmids were recovered from cells by using the Wizard® Plus SV Minipreps DNA Purification System (Promega).
Production and purification of RumC1-Ala18/Ala19 variant were performed as previously described for RumC1 (2).

Leader-peptide chemical synthesis

Chemicals

The N-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, rink amide MBHA resin (100 - 200 mesh) and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Novabiochem; N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), anisole, thioanisole, 1,2-ethandithiol, acetic anhydride, piperidine and triethylamine (TEA) were from Sigma-Aldrich. All the other chemicals and solvents (N,N-dimethylformamide

(DMF), diethyl ether, dichloromethane (DCM), acetonitrile (ACN) and N-methyl-2-pyrrolidone (NMP) were from different commercial sources (highest available grade) and used without further purification.

Peptide synthesis

The leader sequence of RumC1 peptide (H_2N-MRKIVAGKLQTGADFEGSK-NH_2) was prepared by solid phase peptide synthesis in an Initiator$^+$ Alstra automated microwave assisted synthesizer (Biotage). The peptide was assembled on a rink amide MBHA resin (0.25 mmol scale, 0.59 mmol/g) using standard Fmoc methodologies (9). Namely, the amino acids (4 equiv) were coupled using HBTU (3.9 equiv) as coupling agent, DIEA as base (8 equiv) and DMF as solvent. The removal of the Fmoc protecting groups was always done by treating the resin with 20% piperidine in DMF solution. After assembling, the peptide was manually deprotected and cleaved from the resin by treatment with the mixture TFA/thioanisole/anisole/1,2-ethandithiol (%v/v = 90:5:3:2) for 2 h at room temperature and under nitrogen. The resin was filtered out and rinsed with TFA. The filtrate and rinses were combined, reduced under a nitrogen stream and slowly added to cold diethyl ether with magnetic stirring to precipitate the crude peptide. The suspension was transferred to a centrifuge to recover the peptide which was washed with cold diethyl ether and centrifuged again. This step was repeated several times and finally the crude peptide was dissolved in the minimum amount of water and lyophilized. The crude peptide was purified by preparative reversed-phase HPLC in a Phenomenex Jupiter column (250 mm × 21.20 mm, 15 μm, 300 Å) using solvent A (99.9% water/0.1 % TFA) and solvent B (90% ACN/9.9% water/0.1 % TFA). The leader sequence of RumC1 peptide was eluted with a linear gradient from 15% to 35% B in 30 min at a flow rate of 10 mL/min (R_t = 14 min). Its purity was checked by analytical reversed-phase HPLC (Phenomenex Jupiter column, 250 mm × 4.6 mm, 15 μm, 300 Å) and it was greater than 95%. The peptide was characterized by Electrospray Ionization-Mass spectrometry (ESI-MS) in positive mode using a Waters Synapt G2 HDMS (Manchester, UK) equipped with an ESI source employing the following parameters: ESI capillary voltage: +2.8 kV; extraction cone voltage: +20 V; desolvation gas (N2) flow: 100 $L.h^{-1}$; source temperature: 35 °C. Sample solutions were introduced in the ionization source at a 10 $\mu L.min^{-1}$ flow rate using a syringe pump.

***In Vitro* Enzyme Assay.** The *in vitro* enzymatic assays were performed in 100 µl of 100 mM HEPES, pH 7.5, in the presence of 100 µM of the desired peptide substrate, 25 µM of RumMc1 protein, 0.25 mM SAM and 1 mM dithionite. The assays were carried out at 37 °C during 3h under anaerobic conditions. The reactions were stopped by air exposure and were flash frozen in liquid nitrogen.

EPR and Mössbauer spectroscopies. EPR and Mössbauer samples (400 µM) were prepared and flash frozen under anaerobic conditions. When needed, cluster reduction was achieved in 1 hour by addition of 10 mM dithionite. Samples in the presence of SAM were prepared with 3 mM SAM. EPR spectra were recorded on a Bruker EMX spectrometer operating at X-band frequency equipped with an Oxford instrument ESR 900 flow cryostat. Spectra were recorded with a microwave frequency 9.65 GHz under saturated (10K, 39dB, modulation amplitude 10 G) and non-saturated (6K, 13dB, modulation amplitude 10 G) conditions for the g = 2 region. Simulations were performed using Easy Spin toolbox for matLAB. Mössbauer spectra were recorded in transmission mode with a conventional Mössbauer spectrometer which was operated in the constant acceleration mode in conjunction with a multi-channel analyzer in the time-scale mode (WissEl GmbH). The spectrometer was calibrated against α-iron at room temperature. Experiments at 77 K were conducted using a flow cryostat (Optistat^{DN}, Oxford Instruments). Field-dependent Mössbauer spectra were obtained with a helium closed-cycle cryostat (CRYO Industries of America, Inc.) equipped with a superconducting magnet (10). The magnetic field was aligned parallel to the γ-ray beam. The spectral data were transferred from the multi-channel analyzer to a PC for further analysis employing the public domain program Vinda (11) running on an Excel 2003® platform. Analysis of the spectra was performed by least-squares fits using Lorentzian line shapes with the linewidth Γ. Field-dependent spectra were simulated by means of the spin Hamilton formalism (12).

