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1 The unusual structure of Ruminococcin C1 antimicrobial peptide confers clinical properties

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34  
35 Radical SAM enzyme, RiPP, sactipeptide, *Ruminococcus gnavus*, antibiotic

37 The emergence of superbugs developing resistance to antibiotics and the resurgence of microbial  
38 infections have led scientists to start an antimicrobial arms race. In this context, we have previously  
39 identified an active RiPP, the Ruminococcin C1, naturally produced by *Ruminococcus gnavus* E1,  
40 a symbiont of the healthy human intestinal microbiota. This RiPP, subclassified as a sactipeptide,  
41 requires the host digestive system to become active against pathogenic Clostridia and multidrug  
42 resistant strains. Here, we report its unique compact structure on the basis of four intramolecular  
43 thioether bridges with reversed stereochemistry introduced post-translationally by a specific  
44 radical-SAM sactisynthase. This structure confers to the Ruminococcin C1 important clinical  
45 properties including stability to digestive conditions and physicochemical treatments, a higher  
46 affinity for bacteria than simulated intestinal epithelium, a valuable activity at therapeutic doses on  
47 a range of clinical pathogens, mediated by energy resources disruption and finally safety for human  
48 gut tissues.

49

## 50 **Significance**

51 Since the discovery of penicillin, humans have widely developed and used antibiotics to protect  
52 themselves from microbial infections. However, the intensive use of these compounds has led to  
53 the emergence of pathogens resistant to all classes of antibiotics. This major public health threat  
54 has led scientists to find new molecules with different structures and modes of action to overcome  
55 resistance phenomena. A promising alternative concerns bacteriocins secreted by certain bacteria.  
56 Of a peptidic nature, their ribosomal synthesis differentiates them from conventional antibiotics.  
57 The recently identified RumC1 antimicrobial peptide presents a remarkable bactericidal activity for  
58 multi-drug resistant strains. Added to this efficacy, RumC1 is not toxic against a number of human  
59 cell lines and is safe for human gut tissues.

60

61

## 62 INTRODUCTION

63 Ribosomally synthesized and Post-translationally modified Peptides (RiPPs) are an important  
64 group of compounds that have stimulated research interest, notably as natural antimicrobial agents  
65 with bacteriocins (1). During RiPPs biogenesis, a precursor peptide composed of at least a leader  
66 and a core sequence is synthesized by the ribosome. The core peptide is modified by tailoring  
67 enzymes and then the leader sequence is cleaved by one or two peptidases to produce the final  
68 active product (1–4). Among the RiPPs, sactipeptides (Sulfur-to- $\alpha$  carbon thioether cross-  
69 linked peptides) are a subgroup that has emerged in recent years (5–7). Despite spectacular soaring  
70 made with genomic tools, the sactipeptide subclass is currently limited to only seven members.  
71 They include subtilosin A (SboA) (8, 9), thurincin H (10), the sporulation killing factor (SkfA) (11),  
72 thuricin CD that consists of two peptides, Trn- $\alpha$  and Trn- $\beta$  (12), thuricin Z or huazacin (13, 14) and  
73 the recently characterized Ruminococcin C1 (RumC1) (15, 16).

74 The precise mechanistic details of sactipeptide cross-link formation are not fully understood.  
75 However, from a chemical point of view, the thioether bonds in these natural products are distinct  
76 from those of the well-studied lanthipeptides, such as nisin, in which they are formed between a  
77 Cys residue and a  $\beta$ -carbon of a dehydrated Thr/Ser residue (17). In contrast to the two-step redox  
78 neutral mechanism used for the maturation of lanthipeptides, radical-SAM sactisynthase enzymes  
79 introduce chemically equivalent thioether bonds by a one-step radical-based mechanism (5–7). The  
80 enzymes within this superfamily contain the canonical CysX<sub>3</sub>CysX<sub>2</sub>Cys motif, that binds the  
81 radical-SAM [4Fe-4S]<sup>2+/1+</sup> cluster (referred to as the RS cluster), in which the fourth, unique iron,  
82 is used to bind S-adenosylmethionine (SAM) cofactor (18). In its reduced state, the [4Fe-4S]<sup>1+</sup>  
83 cluster catalyzes the reductive cleavage of SAM to generate a 5'-deoxyadenosyl radical (5'-Ado•).  
84 This radical abstracts a H-atom from the cognate substrate to initiate catalysis (18). In the case of  
85 the characterized radical-SAM enzymes involved in the sactipeptide biosynthesis, H-atom  
86 abstraction was shown to occur from the  $\alpha$ -carbon of the acceptor residue (5–7). A survey of  
87 available structural and functional data indicate that all radical-SAM enzymes involved in the  
88 sactipeptides biosynthesis contain a C-terminal extension appended to the radical-SAM domain  
89 called SPASM/Twitch domain that houses additional [4Fe-4S] clusters (19, 20). In contrast to the  
90 Twitch domains, which bind only one additional cluster, SPASM domains present a conserved  
91 cysteine-rich motif that coordinates two additional iron-sulfur clusters. The role of these clusters  
92 remains to be clarified, but it was suggested that they possibly interact with the substrate during  
93 catalysis or may also be implicated in electrons transfer (5–7, 21).

94 Among the seven sactipeptides reported to date, four are structurally characterized  
95 (SboA, thurincin H, Trn- $\alpha$  and Trn- $\beta$ ) and all of them have been purified from the genus

96 *Bacillus*. SboA is a cyclic peptide with three thioether bridges involving two phenylalanines and  
97 one threonine. Trn- $\alpha$ , Trn- $\beta$  and thurincin H are not cyclic, they present three and four thioether  
98 bridges, respectively. One common feature of SboA, Trn- $\alpha$ , Trn- $\beta$  and thurincin H is that they all  
99 present Cys residues in the N-terminal half and the corresponding partners at the C-terminal part, a  
100 property that folds the peptide in a single hairpin-shaped form with the hydrophobic residues facing  
101 outwards as showed by the available structures (8–10, 12, 22). Sequence analysis of the last  
102 identified sactipeptide, namely RumC1, suggests a new fold. Indeed, one pair of cysteines is located  
103 at the N-terminal part of the sequence while the other one is in the C-terminal end (Fig. 1A). Recent  
104 data on the characterization of mature RumC1 by mass spectrometry, strongly suggests that the  
105 thioether network folds the peptide in a double hairpin like structure that differs from the currently  
106 reported ones (16). Furthermore, sactipeptides and by extension antimicrobial peptides (including  
107 RiPPs but not only) have emerged as a potential trove of new weapons and alternatives to  
108 conventional antibiotics to fight multidrug-resistant (MDR) bacteria. However, their clinical use  
109 remains a challenge due to high cost of production, sensitivity to physiological or manufacturing  
110 conditions, as well as toxicity for human tissues (23, 24). In this context, we previously showed  
111 that RumC1 has a potent activity against Gram positive bacteria and is harmless for human cells  
112 (16). Obviously, the next step was to study RumC1 in a clinical context and to address the above-  
113 mentioned reasons that prevent antimicrobial peptides from being considered for pharmaceutical  
114 development.

115 In this work, we sought to determine the three-dimensional structure of RumC1. The rapid  
116 and large-scale production of  $^{13}\text{C}$ - and  $^{15}\text{N}$ -labelled mature RumC1, by heterologous expression in  
117 *Escherichia coli* (16, 25), allowed extensive nuclear magnetic resonance (NMR) analyses to solve  
118 the structure of RumC1 and to propose the thioether network stereochemistry. In the meantime,  
119 combined EPR and Mössbauer spectroscopies enabled us to characterize the Fe-S clusters in  
120 RumMc1 sactisynthase. Finally, we point out that the fold of RumC1 confers resistance to physical,  
121 chemical and digestive constraints, features essential for consideration in pharmaceuticals. The  
122 clinical properties of RumC1 also covers activity against clinical pathogens, including resistant  
123 strains, maintained in a mammalian environment and mediated through energy resources depletion,  
124 without any impact to human tissues.

125

## 126 **Results**

127 **RumC1 sactipeptide displays a double hairpin-like structure.** The two-dimensional [ $^1\text{H}$ ,  $^{15}\text{N}$ ]  
128 HSQC spectrum of RumC1 is well-resolved with 39 peptide amide peaks out of 42 expected, thus  
129 attesting for the folding of the protein (fig. S1). No NH peaks were observed for residues C26, G27

130 and N28, probably due to fast amide exchange with the solvent and/or high flexibility of the region.  
131 We assigned the backbone carbon, nitrogen and proton resonances using a combined strategy of  
132 sequential residue correlations based on HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO,  
133 HN(CA)CO triple resonance experiments, and through-space nOe connectivities using 2D [<sup>1</sup>H, <sup>1</sup>H]  
134 NOESY, 3D [<sup>1</sup>H, <sup>15</sup>N, <sup>1</sup>H] and 3D [<sup>1</sup>H, <sup>13</sup>C, <sup>1</sup>H] NOESY experiments. NMR studies of Subtilosin  
135 A, Thuricin CD and Thurincin H, by Vederas *et. al.* have shown that cysteine sulfur to  $\alpha$ -carbon  
136 thioether linkages induce a 10 to 15 ppm downfield shift for the  $\alpha$ -carbon atoms of bridged residues  
137 (8–10, 12, 22). Moreover, through-space nOe interactions were observed between the  $\beta$ -protons of  
138 cysteines and the amide proton (NH) of modified residues (8–10, 12, 22). In the same manner, we  
139 have demonstrated that RumC1 contains four sulfur to  $\alpha$ -carbon thioether cross-links between Cys3  
140 and Asn16, Cys5 and Ala12, Cys22 and Lys42, and Cys26 and Arg34 (16). Since there are four  
141 thioether bridges, each can adopt one of the two possible configurations at the  $\alpha$ -carbon atom (L or  
142 D). Consequently, 16 stereoisomers must be considered to establish the three-dimensional structure  
143 of RumC1. Calculations for all 16 stereoisomers were carried out using the CYANA software and  
144 seven rounds performed on each stereoisomer using the same NMR restraints file (26). The  
145 structural statistics and constraint violations (table S1) allowed to identify the stereoisomer with the  
146 D stereochemistry at Ala12 ( $\alpha$ -S), Asn16 ( $\alpha$ -S), Arg34 ( $\alpha$ -S) and Lys42 ( $\alpha$ -S) as a representative  
147 structure given: i) the absence of thioether bridge constraint violations, ii) the great number of nOe  
148 connectivities used in the structure calculation, iii) the lowest average target function value and a  
149 low root mean square deviation (*rmsd*). To improve the structure of the DDDD stereoisomer, an  
150 additional refinement step by returning to the NOESY spectra to eliminate the ambiguities found  
151 during the structure calculation by the CYANA software was added. The resulting structural  
152 statistics of the 20 conformers for the DDDD isomer of RumC1 are summarized in table S2. The  
153 backbones of the 20 lowest target function value conformers for the DDDD isomer of RumC1  
154 superimpose quite well with a *rmsd* value of 0.81 Å for the backbone (Fig. 1B). The three-  
155 dimensional structure of RumC1 is thus composed on both sides by two  $\alpha$ -helices and in the middle  
156 by a 2-stranded parallel  $\beta$ -sheet fragment and the whole stiffened by four cysteine sulfur to  $\alpha$ -  
157 carbon thioether cross-links (Fig. 1C).

158 The electrostatic surface potentials present an overall positive charge (Fig. 1D) and the surface  
159 hydrophobicity of the DDDD stereoisomer shows a majority of hydrophilic residues (Fig. 1E). The  
160 compact three-dimensional solution structure of RumC1 reveals a new sactipeptide fold and by  
161 extension a new antimicrobial peptide fold (fig. S2A). Four sulfur to  $\alpha$ -carbon thioether bridges  
162 with a DDDD stereochemistry have been already reported for thurincin H (10). However, the

163 presence of an additional 2-stranded parallel  $\beta$ -sheet fragment in the middle of RumC1 induces a  
164 new fold, thus resulting in a different location of the thioether crosslinks (fig. S2, B-E) (9, 10, 22).

165

166 **Expression, purification and characterization of the RumMc1 sactisynthase.** RumMc1 is  
167 predicted to contain accessory Fe-S clusters, in addition to the RS cluster, thus we decided to co-  
168 express the *rumMc1* gene with the pDB1282 plasmid, which encodes for a set of proteins involved  
169 in iron-sulfur cluster biogenesis (IscS, IscU, IscA, HscB, HscA and Fdx) (27). After the final step  
170 of anaerobic purification, the purity was evaluated by SDS-PAGE to be over 95% (fig. S3). As  
171 expected, the holo-RumMc1 protein was dark brown in color and the iron titration revealed, on  
172 average, ten metal centers per monomer. In good agreement, the UV-vis spectrum of holo-RumMc1  
173 suggests the presence of [4Fe-4S] clusters (Fig. 2A, solid line). Mössbauer spectroscopy on  $^{57}\text{Fe}$ -  
174 enriched oxidized holo-RumMc1 was then used to deeply investigate the nature of the Fe-S clusters.  
175 The experimental spectrum recorded at  $T = 77\text{ K}$  (dashed line) could be simulated (red line) with  
176 three components **1**, **2** and **3** in a 2:1:1 ratio (Fig. 2B). **1** and **2** are characterized by similar isomer  
177 shifts ( $\delta$ ) with  $\delta = 0.42\text{ mms}^{-1}$  for **1** and  $\delta = 0.44\text{ mms}^{-1}$  for **2** while the quadrupole splitting ( $\Delta E_q$ )  
178 varies with  $\Delta E_q = 0.98\text{ mms}^{-1}$  for **1** and  $\Delta E_q = 1.30\text{ mms}^{-1}$  for **2**. These values are in a typical range  
179 for  $\text{Fe}^{2,5+}$  ions in a diamagnetic  $[\text{4Fe-4S}]^{2+}$  cluster (28–30). This is supported by simulations of the  
180 spectra recorded at  $T = 4.2\text{ K}$  in external fields of  $B = 0.1\text{ T}$  and  $B = 5\text{ T}$  (fig. S4, A and B) based  
181 on the spin Hamilton formalism assuming a total spin of  $S = 0$ . The 2:1 ratio suggests that holo-  
182 RumMc1 contains three  $[\text{4Fe-4S}]^{2+}$  clusters. This is supported by the sequence alignment of  
183 RumMc1 with other related proteins that contain conserved cysteine residues localized at the C-  
184 terminal half of the protein (fig. S5). Hence, we can conclude that **1** represents 8  $\text{Fe}^{2,5+}$  ions in the  
185 same tetrahedral sulfur environment belonging to two  $[\text{4Fe-4S}]^{2+}$  clusters while **2** corresponds to  
186 four indistinguishable  $\text{Fe}^{2,5+}$  ions present in the single  $[\text{4Fe-4S}]^{2+}$  cluster supposed to contain the  
187 SAM-binding iron site (RS-cluster). The difference in  $\Delta E_q$  between **1** and **2** points to a slightly  
188 different distribution of electron density. **3** is characterized by  $\delta = 0.33\text{ mms}^{-1}$  and  $\Delta E_q = 0.49\text{ mms}^{-1}$   
189 typical for  $\text{Fe}^{3+}$  high spin ( $S = 5/2$ ) in a tetrahedral sulfur environment (28). This component was  
190 adequately simulated (fig. S4A, with an external magnetic field) by combining contributions from  
191 three distinct, but antiferromagnetically coupled  $\text{Fe}^{3+}$  centers, thus resulting in a global  $S = 1/2$  spin  
192 state as described for a paramagnetic  $[\text{3Fe-4S}]^{1+}$  cluster (31, 32). The respective hyperfine  
193 parameters are characteristic for  $[\text{3Fe-4S}]^{1+}$  clusters (fig. S4B) (31, 32). Upon addition of dithionite  
194 on the holo-RumMc1, the absorption decreased over the entire 310-420 nm range as expected for  
195 the conversion of the  $S = 0$   $[\text{4Fe-4S}]^{2+}$  chromophore to the  $S = 1/2$   $[\text{4Fe-4S}]^{1+}$  level (Fig. 2A, dashed  
196 line). This reduced form has been investigated by X-band EPR spectroscopy in order to gain

197 insights into the individual features of the three [4Fe-4S] clusters involved. The experimental EPR  
198 spectrum of holo-RumMc1 is extremely rich, suggesting the presence of multiple  $S = 1/2$  [4Fe-  
199 4S]<sup>1+</sup> clusters (fig. 2C, black line). It was satisfactorily simulated considering the three following  
200 sets of g-tensors [2.034, 1.913, 1.870] (**A**), [2.050, 1.937, 1.902] (**B**) and [2.074, 1.932, 1.885] (**C**)  
201 (Fig. 2, C and E). Addition of the natural SAM cofactor then induced a substantial change in the  
202 spectrum that was well simulated with four sets of g-tensors [2.034, 1.910, 1.860] (**A**), [2.049,  
203 1.937, 1.904] (**B**), [2.073, 1.932, 1.885] (**C**) and [2.076, 1.930, 1.852] (**A'**) instead of three (Fig. 2,  
204 D and E). As the contribution of **A** has to be lowered to achieve a reliable simulation, these  
205 experiments support that **A** originates from the RS cluster while **B** and **C** rely to two additional  
206 clusters. The new component **A'** was assigned to the amount of the RS cluster, which is bond to the  
207 SAM as previously described (33).

208  
209 **Efficient maturation of RumC1: both the leader and the core sequences are crucial.** Here we  
210 sought to gain insights into the features of RumC1 that are required for its efficient maturation by  
211 RumMc1. It is established that the post-translational modification of RiPPs is an event that is  
212 dependent on the presence of the leader peptide (2). For example, in the case of the sactipeptide  
213 subclass, Marahiel and co-workers have shown that AlbA failed to transform a variant of the SboA  
214 peptide without its leader sequence in a mature sactipeptide (34). We investigated the maturation  
215 of RumC1 by the way of an *in vitro* assay in the presence of RumMc1, the SAM cofactor and  
216 dithionite as an external electron source. These experiments were performed with a full-length  
217 RumC1 precursor peptide, a leader less variant of 44 amino acids (RumC1-44) and a third condition  
218 in which the leader sequence and the core peptide were dissociated but both present in the solution  
219 (RumC1-44-LS). The reaction mixtures were analyzed by LC-MS to detect the formation of  
220 thioether bonds within the three substrates (fig. S6). Since the full maturation of RumC1 leads to  
221 the formation of four thioether bonds, the percentages of maturation (fig. S6A) account for the  
222 species that present a loss of 8 Da in mass compared to the unmodified substrate. The data clearly  
223 indicate that only the RumC1 peptide is fully modified, both RumC1-44 and RumC1-44-LS led to  
224 less than one and ten percent of species with four thioether linkages, respectively (fig. S6, A and  
225 B). It has to be noted that attempts to yield mature peptides from the latter two substrates lead  
226 mainly to a mixture of peptides harboring partial maturation with 2 and 3 thioether bonds (fig. S6,  
227 C and D). In good agreement with the lack of maturation of RumC1-44 during the *in vitro* enzymatic  
228 assay, we also observed that no mature peptide is obtained when this leader less form of RumC1 is  
229 produced *in vivo* using the heterologous expression and maturation protocol in *E. coli* (fig. S7A).

230 In contrast to the sactipeptides identified so far, the thioether connectivity in RumC1 folds  
231 the peptide in a double hairpin like structure. Sequence alignments of the five RumC isoforms  
232 shows a strictly conserved Gly18/Pro19 motif in the loop region between the two hairpins (16). We  
233 therefore sought to determine whether this motif is necessary for inducing a turn in the sequence  
234 and allowing the peptide maturation by RumMc1. For this, we replaced the Gly18/Pro19 motif by  
235 Ala18/Ala19. LC-MS analysis of the heterologously expressed Ala18/Ala19 RumC1 variant clearly  
236 demonstrate the lack of maturation thus suggesting that the loop alteration in RumC1 may disturb  
237 substrate-enzyme interactions that are crucial for the formation of the thioether network (fig. S7B).  
238 As reported by Grove and coworkers for CteA (21), these results suggest that the binding of RumC1  
239 by RumMc1 involves determinants from both the leader and the core sequences.

240  
241 **The compact structure confers a high stability to RumC1.** Studies have proven the potent  
242 activity of sactipeptides (12, 35), however there is a lack of evidence showing that they possess the  
243 physiochemical properties necessary for *in vivo* administration and, from an applied point of view,  
244 for pharmaceutical development (35). Consequently, we assayed the tolerance of RumC1 to such  
245 properties. RumC1 showed no loss of activity when exposed to acidic or basic pH from 2 to 11  
246 (Fig. 3A). Furthermore, RumC1 was resistant to 70 and 100°C for up to 1 hour and more than 15  
247 minutes respectively, meaning that RumC1 possesses the intrinsic thermal resistance that are  
248 required for drug formulation processes (Fig. 3B). Because of their sensitivities to enzymatic  
249 digestion or to physiological salts concentrations and blood enzymes, the administration of  
250 antimicrobial peptides by oral route and/or systemic injection is limited. Interestingly, the activity  
251 of RumC1 wasn't impaired by salts concentrations higher than 150 mM for NaCl and 2 mM for  
252 MgCl<sub>2</sub> (Fig. 3C), which are considered as physiological saline conditions. Likewise, incubation in  
253 human serum at 37°C up to 24 hours did not affect the activity of RumC1 (Fig. 3D). RumC1 was  
254 finally treated with pepsin at pH 2.5 for 2 hours at 37°C, and with pancreatin at pH 6.5 for 5 hours  
255 at 37°C in order to mimic the human gastric and intestinal compartments, respectively. RumC1  
256 showed no loss of activity and its integrity was revealed by MS analysis after these treatments  
257 whereas the lanthipeptide nisin used as a positive control for pancreatin activity showed reduced  
258 antimicrobial potency (Fig. 3E, fig. S8, A and B). Pepsin activity was confirmed as well by  
259 hydrolysis of Bovine Serum Albumine (BSA) (fig. S8A). Therefore, the thioether network leading  
260 to a compact structure of RumC1 confers to the sactipeptide high resistance to physico-chemical  
261 treatments and to the physiological but harsh conditions encountered in blood or in the digestive  
262 tract after systemic or oral administration.

264 **RumC1 is able to act on a simulated infected intestinal epithelium.** Many studies on  
265 antimicrobial peptides show their direct activity on bacterial cultures, without considering their  
266 efficiency in a mammalian environment, such as an infected epithelium. Indeed, antimicrobial  
267 peptides can act on bacteria but can also insert into eukaryotic cells or merely bind to their surface  
268 (24, 36), which could cause a partial loss of activity against extracellular pathogens. On the  
269 contrary, some peptides have higher affinity for bacterial cells than host cells (37). Therefore, we  
270 assayed the potency of RumC1 against *Bacillus cereus*, an aerotolerant human intestinal  
271 opportunistic pathogen, on simulated gut epitheliums using Caco2 and T84 cells as models of  
272 human small intestinal and colonic epithelium, respectively. *B. cereus* was able to grow on  
273 untreated monolayers of Caco2 and T84 whereas treatment with RumC1 was effective to clear the  
274 infection (fig. S9). The MIC of RumC1 against *B. cereus* was measured in the eukaryotic cell line  
275 culture medium in the presence or the absence of human intestinal cells. The MIC value did not  
276 increase in the presence of intestinal cells when RumC1 was added 30 min before, concomitantly  
277 or 30 min after the bacterial cells (Fig. 4A). Indeed, no loss of activity was detected as well when  
278 *B. cereus* was allowed to colonize the epithelium for 30 min before adding RumC1 (Fig. 4A).  
279 Therefore, it appears that RumC1 has a preferential affinity for bacterial cells rather than host cells.  
280 Finally, these results suggest that the physiological environment of the two cell lines did not affect  
281 significantly the activity of RumC1.

282  
283 **RumC1 displays a potent activity against Gram-positive pathogenic clinical isolates.** We  
284 previously showed that RumC1 is active against a broad range of Gram-positive bacteria including  
285 resistant and multi-resistant strains (16), but focusing only on collection strains. In order to evaluate  
286 the potentiality of RumC1 being considered for pharmaceutical development, we investigated its  
287 activity against Gram-positive strains isolated in a clinical context from humans or animals (i.e.  
288 broiler chickens). As RumC1 was first identified for its anti- *Clostridium perfringens* activity (16,  
289 38, 39), we measured for the first time the MIC of the sactipeptide against a large panel of *C.*  
290 *perfringens* clinical isolates (Fig. 4B). RumC1 was active under the micromolar range (between 0.4  
291 and 0.8  $\mu\text{M}$ ) on all the ten strains tested. In comparison with the reference molecules, usually used  
292 to eradicate *C. perfringens*, RumC1 showed a higher activity than metronidazole (12 to 23  $\mu\text{M}$ ) and  
293 an activity similar to vancomycin (0.2 to 0.4  $\mu\text{M}$ ). Interestingly, in a livestock context, RumC1 was  
294 not only active against the CP24 *C. perfringens* strain isolated from a healthy broiler chicken, but  
295 also against strains isolated from animals suffering from dysbiosis or necrotic enteritis, respectively  
296 CP56 and CP60 (40). In addition, RumC was also active against *C. perfringens* strains isolated from  
297 humans suffering from bacteremia ( $n=6$ ) (Fig. 4B).

298 Then, we studied the effect of RumC1 on another main intestinal pathogen from the  
299 *Clostridium* genus, *Clostridium difficile*. RumC1 showed activity against collection and clinical  
300 strains of *C. difficile* with lower MIC (0.3 to 0.6  $\mu\text{M}$ ) than two of the most common antibiotics used  
301 for *C. difficile* intestinal infections (CDI), i.e. metronidazole (1.5  $\mu\text{M}$ ) and vancomycin (0.3 to 0.7  
302  $\mu\text{M}$ ) (Fig. 4B). Moreover, RumC1 is also efficient at the micromolar range against other intestinal  
303 pathogens (clinical and collection strains) of main importance such as *Listeria monocytogenes*, *B.*  
304 *cereus*, *Enterococcus faecalis* and *Enterococcus faecium* including strains resistant to amoxicillin  
305 and/or vancomycin (Fig. 4B). Beside its activity against pathogens causing gut infections, RumC1  
306 is also active against a clinical *Streptococcus pneumoniae* strain at a low MIC (0.3  $\mu\text{M}$ ), a pathogen  
307 responsible of multiple types of infection (including respiratory tract infection, meningitis and  
308 septicemia) (Fig. 4B).

309  
310 **The killing mechanism of RumC1 involves energy resource shortage.** Unlike many bacteriocins  
311 RumC1 does not have a pore forming action (16), we therefore investigated its effects on the main  
312 macromolecules synthesis pathways usually targeted by antibiotics. *C. perfringens* cells were  
313 exposed to RumC1 and the synthesis of DNA, RNA, proteins and peptidoglycan were followed by  
314 measuring the incorporation of radio-labelled precursors. Remarkably, RumC1 was able to inhibit  
315 the synthesis pathways of the four macromolecules with efficacies similar to conventional  
316 antibiotics targeting each of these pathways (Fig. 5A). As these synthesis pathways are all energy-  
317 dependent, we assayed the potency of RumC1 to break such energy resources. Thus, we measured  
318 by bioluminescent assay the ATP released by *C. perfringens* cells in the extracellular medium, and  
319 then lysed cells to derive the intracellular ATP content (Fig 5B). Upon treatment with RumC1 for  
320 15 min, outer ATP was not increased compared to cells untreated whereas inner ATP decreased in  
321 a dose-dependent manner. Treatment with nisin, a pore-forming bacteriocin led to drastic increase  
322 of outer ATP whereas metronidazole does not impact ATP synthesis pathway. Since the synthesis  
323 of DNA, RNA, proteins and peptidoglycan accounts for the consumption of approximately 70 %  
324 of the total ATP content of bacteria, their observed inhibition is likely linked to ATP depletion and  
325 therefore the specificity of RumC1 could be related to an inhibition of the ATP synthesis pathway  
326 (41).