Nano-LC−MS/MS Analyses. RumC1 samples were generally injected at a concentration of 0.1 µM. Samples were diluted in 5% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and analysed by online nano-LC−MS/MS (NCS HPLC, Dionex, and Qexactive HF, Thermo Fisher Scientific). Peptides were sampled on a 300 µm × 5 mm PepMap C18 precolumn and separated on a 75 µm × 250 mm C18 column (PepMap, Dionex). The nano-LC method consisted of a 40 min gradient at a flow rate of 300 nL/min, and MS and MS/MS data were acquired using Xcalibur (Thermo Fisher Scientific). In order to improve the quality of the MS/MS spectra we performed parallel reaction monitoring (PRM) experiments to characterize RumC1 species. According to our previous characterization of RumC1 (Chiumento et al., 2019), we decided to focus these analyses on the highest m/z ions for the long (with leader peptide) and short (without leader peptide) peptides (respectively m/z = 1120 (6^+) and m/z = 1083 (4^+)). The m/z window was open at 4 units in order to consider modified species. The collision energy was set to 27.

The MS interpretations were done on the basis of previously annotated spectra and specific fragmentation pattern of thioether bridges using HCD (2). Mascot (version 2.6) was also used for the confirmation of the modifications, as previously described (2).

Stability assays. Stability of RumC1 was evaluated after incubation varying pH, temperatures, and in human serum. RumC1 was incubated in phosphate-buffered saline (PBS) at pH range from 2 to 11 at a volume ratio of 1:1 for 1 hour at room temperature. In a second assay RumC1 was incubated at temperatures of 70, 80, 90 and 100 °C for 5, 15, 30 or 60 minutes before being cooled on ice. Finally, RumC1 was incubated in human serum (Sigma Aldrich) at a volume ratio of 1:1 for 4 or 24 hours at 37°C with stirring (180 rpm). After each of this treatment, Minimal Inhibitory Concentrations (MIC) were determined against *Clostridium perfringens* ATCC13124 in Brain Heart Infusion broth supplemented with yeast extract (5 g/L) and hemin (5 mg/L) (BHI-YH) as described (2). MIC of untreated RumC1 was used to set the maximum of antimicrobial activity and calculate the residual activity of each treated RumC1. Stability and activity of RumC1 was also measured in physiological and higher concentration of salts: MIC of RumC1 was determined as above but the BHI-YH medium was replaced with Mueller Hinton (MH) broth supplemented in either NaCl at 100, 200, and 300 mM or in $MgCl_2$ at 1, 2 and 3 mM. For the stability to salts assays, MIC of untreated RumC1 in MH broth was used as the maximum of antimicrobial activity.

Simulated gastro-intestinal digestion of RumC1. RumC1 was treated with pepsin (Sigma-Aldrich) with a molecular ratio of 1:2.5 (RumC1:pepsin) at 37°C in sodium acetate 50 mM pH 2.5 for 2 hours with stirring (180 rpm) to mimic the digestive conditions occurring in the human stomach. To stop the enzymatic reaction of pepsin, $NaHCO_3$ 1M was added until pH 7 was reached. To simulate the intestinal compartment, RumC1 was incubated with pancreatin (Sigma-Aldrich) with a molecular ratio of 1:5 (RumC1:pancreatin) at 37°C in sodium acetate 50 mM pH 6.5 for 5 hours with stirring (180 rpm). Then Phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) was added to a final concentration of 0.1 mM to inhibit the action of pancreatin. MIC of treated RumC1 was determined against *C. perfringens* ATCC13124 in BHI-YH as described (2) after controlling that the enzymes in the above conditions without RumC1 showed no anti-*C. perfringens* activity. MIC of untreated RumC1 was used to set the maximum of antimicrobial activity and calculate the residual activity of each treated RumC1. Furthermore, RumC1 was detected by RP-C18-HPLC using an analytical column Jupiter 15-µm C18 300 Å (250 mm by 21.2 mm; Phenomenex). Elution was performed at 1 ml/min with a 0 to 40% linear gradient of 90% ACN and 0.1% TFA for 30 min. Finally, mass spectrometry analysis were conducted to compare the molecular masses of digested

RumC1 and untreated RumC1. As it has been reported that the bacteriocin nisin is digested by pancreatin but not by pepsin (13), nisin (from Sigma-Aldrich) was used as a positive control of the enzymatic activity of pancreatin only. To validate pepsin activity, Bovine Albumine Serum (BSA, Sigma-Aldrich) was used as a positive control.