327  
328 **Integrity of human tissues treated with RumC1.** We previously reported that RumC1 was a safe  
329 new antimicrobial peptide because of its lack of *in vitro* toxicity on gastric and intestinal cell lines  
330 culture (16). To further characterize the safety of RumC1, we assayed the toxicity of the sactipeptide  
331 *ex vivo*, directly on human ileocecal tissues. Intestinal explants were isolated from unaffected area

332 of ileocecal resection from two patients diagnosed with intestinal carcinoma and were incubated  
333 with RumC1 at 100  $\mu$ M or the detergent CTAB at 300  $\mu$ M for 4 hours. Control and RumC1 exposed  
334 human intestinal explants displayed normal tissue organization with normal crypt and villosity  
335 lengths and no sign of epithelial desquamation or erosion, in accordance with data we obtained  
336 previously *in vitro* on human intestinal cell lines. Conversely, exposure to CTAB, used as positive  
337 control of tissue damages, caused important lesions to the human intestinal epithelium with cell  
338 desquamation and shortening of the crypts and villousities (Fig. 6).

## 340 Discussion

341 In this study, we elucidated the three-dimensional structure of mature Ruminococcin C1 from  
342 *Ruminococcus gnavus* E1. NMR experiments clearly showed that the thioether network of RumC1,  
343 involving four sulfur to  $\alpha$ -carbon bridges, folds the peptide into a double hairpin-like motif, which  
344 differs from the currently reported structures within the sactipeptide family. By extension, the fold  
345 not described so far includes two-stranded parallel  $\beta$ -sheet into the core enclosed between two  
346 parallel  $\alpha$ -helices. In contrast to a recent report by Berteau *et al.*, where the residues involved in  
347 the thioether bridges were identified to be L-configured, after hydrolysis and derivatization (15),  
348 here we found that the stereoisomers that fits best with the NMR data featured D-configurations at  
349 Ala12, Asn16, Arg34 and Lys42. RumC1 has a global positive charge at physiological pH and  
350 displays a constrained and rigid backbone structure that presents a mostly hydrophilic surface  
351 unlike sactipeptides with known structure presenting mostly hydrophobic surface. These  
352 sactipeptides, like most of the bacteriocins that target Gram-positive strains, have a pore-forming  
353 mode of action. Despite an overall cationic charge, RumC1 is unable to insert into lipids extracts  
354 most likely due to its hydrophilic surface and exerts probably an intracellular mode of action  
355 reaching one or more specific targets by active transport.

356 The double hairpin-like structure of RumC1 provides stability to pH and high-temperatures,  
357 which could facilitate pharmaceutical manufacturing processes. We also showed that RumC1 is  
358 resistant to digestive proteolytic conditions, most likely because the sites of cleavage are protected  
359 by the four thioether bonds and the tridimensional folding of the peptide. Many RiPPs including  
360 sactipeptides, such as Subtilisin A and Thuricin CD, are not resistant to both pepsin and pancreatic  
361 proteases (42, 43), implying that the original folding of RumC1 offers additional protection.  
362 Moreover, RumC1 is also resistant to physiological salts conditions as well as to human serum,  
363 which can both be detrimental for an exogenous compound. Based on these considerations, we  
364 followed our investigations of RumC1 in a preclinical context. The first step was to ensure that  
365 RumC1 could cure infections in the mammalian environment. Therefore, we treated with RumC1

366 a *B. cereus* infected simulated epitheliums. RumC1 remained active at very low doses, exactly at  
367 the same level as the MIC obtained in the absence of eukaryotic cells. This observation confirms  
368 the stronger affinity of RumC1 for bacterial versus host cells, and the specific primary function of  
369 RumC1 as antibiotic compound. Then we checked that RumC1 has potent activity on Gram-positive  
370 clinical pathogens and not only on collections strains. RumC1 showed strong activity under the  
371 micromolar range toward *Clostridia* pathogens from both human and animal origins. The measured  
372 MICs are equivalent to vancomycin, one of the reference antibiotics used in therapy against these  
373 pathogens, and lower than metronidazole, another reference antibiotic known to generate resistance  
374 (16). Therefore, the use of RumC1 to treat infections caused by *C. perfringens* or *C. difficile* should  
375 be considered. Indeed, from a livestock point of view, *C. perfringens* can cause necrotic enteritis,  
376 which is one of the most common infection in broiler chickens with mortality rates as high as 50%  
377 and resulting in dehydration, lesions on intestinal mucosa or other organs like the liver, spleen,  
378 heart or kidneys (44). On the other hand, *C. difficile* is a major human pathogen causing nosocomial  
379 infections, community-associated diarrhea and is responsible of 250,000 intestinal infections per  
380 year in the US, associated with a mortality rate of 15 to 20%. In 2019, *C. difficile* has been classified  
381 by the Centre for Diseases Control and Prevention as an urgent threat for which new antimicrobials  
382 are needed (45, 46). In the same line, RumC1 is active against amoxicillin and vancomycin resistant  
383 *E. faecium*, registered on all priority lists of human pathogens for which new antimicrobials are  
384 urgently needed. It is registered by the CDC as a serious concern that requires prompt action and  
385 by the WHO on the “antibiotic-resistant priority pathogens” as high concern. *E. faecium* is also  
386 pointed by the Infectious Diseases Society of America (IDSA) across the “ESKAPE pathogens”  
387 list of bacteria, which includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*  
388 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., (47) for  
389 its ability to escape the effects of the commonly used antibiotics through evolutionarily developed  
390 mechanisms of resistance (48). *E. faecium*, characterized by drug resistance mechanisms,  
391 commonly causes life-threatening nosocomial infections amongst critically ill and  
392 immunocompromised individuals. Consequently, it is important to note that RumC1 is particularly  
393 active against this pathogen with a MIC of 1,2  $\mu$ M, thus it could be considered as a potential  
394 therapeutic solution. In the case of *S. pneumoniae*, with a prevalence of 1.2 million of infections  
395 per year in the US, leading to its inclusion into the CDC and the WHO lists of priority pathogens,  
396 the use of RumC1 with a MIC of 300 nM may also be relevant. It should be noted that RumC1 is a  
397 broad-spectrum anti-Gram-positive molecule directed against resistant anaerobic and aerobic  
398 clinical pathogens, which is rare in the antibiotics market with a few exceptions such as  
399 vancomycin, thus enhancing its clinical potential. Moreover, we showed that RumC1 inhibits the

400 synthesis of macromolecules including DNA, RNA, proteins or peptidoglycan and the production  
401 of ATP. At this stage, since the main biosynthetic pathways are inhibited, it is conceivable that  
402 RumC1 applies its antimicrobial activity intracellularly through a non-specific mechanism, or  
403 conversely, targeting specifically ATP synthases and thus disrupting all necessary energy resources,  
404 such as bedaquiline, the only antibiotic currently on the market targeting ATP synthases but used  
405 only for the treatment of infections caused by Mycobacterium (49). Otherwise, we have previously  
406 identified that the phenotype induced by RumC1 treatment resembles the one induced by  
407 metronidazole in *C. perfringens* (16). However, we have shown here that metronidazole only  
408 inhibits DNA synthesis and, to a lesser extent, protein synthesis. Metronidazole is known to impact  
409 DNA synthesis and repair systems, and most likely inhibits the activity of the strictly ATP-  
410 dependent ribonucleotide reductase by modulating the redox state of cells, but its precise  
411 mechanism remains elusive (50). Here we suggest that the phenotypic similarities induced by  
412 RumC1 and metronidazole might arise from a DNA synthesis inhibition and possibly other  
413 common events, although they certainly do not share the same mechanism. Therefore, further in-  
414 depth investigations are needed to resolve the potentially unique intracellular mode of action of  
415 RumC1. Finally, we followed our preclinical studies by assessing the safety of RumC1 on gut  
416 tissues directly sourced from patients. RumC1 did not induce epithelial lesions at a dose about a  
417 hundred times higher than the effective antimicrobial dose. This observation could be expected as  
418 humans have been exposed to RumC1 through evolution as this peptide is produced by a symbiotic  
419 bacterium present in the gut microbiota of healthy adults. Overall, RumC1 encompass properties  
420 essential for a drug candidate to cure intestinal infections, especially since i) it can be administrated  
421 by oral route, ii) it is active in the intestinal epithelium environment, iii) RumC1 shows activity at  
422 therapeutic doses against clinical intestinal resistant pathogens, and finally iv) since it is safe for  
423 gut tissues. Only few AMPs meet all the conditions that are necessary for reaching the marketing  
424 step, i.e. respecting the conditions of stability, presenting activity under physiological conditions  
425 (including in the presence of eukaryotic cells), owning antimicrobial effect against clinical  
426 pathogens at very low doses including resistant or MDR, and finally retaining safety for human  
427 cells and tissues. It is interesting to note that RumC1 fulfils all these conditions, except one  
428 remaining bottleneck for the industrial scale use, which concerns the cost of production. RumC-  
429 like synthetic molecule development could be addressed to solve this concern and highlight the  
430 great potential of this original sactipeptide.

431

## 432 **Materials and Methods**

433 Detailed descriptions of materials and methods, including expression, purification of (<sup>13</sup>C, <sup>15</sup>N)-  
434 labelled mature RumC1, NMR studies, structure calculations, EPR and Mössbauer analyses of  
435 RumMc1, stability assays of RumC1 and MIC determinations are given in *SI Appendix*.

#### 437 **Supporting Information**

438 Supplementary figures Fig. S1 to Fig. S9 as well as Tables S1 and S2 are provided in *SI Appendix*.

#### 440 **Data Availability**

441 All data needed to evaluate the conclusions in the paper are present in the paper and the *SI Appendix*.  
442 All of the peak lists and the complete <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N backbone and side chain chemical shift  
443 assignments of RumC1 have been deposited into the Biological Magnetic Resonance Databank  
444 (<http://www.bmrb.wisc.edu>) under ascension code 50027. Coordinates of the twenty conformations  
445 of DDDD stereoisomer of RumC1 have been deposited into the PDB under ascension code 6T33.

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462  
463 **Author contributions:** CR, SC, KJ and CB performed the *in vitro* assays for RumC1, RumMc1  
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465 RumC1 structural analysis by RMN; CSM and VS performed the Mössbauer analyses on RumMc1  
466 and wrote the corresponding parts; ST and LLP performed the EPR analysis on RumMc1; SKJ and

467 YC performed nanoLC-MS/MS characterizations; BDLV, PS, EDP, MM and CN performed the  
468 surgical biopsy, the human explant treatment, the cytotoxicity assays and the microscopy  
469 analysis; HO, NV and LP were involved in biosynthetic pathways and ATP assays; OI performed  
470 the peptide chemical synthesis; TG, MF and ED were involved in study design; PP, JP, MA, FG,  
471 ML and VD conceptualized the study, designed experiments, interpreted the data and wrote the  
472 manuscript.

473

474 **Competing interests:** The authors declare that they have no competing interests.

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585 **Figure legends**

586 **Fig. 1. Sequence and three-dimensional structure of Ruminococcin C1.** (A) Sequence of  
587 RumC1 containing leader peptide (*italics*) and core peptide (RumC1-44), cysteine residues are  
588 underlined. (B) Backbone overlay of the 20 lowest target function value conformers for the DDDD  
589 stereoisomer of RumC1. (C) Cartoon backbone representation of the three-dimensional solution  
590 structure of RumC1 with the D stereochemistry at Ala12 ( $\alpha$ -S), Asn16 ( $\alpha$ -S), Arg34 ( $\alpha$ -S) and Lys42  
591 ( $\alpha$ -S). Cysteine sulfur to  $\alpha$ -carbon thioether cross-links are colored in orange and the position are  
592 indicated. (D) Electrostatic surface potential of RumC1, where blue indicates positive charge and  
593 red indicates negative charge. (E) Surface hydrophobicity of RumC1, where yellow represents  
594 hydrophobic residues and white represents hydrophilic residues.

595 **Fig. 2. Spectroscopic characterizations of holo-RumMc1.** (A) UV-vis spectrum of holo-  
596 RumMc1 in the absence (solid trace) or the presence of dithionite (dashed trace). (B) Mössbauer  
597 spectrum of holo-RumMc1 taken at  $T = 77$  K with the simulation (red solid line) representing the  
598 sum of the subcomponents **1**, **2** and **3** (black lines). **1** and **2** are simulated in a ratio of 2:1 and  
599 represent two diamagnetic  $[4\text{Fe}4\text{S}]^{2+}$  clusters. One  $[4\text{Fe}-4\text{S}]^{2+}$  cluster is supposed to bind SAM. **3**  
600 was assigned to  $\text{Fe}^{3+}$  ions present in a  $[3\text{Fe}-4\text{S}]^{1+}$  cluster in a  $S = 1/2$  state. The respective Mössbauer  
601 parameters obtained from the simulation of the spectra at 77 K are summarized below the spectrum.  
602 (C) X-band CW EPR spectra of dithionite-reduced holo-RumMc1. The black line represents the  
603 experimental spectrum, while the red trace is the simulated spectrum by using three components A,  
604 B and C. (D) X-band CW EPR spectra of dithionite-reduced holo-RumMc1 in the presence of SAM.  
605 The black trace represents the experimental spectrum, while the red trace is the simulated spectrum  
606 by using four components A, B, C and A'. (E) g-values of components A, B, C and A' used to  
607 simulate the experimental EPR spectra.

608 **Fig. 3. Stability of RumC1.** (A) RumC1 was exposed to a range of pH for 1 hour, (B) to high  
609 temperatures up to 1 hour, (C) to human serum up to 24 hours, before measuring its MIC. (D) MIC  
610 of RumC1 was determined in MH medium supplemented with NaCl or  $\text{MgCl}_2$ . (A to D) All MICs  
611 were determined against *C. perfringens*. Residual antimicrobial activity was calculated based on  
612 the MIC of untreated RumC1. (E) Stability of RumC1 in conditions mimicking the GI tract. MIC  
613 of RumC1 against *C. perfringens* was determined after exposure to digestive enzymes. Nisin was  
614 used as a positive control of pancreatin activity. Residual antimicrobial activity was calculated  
615 based on the MIC of untreated bacteriocins. <sup>a</sup> Residual antimicrobial activity were measured in this  
616 study. <sup>b</sup> Published by Jarvis and Mahoney, 1969.

617 **Fig. 4. Antimicrobial activity of RumC1.** (A) Activity assays of RumC1 on *Bacillus cereus* grown  
618 in eukaryotic cell culture medium in the absence or the presence of a simulated intestinal

619 epithelium. Small and colic intestine compartments were simulated by Caco2 and T84 culture cells  
620 monolayer respectively, whereas RumC1 was added before (30 min), concomitantly and after  
621 infection (30 min) with  $5 \times 10^5$  CFU/mL *B. cereus* culture. **(B)** Activity spectrum of RumC1 against  
622 laboratory and clinical Gram-positive pathogens. Collection strains and clinical isolates are  
623 indicated. The table includes MIC of conventional antibiotics commonly used for clinical treatment  
624 and considered here as references (i.e. metronidazole, vancomycin and amoxicillin). Acquired  
625 resistance to antibiotics were determined following the EUCAST 2019 clinical breakpoint tables  
626 and are indicated by "R", whereas "i.r." refers to "intrinsic resistance", and \* to the laboratory  
627 isolate references.

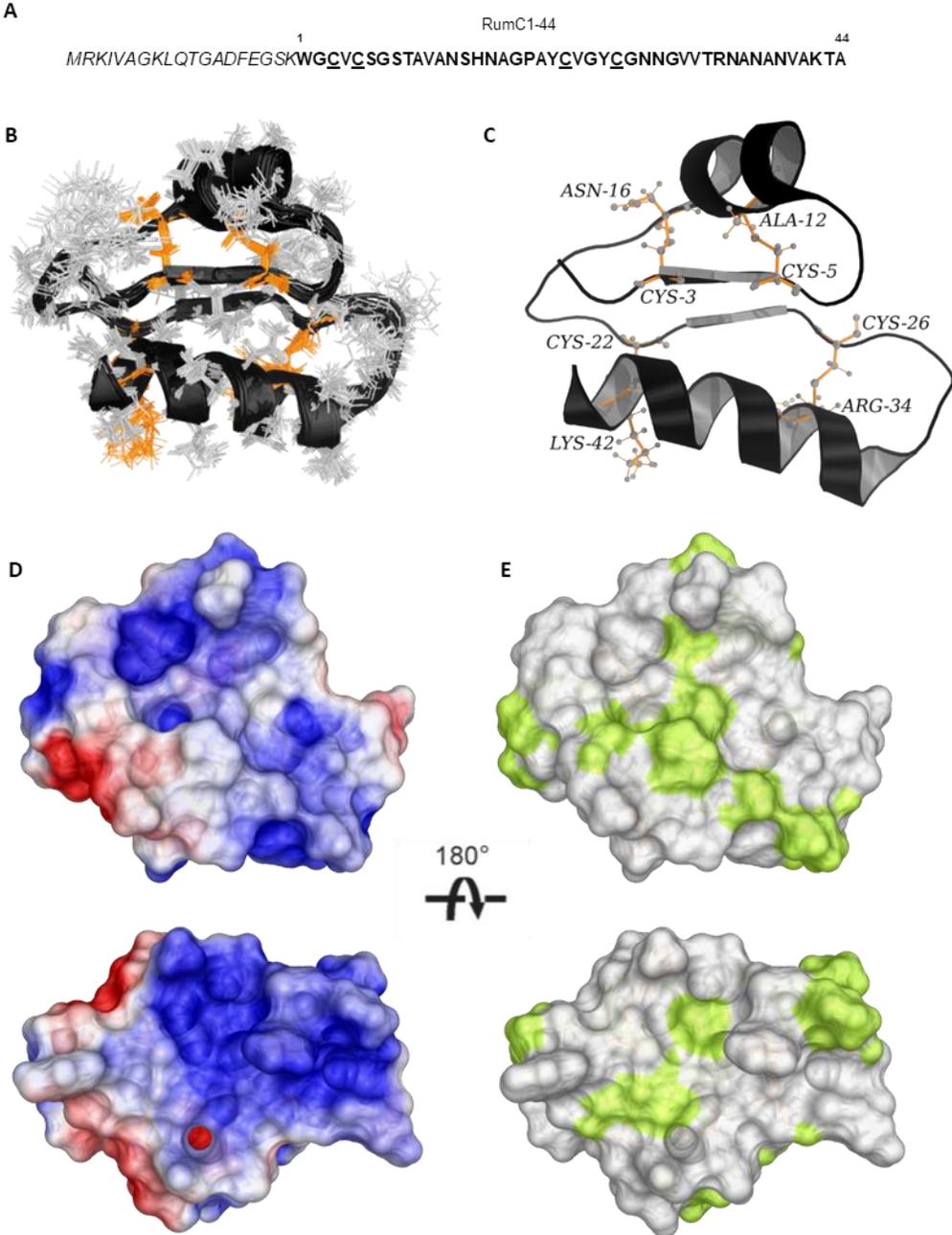
628 **Fig. 5. Antibacterial mode of action of RumC1. (A, B)** Cells of *C. perfringens* in early log phase  
629 were either treated with RumC1, control antibiotics, metronidazole, nisin or left untreated. Each  
630 experiment was done in independent triplicates. **(A)** After 15 min of treatment at 5xMIC, *C.*  
631 *perfringens* cells were incubated with radio-labelled precursors of DNA, RNA, proteins and  
632 peptidoglycan for 45 min. The synthesis of each pathway was measured by radioactivity counts.  
633 Gemifloxacin, rifampicin, tetracycline and vancomycin were used as controls antibiotics for the  
634 inhibition of DNA, RNA, proteins and peptidoglycan, respectively. Radio-labelled precursor  
635 incorporation is expressed as a percentage of maximum incorporation determined with the untreated  
636 condition. **(B)** After 15 min of treatment, the ATP present in the extracellular media (outer ATP)  
637 was measured by bioluminescence, then cells were lysed and the ATP content in the extracellular  
638 media was measured again. The inner ATP content was deduced from the difference between ATP  
639 content in the extracellular media before and after cell lysis. Cells were treated with 2.5x, 5x and  
640 10xMIC for each condition. Relative Light Units (RLU) are expressed as percentages normalized  
641 by the value of the inner ATP content of untreated cells.

642 **Fig. 6. Histological analysis of human intestinal tissue treated with RumC1.** Human intestinal  
643 explants were left untreated or treated with RumC1 (100  $\mu$ M) or CTAB (300  $\mu$ M) for 4 h before  
644 H&E staining and microscopic observations as described in the Materials and Methods section.  
645 Images are representative of overall observed effects. Scale bar = 50  $\mu$ m.

646

647 Figure 1

648

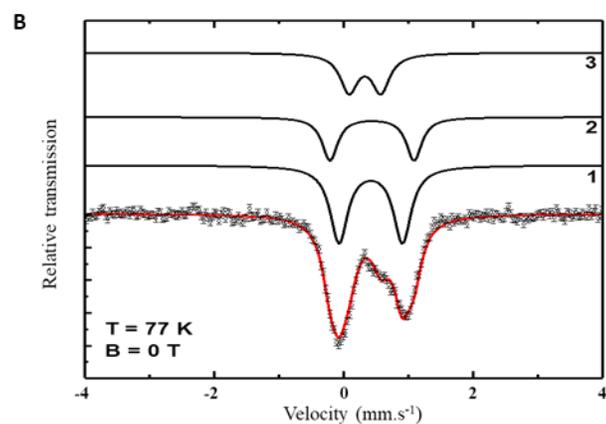
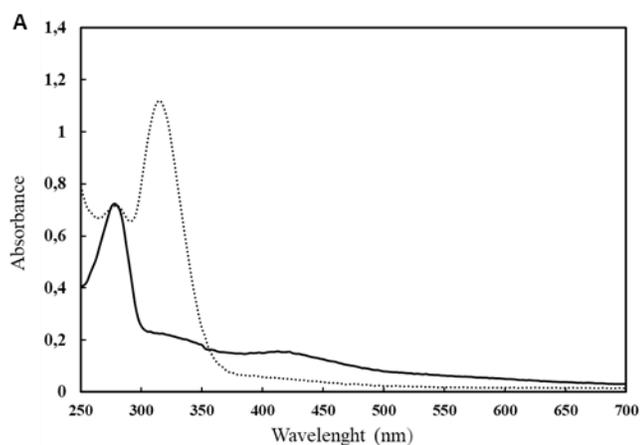


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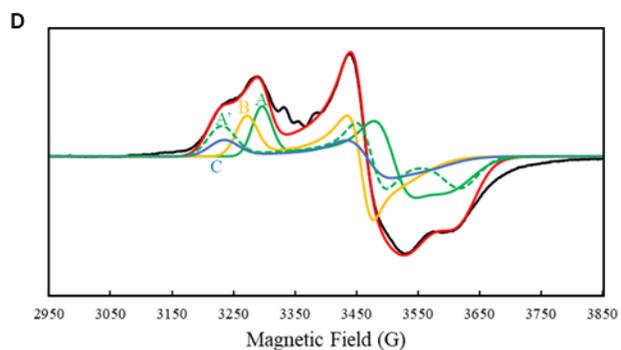
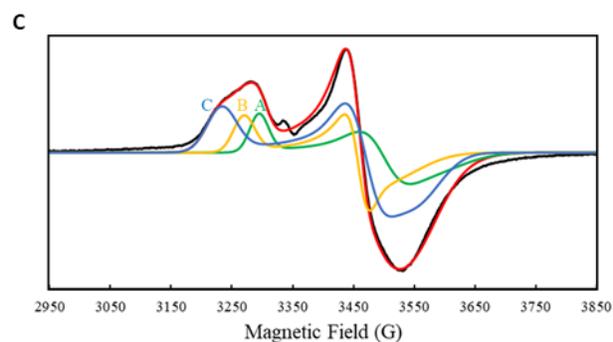
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651 Figure 2

652



	1	2	3
$\delta$ (mms <sup>-1</sup> )	0.42 ( $\pm$ 0.01)	0.44 ( $\pm$ 0.01)	0.33 ( $\pm$ 0.02)
$\Delta E_q$ (mms <sup>-1</sup> )	0.98 ( $\pm$ 0.02)	1.30 ( $\pm$ 0.02)	0.49 ( $\pm$ 0.02)
$\Gamma$ (mms <sup>-1</sup> )	0.33 ( $\pm$ 0.01)	0.29 ( $\pm$ 0.01)	0.33 ( $\pm$ 0.02)
Area (%)	50 ( $\pm$ 1)	25 ( $\pm$ 1)	25 ( $\pm$ 1)



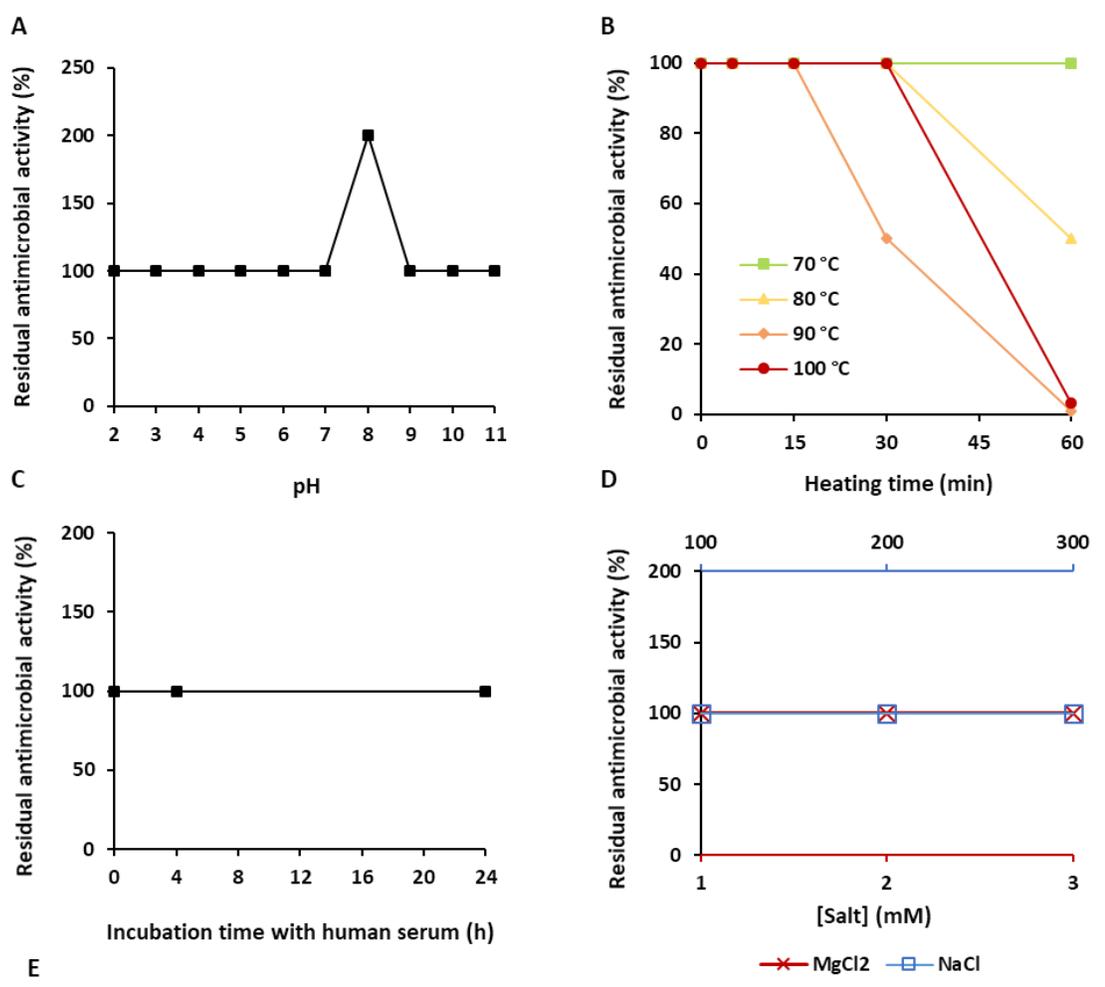
Proteins	SAM	A		B		C		A'	
		$g_x$	$g_y$	$g_x$	$g_y$	$g_x$	$g_y$	$g_x$	$g_y$
RumMc1	-	2,034(2)	1,913(2)	2,050(2)	1,937(2)	2,074(2)	1,932(2)		
			1,870(4)		1,902(4)		1,885(4)		
RumMc1	+	2,034(2)	1,910(2)	2,049(2)	1,937(2)	2,073(2)	1,932(2)	2,076(2)	1,930(2)
			1,860(4)		1,904(4)		1,885(4)		1,852(3)