Antimicrobial activity on human intestinal epithelium models. Caco-2 (ATCC HTB-37) and T84 (ATCC CCL-248) cells were being used as models of small intestinal and colonic epithelial cells, respectively. Cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10 % foetal calf serum (FCS), 1 % L-glutamine and 1 % Streptomycin-Penicillin antibiotics (all from Invitrogen). Cells were routinely seeded and grown onto 25 cm^2 flasks maintained in a 5 % CO_2 incubator at 37 °C. To test the influence of human intestinal epithelial cells on RumC1 activity, cells grown on 25 cm^2 flasks were detached using trypsin–EDTA solution (from Thermo Fisher Scientific), counted using Mallasez counting chamber and seeded into 96-well cell culture plates (Greiner bio-one) at approximately 10^4 cells per well. Cells were grown for 7-10 days until differentiation. The culture medium was then replaced twice with DMEM supplemented with 10% FCS but free of phenol red and of antibiotics 24h and 48h to make sure any trace of antibiotics was removed prior to the infection with *B. cereus.* A suspension of *B. cereus* DSM31 was prepared in the same media at a final concentration of 5.10^5 CFU/mL and added or not to Caco-2 and T84 monolayers. RumC1 was added 30 min before or at the same time or after the bacterial cells. Microplates were incubated for 24h at 37°C with 5% CO_2. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide that inhibited the growth of bacteria. Independent triplicate were made and sterility and growth controls were prepared for each assay.

MIC determination. Strains tested were acquired from commercial collections (the American Type Culture Collection (ATCC), www.atcc.org; the Collection de l'Institut Pasteur (CIP), www.pasteur.fr), from a laboratory collection (Centre National de Référence de la résistance aux antibiotiques, www.cnr-resistance-antibiotiques.fr) or from clinical sampling. *C. perfringens* CP24, 56, 60 were isolated from chickens and provided by UGent (14). Human clinical strains were mostly isolated from bloodstream infections, and in bone and joint infection at the University hospital of Besançon (France). The MIC were determined by broth microdilution for fastidious organisms (*Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Bacillus cereus*), and non-fastidious organisms (*Enterococcus* species) by independent triplicates according to the EUCAST 2019 recommendations except for the Clostridia (15). Briefly, a bacterial suspension of Clostridia

was grown in anaerobic conditions (in a Trexler-type anaerobic chamber without stirring) and adjusted in MH broth supplemented with 5% lysed horse blood and 20 mg/L β-nicotinamide adenine dinucleotide at 5.10^6 UCF/mL. Ninety microliters of cell suspension were transferred in a sterile F-bottom polypropylene 96-well microplates. Thus, ten microliters of sterile RumC1 or antibiotics used as control from 100 to 0.1 µM by two-fold serial dilutions were added to the bacterial suspension to obtain a final concentration of 5.10^5 CFU/mL. Microplates were incubated 48h at 37°C in anaerobic conditions. MIC was defined as the lowest concentration of peptide that inhibited the growth of bacteria after 48h incubation at 37°C. Sterility and growth controls were prepared for each assay.

Macromolecules synthesis studies. *C. perfringens* ATCC 13124 was grown in BHI-YH broth (2) in airtight jars in the presence of anerobic atmosphere generation bags (Sigma-Aldrich) without stirring at 37°C until $OD_{600\ nm}$ reached 0.2. Then RumC1 or metronidazole or antibiotics with known mechanisms of action were added at 5xMIC. The antibiotics gemifloxacin, rifampicin, tetracycline and vancomycin were used as controls for the inhibition of DNA, RNA, protein and peptidoglycan synthesis, respectively. After 15 min of incubation at 37°C in anaerobic conditions, samples were labelled with [methyl-3H]thymidine or [5,6-3H]uridine or L-[4,5-3H(N)]-leucine or D-[1-3H] HCl glucosamine (all from Hartmann Analytic) at 10 µCi/mL to follow the synthesis of DNA, RNA, proteins and peptidoglycan respectively. After 45 min of incubation at 37°C in anaerobic conditions, bacterial cells were lysed and the macromolecules were precipitated with ice cold trichloroacetic acid (TCA, Sigma-Aldrich) at a final concentration of 20%; samples were kept on ice for an hour. Then the precipitates were filtered on Whatman glass microfiber filters pre-soaked in ice cold TCA 5%. After washing the filters with ice cold TCA 5% twice and then ice cold absolute ethanol once, they were soaked in 10 mL of scintillation liquid (Ultima Gold, PerkinElmer). Radioactivity was measured by liquid scintillation counting (TriCarb2800, PerkinElmer). As each condition displayed different growth rates, the radioactivity counts were normalized by the $OD_{600\ nm}$ of the samples. Results were expressed as a percentage of total macromolecule synthesis that was measured for each macromolecule with untreated cells. All experiments were done in independent triplicates.