653

654

655 Figure 3

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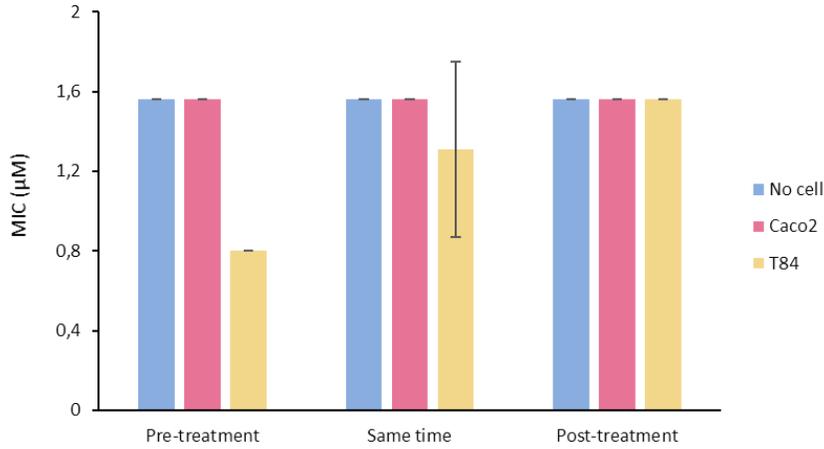


Simulated digestive compartment	Enzymes	pH	Temperature (°C)	Time (h)	Residual antimicrobial activity (%)	
					RumC1	Nisin
Stomach	Pepsin	2.5	37	2	100 <sup>a</sup>	100 <sup>b</sup>
Intestin	Pancreatin	6.5	37	5	100 <sup>a</sup>	12.5 <sup>a</sup>

657

658

A

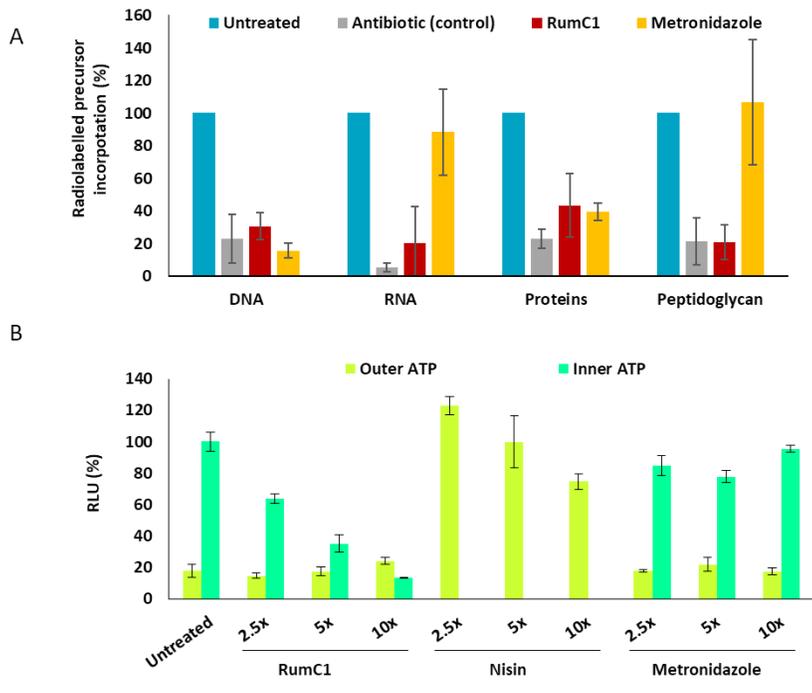


B

Organism	Source	Clinical isolate	Strain number	RumC1 MIC (µM)	Conventional antibiotics MIC (µM)		
					Metronidazole	Vancomycin	Amoxicillin
<i>Clostridium perfringens</i>	Laboratory	/	ATCC 13124	<b>0.8</b>	12	0.2	2.2
	Broilers	"	CP24*	<b>0.4</b>	12	0.2	2.2
	"	"	CP56*	<b>0.8</b>	12	0.2	2.2
	"	"	CP60*	<b>0.4</b>	12	0.2	2.2
	Human	Haemoculture	1*	<b>0.6</b>	23	0.4	≤ 0.3
	"	"	2*	<b>0.6</b>	23	0.4	≤ 0.3
	"	"	1600366478*	<b>0.6</b>	12	0.2	0.3
	"	"	1500022151*	<b>0.6</b>	12	0.2	0.7
<i>Clostridium difficile</i>	Laboratory	/	ATCC 700057	<b>0.6</b>	1.5	0.7	i.r.
	Human	Sacrum tissue	1600597867*	<b>0.3</b>	1.5	0.3	i.r.
<i>Listeria monocytogenes</i>	Human	Haemoculture	1*	<b>0.8</b>	i.r.	0.7	≤ 0.3
<i>Bacillus cereus</i>	Laboratory	/	CIP 5257	<b>1.6</b>	i.r.	0.7	i.r.
	Human	Haemoculture	1900276321*	<b>1.2</b>	i.r.	R	11
<i>Streptococcus pneumoniae</i>	Laboratory	/	ATCC 49619	<b>0.6</b>	i.r.	≤ 0.1	0.2
	Human	Haemoculture	1900304262*	<b>0.3</b>	i.r.	≤ 0.1	0.2
<i>Enterococcus faecalis</i>	Laboratory	/	JH2-2	<b>2.5</b>	i.r.	0.7	0.7
	Human	Haemoculture	1900302094*	<b>2.5</b>	i.r.	1.4	0.7
<i>Enterococcus faecium</i>	Laboratory	/	BM4147	<b>1.2</b>	i.r.	R	R
	Human	Haemoculture	1900300992*	<b>1.2</b>	i.r.	0.7	11

663 Figure 5

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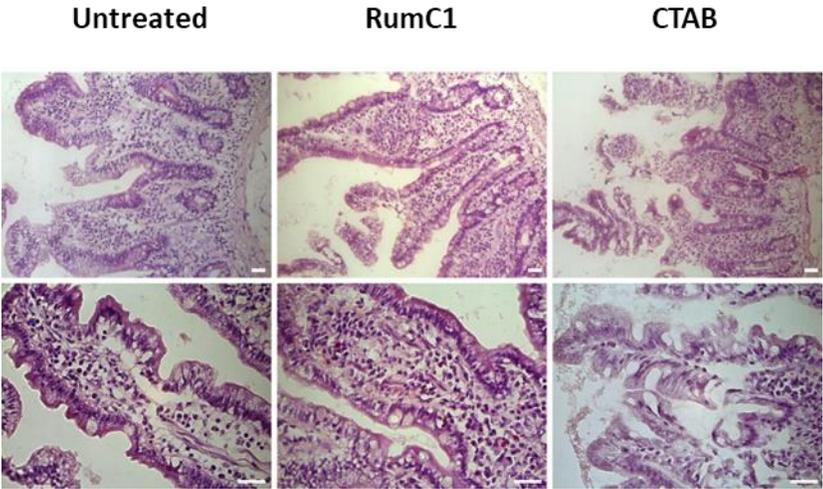


665

666

667 Figure 6

668



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671

672 **Supporting Information for**

673  
674  
675 **The unusual structure of Ruminococcin C1 antimicrobial peptide confers clinical properties**

676  
677 Clarisse Roblin<sup>†</sup>, Steve Chiumento<sup>†</sup>, Olivier Bornet\*, Matthieu Nouailler, Christina S. Müller, Katy  
678 Jeannot, Christian Basset, Sylvie Kieffer-Jaquinod, Yohann Couté, Stéphane Torelli, Laurent Le  
679 Pape, Volker Schünemann, Hamza Olleik, Bruno De La Villeon, Philippe Sockeel, Eric Di  
680 Pasquale, Cendrine Nicoletti, Nicolas Vidal, Leonora Poljak, Olga Iranzo, Thierry Giardina, Michel  
681 Fons, Estelle Devillard, Patrice Polard, Marc Maresca, Josette Perrier, Mohamed Atta, Françoise  
682 Guerlesquin, Mickael Lafond\*, Victor Duarte\*

683  
684 \* Correspondence to: bornet@imm.cnrs.fr; mickael.lafond@univ-amu.fr; victor.duarte@cea.fr

685 <sup>†</sup> The following authors contributed equally to this work: Clarisse Roblin & Steve Chiumento

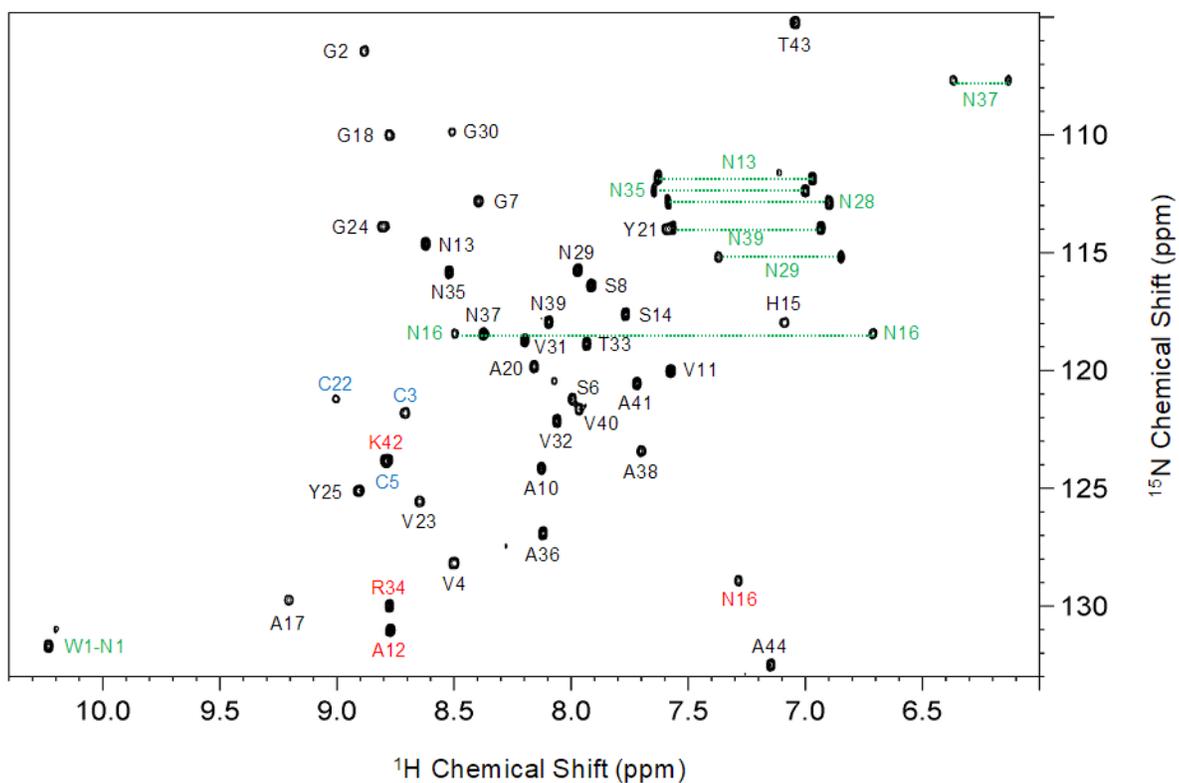
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687 **This PDF file includes:**

688       Figures S1 to S9

689       Tables S1 and S2

690       SI Materials and Methods

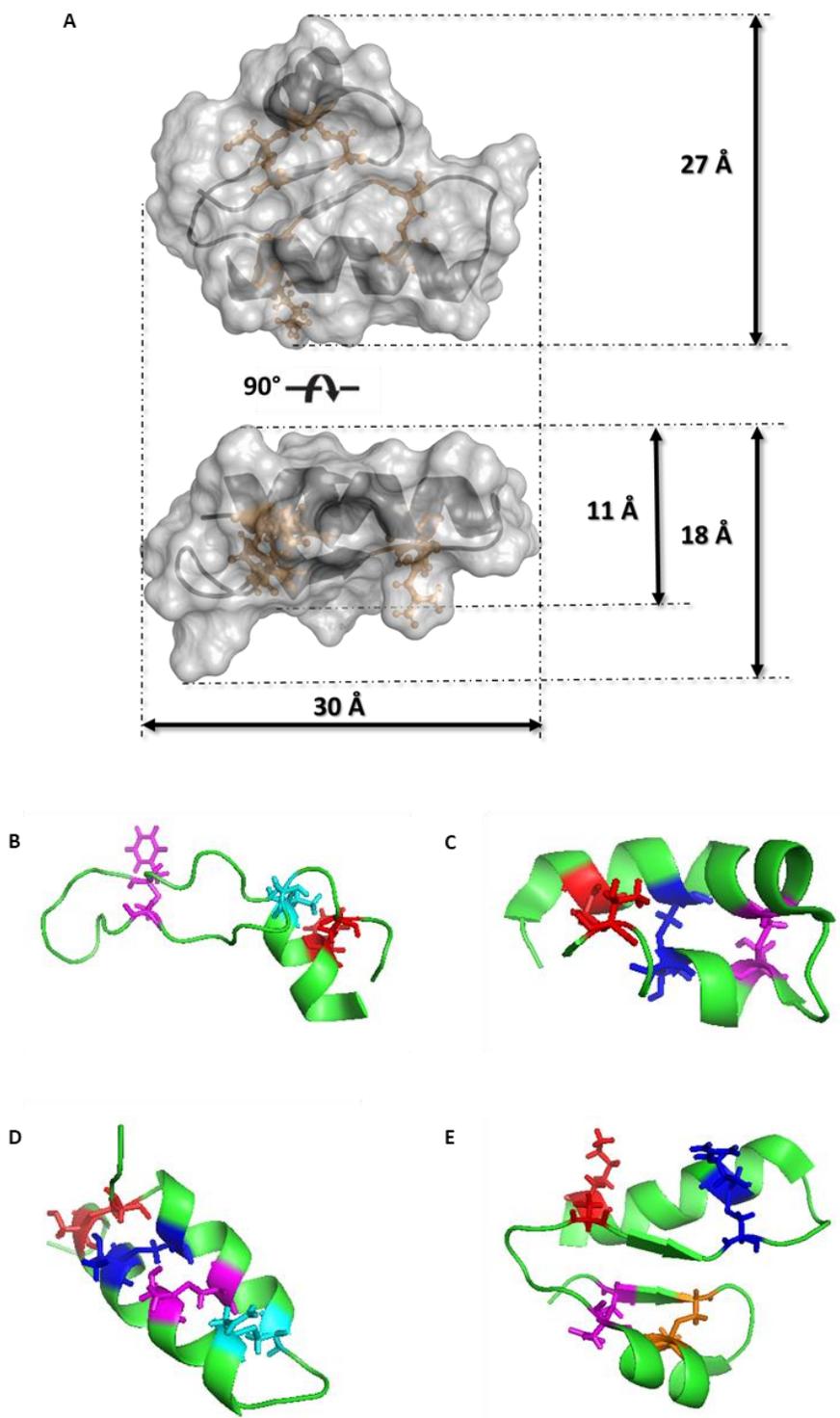
691       SI References



**Fig. S1. Two-dimensional [ $^1\text{H}$ ,  $^{15}\text{N}$ ] 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  $^{13}\text{C}$  and  $^{15}\text{N}$ -isotopically enriched RumC1 in 10 mM phosphate buffer, pH 6.8 in 90%  $\text{H}_2\text{O}$ /10%  $\text{D}_2\text{O}$  at 27°C.

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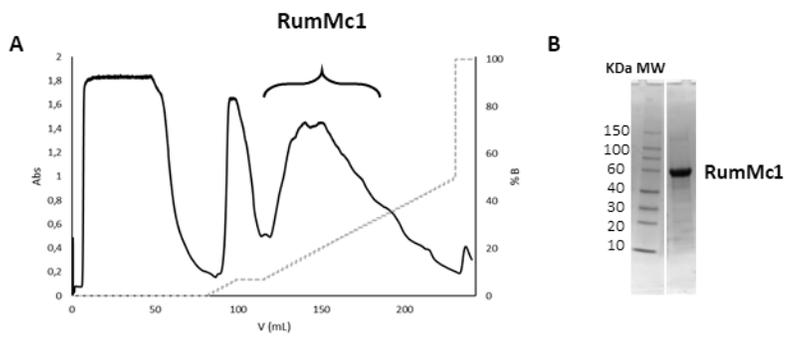
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**Fig. S2. Dimensions of the NMR structure of RumC1. Comparison of sactipeptides structures. (A)** Dimensions of the NMR structure of the DDDD stereoisomer of RumC1. NMR Structures of: **(B)** Subtilosin A, **(C)** Thuricin CD, **(D)** Thurincin H and **(E)** RumC1. Stereoisomers at the  $\alpha$ -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.

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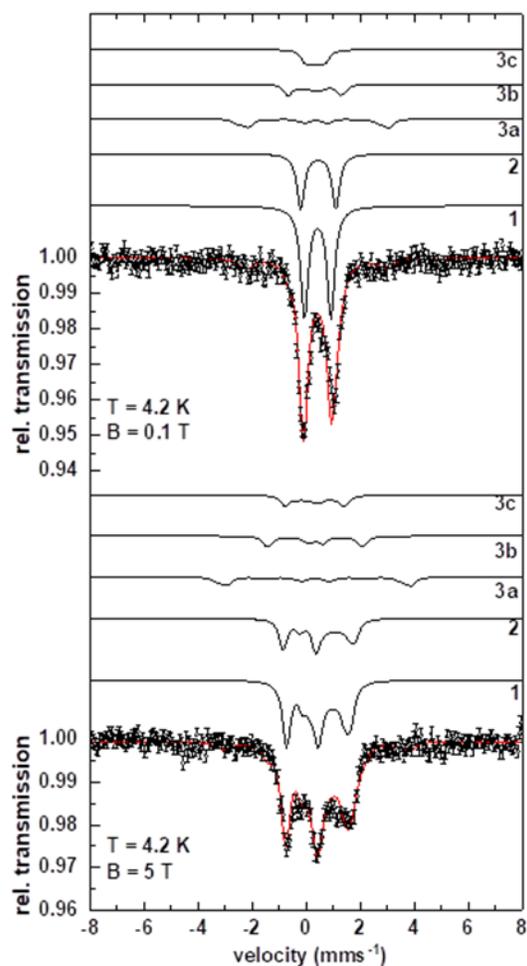


**Fig. S3. Anaerobic purification of RumMc1.** (A) FPLC chromatogram of overexpressed RumMc1 on a nickel-charge IMAC column. %B is the % of a 50 mM HEPES, pH 7,5, 300 mM NaCl, 500 mM imidazole buffer solution. (B) SDS-PAGE analysis of purified RumMc1

697

698

A



B

	1	2	3a	3b	3c
$\bar{\delta}$ (mm/s)	0.42 ( $\pm 0.02$ )	0.44 ( $\pm 0.02$ )	0.33 ( $\pm 0.03$ )	0.33 ( $\pm 0.03$ )	0.33 ( $\pm 0.03$ )
$\Delta E_Q$ (mm/s)	0.98 ( $\pm 0.02$ )	1.30 ( $\pm 0.02$ )	0.49 ( $\pm 0.03$ )	0.49 ( $\pm 0.03$ )	0.49 ( $\pm 0.03$ )
$\Gamma$ (mm/s)	0.35 ( $\pm 0.02$ )	0.33 ( $\pm 0.02$ )	0.33 ( $\pm 0.03$ )	0.33 ( $\pm 0.03$ )	0.33 ( $\pm 0.03$ )
$\eta$	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) <sup>1</sup>	(11/11/11) <sup>1</sup>	(2.5/2.5/2.5) <sup>1</sup>
Area (%)	50	25	8.33	8.33	8.33

699

700 **Fig. S4. Field dependent spectra of RumMc1.** (A) Mössbauer spectra of RumMc1 with the simulation (red solid line) representing  
701 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  
702 diamagnetic  $[4\text{Fe}4\text{S}]^{2+}$  clusters. Component 3 is divided into three subcomponents 3a, 3b and 3c that represent three  $\text{Fe}^{3+}$  high  
703 spin ions antiferromagnetically coupled to a total spin of  $S = 1/2$  as present in a  $[3\text{Fe}4\text{S}]^{1+}$  cluster. (B) Mössbauer parameters  
704 obtained from the simulation of the field dependent spectra of RumMc1. <sup>1</sup> Values for  $A_{xyz}/\mu_N g_N$  were taken from B. H. Huynh *et al.*  
705 (1).  
706

AlbA	FPMP LHATFELTHRCNLKCAHCYLESSPEALGTVSIEQ-----FKKTADMLFDN--GVL	167
anSMe	MPPLSLLIKPASSGCNLKCTYCFYHLSLSDNRNVKSYGIMRDEVLES MVKRVLNEANGHCS	60
RumMc1	RYDLQQVILELTEQCNRRCRYCIYNEHNEG YRNFSPKAMTWDVAKRAVEYARDNSGDKVA	169
	: **::* :* .. : *	: .. :: . :
AlbA	---NWVDDFGRGRDIVHPTKDAEQHRKFMEYEQHVIDEFKDLIPII-----P-YERKRAANCGA	346
anSMe	LFDFWYEDFLNGNRV-----SIRYFDGLL---ETILLGKSS-----SC---GMNGTCT-	263
RumMc1	AIEGWALARDLEED-----PKSYVAGIV---ADKLVRIHNRRQTQEPCKDLRRNGCCIP	395
	* . * :: :	. *
AlbA	GWKSIVISPFGEVRF CALFP-KEFSLGNI FHDSYESIFNSPLVHKLWQAQAPRFSEHCMKDKCPFSG	412
anSMe	--CQFVVESDGSVYPCDFYVLDKWRLGNIQDMTMKELFETNKNHEFIKL---SFKVHEE CKKCKWFR	324
RumMc1	GNRRVYVKTDGKFLLEKGTG-DAPDIGNVFEGADLEKIKKYIEEY-----DEKSITRCNECWARN	455
	. :: *.. * . : ** : . : . : . : . :	. : **
AlbA	YCGGCYLKGLNSNKY-----HRKNI CSWAKNEQL-----EDVVQLI-----	448
anSMe	---LCKGGCRRCRDSKEDSALELNYYCQSYK-----EFFEY-----AFPRLINVANNIK--	370
RumMc1	LCGLCYAACYEAE GIDME---RKEKVCGAHRYATK GELISYYSILEEKPEVIEEIDAVPYY	513
	* ... . : * :	:::

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

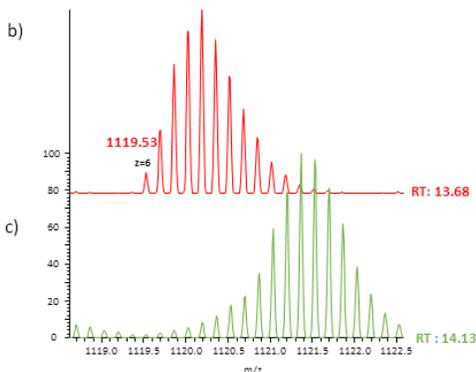
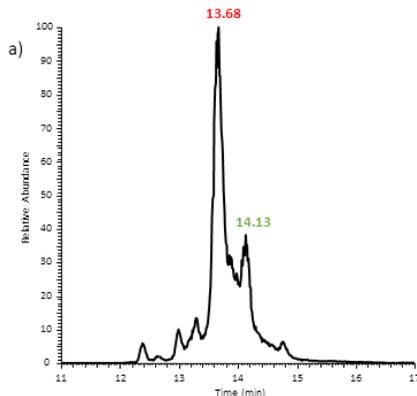
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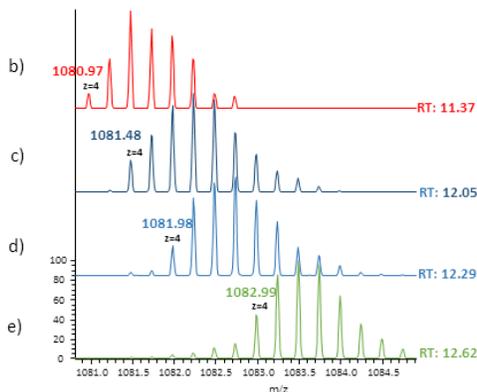
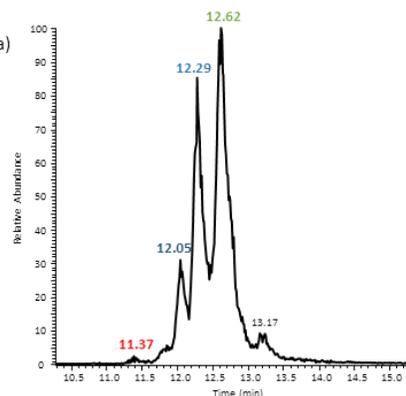
A