ATP bioluminescent assays. *C. perfringens* ATCC 13124 was grown in BHI-YH broth (2) in anaerobic conditions (in a Trexler-type anaerobic chamber without stirring) at 37°C until $OD_{600\ nm}$ reached 0.4. Then *C. perfringens* cells were distributed in white polystyrene Nunc™ 96-well plate (ThermoFisher Scientific) and RumC1, metronidazole or nisin were added at 2.5, 5 or 10xMIC.

After 15 min of incubation in the same conditions, 100 µL of cells were mixed with 10 µL of luciferin-luciferase reagent (Yelen Analytics) prepared in IMI-Yelen Buffer (Yelen Analytics). The mix was homogenized and incubated 30 s before reading of the emitted photon using a microplate reader (Synergy Mx, BioTek). Then, 10 µL of lysis reagent (Yelen Analytics) was added to the mixture, homogenized and incubated 1 min before a new reading of the emitted photon. The intensity of the bioluminescent light was expressed as relative light units (RLU) which is proportional to extracellular ATP concentration (16). The inner ATP concentration was derived from the difference in ATP concentration in the extracellular media before and after cell lysis. Results were expressed as a percentage of total inner ATP concentration that was measured with untreated cells. All experiments were done in independent triplicates.

***Ex-vivo* evaluation of the RumC1 innocuity using human intestinal explants**. In order to evaluate the innocuity of RumC1 peptide for the human gut, *ex-vivo* experiments were performed using human explants as previously described (17). Human intestinal tissues, corresponding to ileocecal area, were obtained from patients undergoing surgery at the unit of gastrointestinal surgery, Hospital Laveran (Marseille, France), according to a collaborative "clinical transfer" project. The procedure was approved by the French ethic committee (CODECOH n° DC-2019-3402). All patients agreed for the use of their tissues for research purposes. Diagnoses leading to surgery were intestinal carcinoma. Samples were taken from macroscopically unaffected area as identified by the surgeons. After resection, the tissues were placed in ice-cold oxygenated sterile DMEM solution containing 1% (w/v) streptomycin/penicillin solution and 50 µg/mL gentamycin and were directly transferred to the laboratory within 15 min. Intestinal tissues were extensively washed and maintained in ice-cold culture media. Tissues were cleaned under binocular microscope from vascular vessels and conjunctive tissue using forceps. Intestinal explants (diameter of 0.5 cm^2) were then isolated from the cleaned resections using surgical punch, the complete procedure being complete in less than 2 h after the resections were obtained from the surgery unit. Finally, the explants were washed 3 times with culture media without antibiotics and transferred into 24-well plates before being incubated at 37°C during 4 h with RumC1 peptide diluted in DMEM media at 100 µM. In parallel, explants were left untreated (negative controls) or were incubated with the 300 µM of detergent cetyl trimethylammonium bromide (CTAB) used as positive control of tissue damages. After incubation, the explants were collected, washed three times with PBS^{2+} and fixed overnight at 4°C with PFA diluted at 4% (v/v) in PBS. The next day, intestinal explants were washed twice with PBS and included in inclusion medium (TFM - EMS), in transverse position to allow cutting respecting the crypt-villosity axis using cryostat (Leica CM3050). Four sections of 5

µm thickness were obtained per explant and each section being separated from the next by 100 µm in order to cover all the tissue. Explants were then stained using hematoxylin and eosin (H&E) staining protocol. Briefly, samples were incubated for 8 min in hematoxylin (from Sigma-Aldrich) and then allowed to stain by incubation with water for 2 min. Then explants were incubated for 1 min in eosin (from Sigma-Aldrich) and then in water for 1 min. This was followed by incubation of explants with ethanol at concentration of 70% then 95% and finally 100% for 2 min each. After blotting excess ethanol, intestinal explants were incubated for 15 min with xylene and mounted with coverslip using Eukitt mounting media (EMS, Hatfield, PA, USA). Finally, explants were left overnight to dry before examination of tissue organization under the microscope (Leitz DMRB microscope (Leïca) equipped with Leïca DFC 450C camera) (18).

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