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

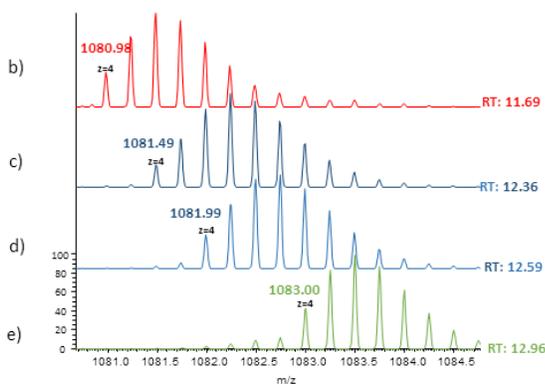
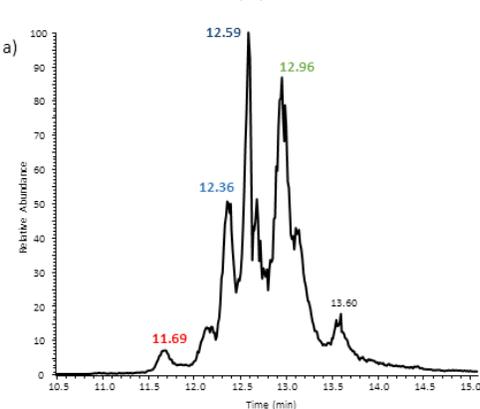
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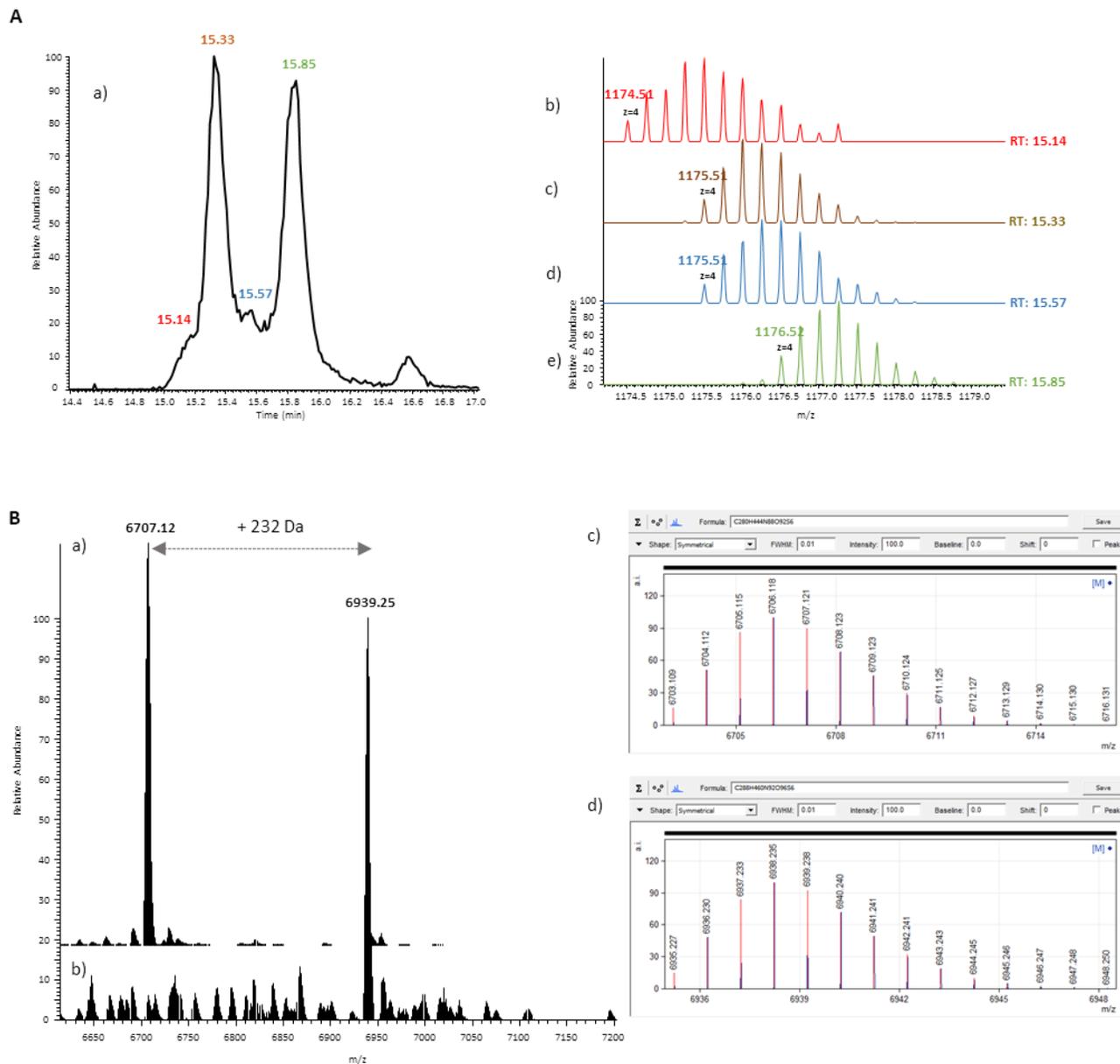
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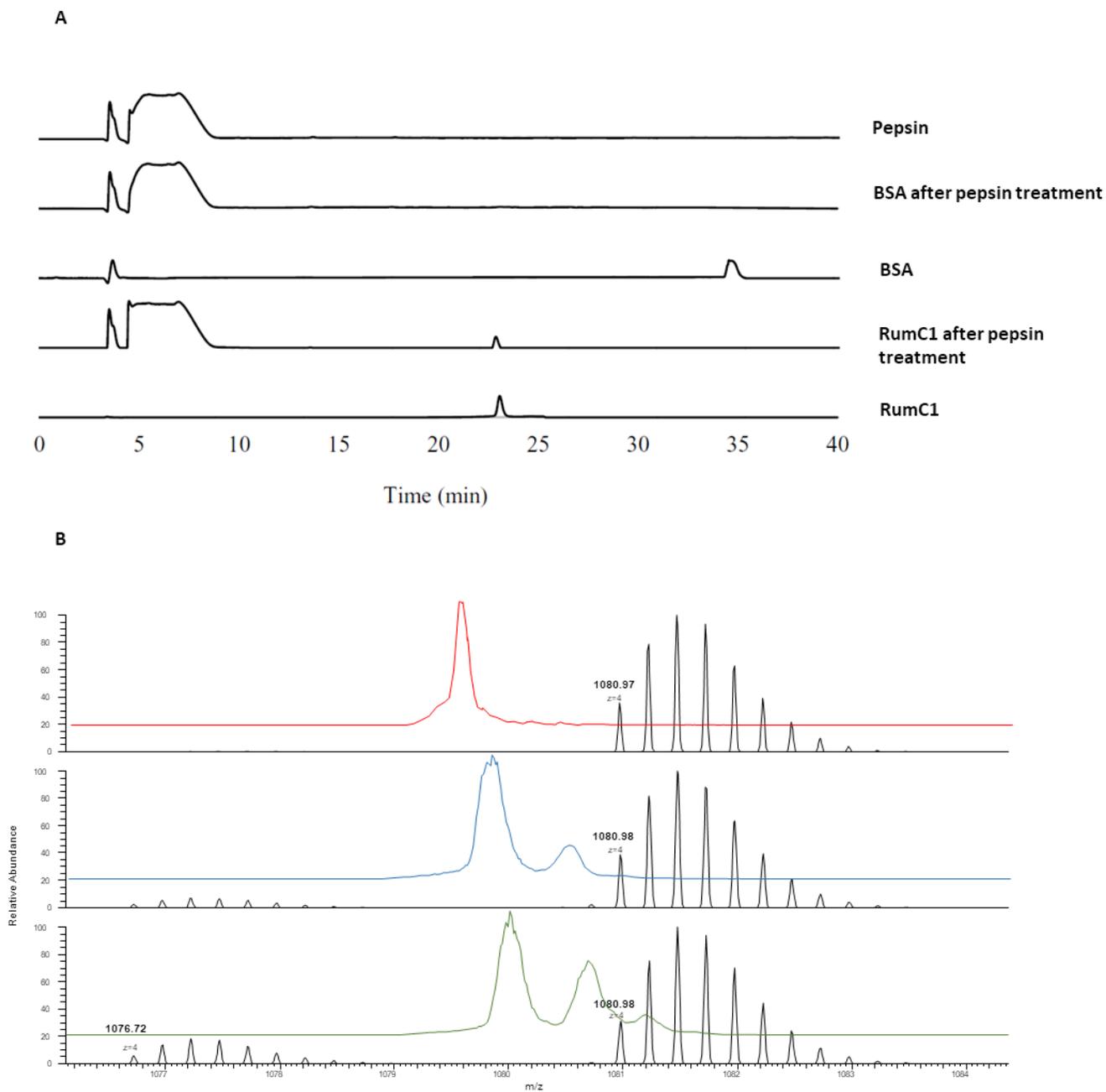
D



**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 matured form containing 4 thioether bonds. (c) A mixture of unmaturation 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 matured 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 matured 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 matured 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 matured form of RumC1-44-LS.



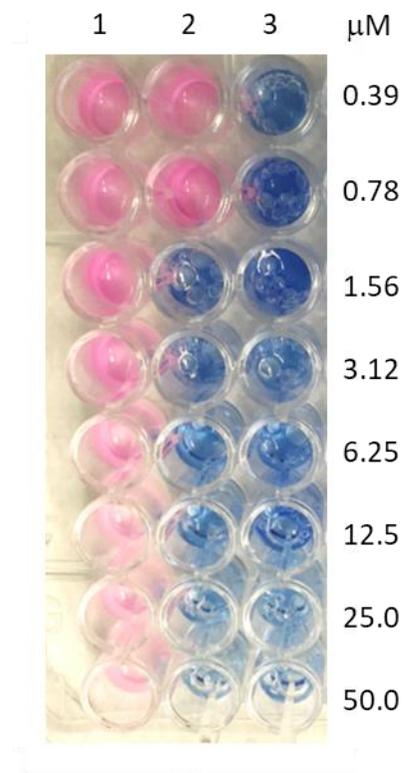
**Fig. S7. Analysis of *in vivo* maturation of RumC1-44 and RumC1-Ala18/Ala19 variant. (A)** LC-MS analysis of *in vivo* matured RumC1-44. (a) LC-MS trace of *in vivo* matured RumC1-44. Zoom-in spectra at RT = 15.14 (b), RT = 15.33 (c), RT = 15.57 (d) and RT = 15.85 (e) for m/z corresponding to RumC1-44. (b) A major ion  $[M+4H]^{4+} = 1174.5$  Da corresponds to the fully matured 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 matured form of RumC1-44. **(B)** MS Analysis of *in vivo* matured 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 (-NH<sub>3</sub>).

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**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.

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717 **Table S1. Comparison of statistics generated by the 16 stereoisomers of RumC1.** Assigned  
718 NOEs represent total number of off-diagonal NOE assignments used by CYANA to perform the  
719 structure calculation.  
720

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

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725**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	

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## SI Materials and Methods

**Heterologous expression and purification of (<sup>13</sup>C, <sup>15</sup>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<sub>4</sub> (1 mM), FeCl<sub>3</sub> (50 µM) and glucose (4 mg/mL) at 37 °C. At an optical density (OD<sub>600</sub>) 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<sub>2</sub>HPO<sub>4</sub> 6 g/L, KH<sub>2</sub>PO<sub>4</sub> 3 g/L, <sup>15</sup>NH<sub>4</sub>Cl 1 g/L) containing kan (50 µg/mL), amp (100 µg/mL), chl (34 µg/mL), vitamin B1 (0.5 µg/mL), MgSO<sub>4</sub> (1 mM), FeCl<sub>3</sub> (50 µM) and labeled glucose-<sup>13</sup>C (4 mg/mL). The culture was grown at 25°C to an optical density (OD<sub>600</sub>) of 0.8. FeCl<sub>3</sub> (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). (<sup>13</sup>C, <sup>15</sup>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 RumC1 was 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 <sup>13</sup>C-<sup>15</sup>N-labeled RumC1 was approximately 0.2 mM in 10 mM phosphate buffer, 90% H<sub>2</sub>O/10% D<sub>2</sub>O 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: [<sup>15</sup>N,<sup>1</sup>H] HSQC and [<sup>13</sup>C,<sup>1</sup>H] HSQC; 3D: [<sup>1</sup>H,<sup>15</sup>N,<sup>13</sup>C] HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO, HN(CA)CO, <sup>13</sup>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 <sup>13</sup>C-TOCSY-HSQC. <sup>1</sup>H assignments for aromatic side chains and asparagines side chain amides were made using 2D [<sup>1</sup>H, <sup>1</sup>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  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  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 [ $^1\text{H}$ ,  $^1\text{H}$ ] NOESY was acquired with a mixing time of 150 ms, using a 2 mM unlabeled RumC1 sample in 10 mM phosphate buffer, 90%  $\text{H}_2\text{O}$ / 10%  $\text{D}_2\text{O}$ , pH 6.8 at  $27^\circ\text{C}$ , 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 [ $^1\text{H}$ ,  $^1\text{H}$ ] NOESY, 3D [ $^1\text{H}$ ,  $^{15}\text{N}$ ,  $^1\text{H}$ ] NOESY, 3D [ $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^1\text{H}$ ] 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  $\mu\text{g}/\text{mL}$ ), amp (100  $\mu\text{g}/\text{mL}$ ) was inoculated with a single colony of BL21 (DE3) cells carrying pET-28a-*rumMc1* and pDB1282. The 100 mL culture was grown overnight at  $37^\circ\text{C}$ , 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  $\text{MgSO}_4$ , 50  $\mu\text{g}/\text{mL}$  kan, 100  $\mu\text{g}/\text{mL}$  amp) and 50  $\mu\text{M}$   $\text{FeCl}_3$ . The fermenter culture was grown at  $37^\circ\text{C}$ , 200 rpm to an  $\text{OD}_{600\text{ nm}} \sim 0.3\text{-}04$  and then supplemented with a final concentration of 50  $\mu\text{M}$   $\text{FeCl}_3$ , 300  $\mu\text{M}$  L-Cys, and 13.3 mM L-arabinose. At an ( $\text{OD}_{600}$ ) of 1.2, the culture was cooled at  $24^\circ\text{C}$ , 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  $4^\circ\text{C}$ ).

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

812  
813 **Site directed mutagenesis of *rumC1* and heterologous expression of RumC1 variant.** Site  
814 directed mutagenesis of the *MBP-rumC1* construct was performed to produce the Ala18/Ala19  
815 variant of RumC1. Mutagenesis was done by following the instructions from the Q5 Site-Directed  
816 Mutagenesis Kit (New England BioLabs®). The NEBaseChanger tool was used to generate the 2  
817 primer sequences (5'-CCATAACGCGGCTGCGGCGTACTGCG, 5'-  
818 CTGTTTCGCAACCGCGGTG) and annealing temperatures. Template plasmids were digested  
819 using DpnI and were transformed into competent Top10 cells. The mutant plasmids were recovered  
820 from cells by using the Wizard® Plus SV Minipreps DNA Purification System (Promega).  
821 Production and purification of RumC1-Ala18/Ala19 variant were performed as previously  
822 described for RumC1 (2).

## 824 **Leader-peptide chemical synthesis**

### 825 *Chemicals*

826 The N-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, rink amide MBHA resin (100 -  
827 200 mesh) and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate  
828 (HBTU) were obtained from Novabiochem; N,N-diisopropylethylamine (DIEA), trifluoroacetic  
829 acid (TFA), anisole, thioanisole, 1,2-ethanedithiol, acetic anhydride, piperidine and triethylamine  
830 (TEA) were from Sigma-Aldrich. All the other chemicals and solvents (N,N-dimethylformamide

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

#### 834 *Peptide synthesis*

835 The leader sequence of RumC1 peptide (H<sub>2</sub>N-MRKIVAGKLQTGADFEGSK-NH<sub>2</sub>) was prepared  
836 by solid phase peptide synthesis in an Initiator<sup>+</sup> Alstra automated microwave assisted synthesizer  
837 (Biotage). The peptide was assembled on a rink amide MBHA resin (0.25 mmol scale, 0.59 mmol/g)  
838 using standard Fmoc methodologies (9). Namely, the amino acids (4 equiv) were coupled using  
839 HBTU (3.9 equiv) as coupling agent, DIEA as base (8 equiv) and DMF as solvent. The removal of  
840 the Fmoc protecting groups was always done by treating the resin with 20% piperidine in DMF  
841 solution. After assembling, the peptide was manually deprotected and cleaved from the resin by  
842 treatment with the mixture TFA/thioanisole/anisole/1,2-ethanedithiol (% v/v = 90:5:3:2) for 2 h at  
843 room temperature and under nitrogen. The resin was filtered out and rinsed with TFA. The filtrate  
844 and rinses were combined, reduced under a nitrogen stream and slowly added to cold diethyl ether  
845 with magnetic stirring to precipitate the crude peptide. The suspension was transferred to a  
846 centrifuge to recover the peptide which was washed with cold diethyl ether and centrifuged again.  
847 This step was repeated several times and finally the crude peptide was dissolved in the minimum  
848 amount of water and lyophilized. The crude peptide was purified by preparative reversed-phase  
849 HPLC in a Phenomenex Jupiter column (250 mm × 21.20 mm, 15 μm, 300 Å) using solvent A  
850 (99.9% water/0.1 % TFA) and solvent B (90% ACN/9.9% water/0.1 % TFA). The leader sequence  
851 of RumC1 peptide was eluted with a linear gradient from 15% to 35% B in 30 min at a flow rate of  
852 10 mL/min (R<sub>t</sub> = 14 min). Its purity was checked by analytical reversed-phase HPLC (Phenomenex  
853 Jupiter column, 250 mm × 4.6 mm, 15 μm, 300 Å) and it was greater than 95%. The peptide was  
854 characterized by Electrospray Ionization-Mass spectrometry (ESI-MS) in positive mode using a  
855 Waters Synapt G2 HDMS (Manchester, UK) equipped with an ESI source employing the following  
856 parameters: ESI capillary voltage: +2.8 kV; extraction cone voltage: +20 V; desolvation gas (N<sub>2</sub>)  
857 flow: 100 L.h<sup>-1</sup>; source temperature: 35 °C. Sample solutions were introduced in the ionization  
858 source at a 10 μL.min<sup>-1</sup> flow rate using a syringe pump.

859

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

865

866 **EPR and Mössbauer spectroscopies.** EPR and Mössbauer samples (400  $\mu\text{M}$ ) were prepared and  
867 flash frozen under anaerobic conditions. When needed, cluster reduction was achieved in 1 hour by  
868 addition of 10 mM dithionite. Samples in the presence of SAM were prepared with 3 mM SAM.  
869 EPR spectra were recorded on a Bruker EMX spectrometer operating at X-band frequency equipped  
870 with an Oxford instrument ESR 900 flow cryostat. Spectra were recorded with a microwave  
871 frequency 9.65 GHz under saturated (10K, 39dB, modulation amplitude 10 G) and non-saturated  
872 (6K, 13dB, modulation amplitude 10 G) conditions for the  $g = 2$  region. Simulations were  
873 performed using Easy Spin toolbox for matLAB. Mössbauer spectra were recorded in transmission  
874 mode with a conventional Mössbauer spectrometer which was operated in the constant acceleration  
875 mode in conjunction with a multi-channel analyzer in the time-scale mode (WissEl GmbH). The  
876 spectrometer was calibrated against  $\alpha$ -iron at room temperature. Experiments at 77 K were  
877 conducted using a flow cryostat (Optistat<sup>DN</sup>, Oxford Instruments). Field-dependent Mössbauer  
878 spectra were obtained with a helium closed-cycle cryostat (CRYO Industries of America, Inc.)  
879 equipped with a superconducting magnet (10). The magnetic field was aligned parallel to the  $\gamma$ -ray  
880 beam. The spectral data were transferred from the multi-channel analyzer to a PC for further  
881 analysis employing the public domain program Vinda (11) running on an Excel 2003<sup>®</sup> platform.  
882 Analysis of the spectra was performed by least-squares fits using Lorentzian line shapes with the  
883 linewidth  $\Gamma$ . Field-dependent spectra were simulated by means of the spin Hamilton formalism (12).

884

885 **Nano-LC–MS/MS Analyses.** RumC1 samples were generally injected at a concentration of 0.1  
886  $\mu\text{M}$ . Samples were diluted in 5% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and analysed  
887 by online nano-LC–MS/MS (NCS HPLC, Dionex, and Qexactive HF, Thermo Fisher Scientific).  
888 Peptides were sampled on a 300  $\mu\text{m} \times 5$  mm PepMap C18 precolumn and separated on a 75  $\mu\text{m} \times$   
889 250 mm C18 column (PepMap, Dionex). The nano-LC method consisted of a 40 min gradient at a  
890 flow rate of 300 nL/min, and MS and MS/MS data were acquired using Xcalibur (Thermo Fisher  
891 Scientific). In order to improve the quality of the MS/MS spectra we performed parallel reaction  
892 monitoring (PRM) experiments to characterize RumC1 species. According to our previous  
893 characterization of RumC1 (Chiumento et al., 2019), we decided to focus these analyses on the  
894 highest  $m/z$  ions for the long (with leader peptide) and short (without leader peptide) peptides  
895 (respectively  $m/z = 1120$  ( $6^+$ ) and  $m/z = 1083$  ( $4^+$ )). The  $m/z$  window was open at 4 units in order  
896 to consider modified species. The collision energy was set to 27.

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

900

901 **Stability assays.** Stability of RumC1 was evaluated after incubation varying pH, temperatures, and  
902 in human serum. RumC1 was incubated in phosphate-buffered saline (PBS) at pH range from 2 to  
903 11 at a volume ratio of 1:1 for 1 hour at room temperature. In a second assay RumC1 was incubated  
904 at temperatures of 70, 80, 90 and 100 °C for 5, 15, 30 or 60 minutes before being cooled on ice.  
905 Finally, RumC1 was incubated in human serum (Sigma Aldrich) at a volume ratio of 1:1 for 4 or  
906 24 hours at 37°C with stirring (180 rpm). After each of this treatment, Minimal Inhibitory  
907 Concentrations (MIC) were determined against *Clostridium perfringens* ATCC13124 in Brain  
908 Heart Infusion broth supplemented with yeast extract (5 g/L) and hemin (5 mg/L) (BHI-YH) as  
909 described (2). MIC of untreated RumC1 was used to set the maximum of antimicrobial activity and  
910 calculate the residual activity of each treated RumC1. Stability and activity of RumC1 was also  
911 measured in physiological and higher concentration of salts: MIC of RumC1 was determined as  
912 above but the BHI-YH medium was replaced with Mueller Hinton (MH) broth supplemented in  
913 either NaCl at 100, 200, and 300 mM or in MgCl<sub>2</sub> at 1, 2 and 3 mM. For the stability to salts assays,  
914 MIC of untreated RumC1 in MH broth was used as the maximum of antimicrobial activity.

915

916 **Simulated gastro-intestinal digestion of RumC1.** RumC1 was treated with pepsin (Sigma-  
917 Aldrich) with a molecular ratio of 1:2.5 (RumC1:pepsin) at 37°C in sodium acetate 50 mM pH 2.5  
918 for 2 hours with stirring (180 rpm) to mimic the digestive conditions occurring in the human  
919 stomach. To stop the enzymatic reaction of pepsin, NaHCO<sub>3</sub> 1M was added until pH 7 was reached.  
920 To simulate the intestinal compartment, RumC1 was incubated with pancreatin (Sigma-Aldrich)  
921 with a molecular ratio of 1:5 (RumC1:pancreatin) at 37°C in sodium acetate 50 mM pH 6.5 for 5  
922 hours with stirring (180 rpm). Then Phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) was  
923 added to a final concentration of 0.1 mM to inhibit the action of pancreatin. MIC of treated RumC1  
924 was determined against *C. perfringens* ATCC13124 in BHI-YH as described (2) after controlling  
925 that the enzymes in the above conditions without RumC1 showed no anti-*C. perfringens* activity.  
926 MIC of untreated RumC1 was used to set the maximum of antimicrobial activity and calculate the  
927 residual activity of each treated RumC1. Furthermore, RumC1 was detected by RP-C18-HPLC  
928 using an analytical column Jupiter 15-µm C18 300 Å (250 mm by 21.2 mm; Phenomenex). Elution  
929 was performed at 1 ml/min with a 0 to 40% linear gradient of 90% ACN and 0.1% TFA for 30 min.  
930 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<sup>2</sup> flasks maintained in a 5 % CO<sub>2</sub> incubator at 37 °C. To test the influence of human intestinal epithelial cells on RumC1 activity, cells grown on 25 cm<sup>2</sup> 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<sup>4</sup> 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<sup>5</sup> 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<sub>2</sub>. 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](http://www.atcc.org); the Collection de l'Institut Pasteur (CIP), [www.pasteur.fr](http://www.pasteur.fr)), from a laboratory collection (Centre National de Référence de la résistance aux antibiotiques, [www.cnr-resistance-antibiotiques.fr](http://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

965 was grown in anaerobic conditions (in a Trexler-type anaerobic chamber without stirring) and  
966 adjusted in MH broth supplemented with 5% lysed horse blood and 20 mg/L  $\beta$ -nicotinamide  
967 adenine dinucleotide at  $5.10^6$  UCF/mL. Ninety microliters of cell suspension were transferred in a  
968 sterile F-bottom polypropylene 96-well microplates. Thus, ten microliters of sterile RumC1 or  
969 antibiotics used as control from 100 to 0.1  $\mu$ M by two-fold serial dilutions were added to the  
970 bacterial suspension to obtain a final concentration of  $5.10^5$  CFU/mL. Microplates were incubated  
971 48h at 37°C in anaerobic conditions. MIC was defined as the lowest concentration of peptide that  
972 inhibited the growth of bacteria after 48h incubation at 37°C. Sterility and growth controls were  
973 prepared for each assay.

974

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

994

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

999 After 15 min of incubation in the same conditions, 100  $\mu$ L of cells were mixed with 10  $\mu$ L of  
1000 luciferin-luciferase reagent (Yelen Analytics) prepared in IMI-Yelen Buffer (Yelen Analytics). The  
1001 mix was homogenized and incubated 30 s before reading of the emitted photon using a microplate  
1002 reader (Synergy Mx, BioTek). Then, 10  $\mu$ L of lysis reagent (Yelen Analytics) was added to the  
1003 mixture, homogenized and incubated 1 min before a new reading of the emitted photon. The  
1004 intensity of the bioluminescent light was expressed as relative light units (RLU) which is  
1005 proportional to extracellular ATP concentration (16). The inner ATP concentration was derived  
1006 from the difference in ATP concentration in the extracellular media before and after cell lysis.  
1007 Results were expressed as a percentage of total inner ATP concentration that was measured with  
1008 untreated cells. All experiments were done in independent triplicates.

1009

1010 ***Ex-vivo* evaluation of the RumC1 innocuity using human intestinal explants.** In order to  
1011 evaluate the innocuity of RumC1 peptide for the human gut, *ex-vivo* experiments were performed  
1012 using human explants as previously described (17). Human intestinal tissues, corresponding to  
1013 ileocecal area, were obtained from patients undergoing surgery at the unit of gastrointestinal  
1014 surgery, Hospital Laveran (Marseille, France), according to a collaborative “clinical transfer”  
1015 project. The procedure was approved by the French ethic committee (CODECOH n° DC-2019-  
1016 3402). All patients agreed for the use of their tissues for research purposes. Diagnoses leading to  
1017 surgery were intestinal carcinoma. Samples were taken from macroscopically unaffected area as  
1018 identified by the surgeons. After resection, the tissues were placed in ice-cold oxygenated sterile  
1019 DMEM solution containing 1% (w/v) streptomycin/penicillin solution and 50  $\mu$ g/mL gentamycin  
1020 and were directly transferred to the laboratory within 15 min. Intestinal tissues were extensively  
1021 washed and maintained in ice-cold culture media. Tissues were cleaned under binocular microscope  
1022 from vascular vessels and conjunctive tissue using forceps. Intestinal explants (diameter of 0.5 cm<sup>2</sup>)  
1023 were then isolated from the cleaned resections using surgical punch, the complete procedure being  
1024 complete in less than 2 h after the resections were obtained from the surgery unit. Finally, the  
1025 explants were washed 3 times with culture media without antibiotics and transferred into 24-well  
1026 plates before being incubated at 37°C during 4 h with RumC1 peptide diluted in DMEM media at  
1027 100  $\mu$ M. In parallel, explants were left untreated (negative controls) or were incubated with the 300  
1028  $\mu$ M of detergent cetyl trimethylammonium bromide (CTAB) used as positive control of tissue  
1029 damages. After incubation, the explants were collected, washed three times with PBS<sup>2+</sup> and fixed  
1030 overnight at 4°C with PFA diluted at 4% (v/v) in PBS. The next day, intestinal explants were  
1031 washed twice with PBS and included in inclusion medium (TFM - EMS), in transverse position to  
1032 allow cutting respecting the crypt-villus axis using cryostat (Leica CM3050). Four sections of 5

1033  $\mu\text{m}$  thickness were obtained per explant and each section being separated from the next by 100  $\mu\text{m}$   
1034 in order to cover all the tissue. Explants were then stained using hematoxylin and eosin (H&E)  
1035 staining protocol. Briefly, samples were incubated for 8 min in hematoxylin (from Sigma-Aldrich)  
1036 and then allowed to stain by incubation with water for 2 min. Then explants were incubated for 1  
1037 min in eosin (from Sigma-Aldrich) and then in water for 1 min. This was followed by incubation  
1038 of explants with ethanol at concentration of 70% then 95% and finally 100% for 2 min each. After  
1039 blotting excess ethanol, intestinal explants were incubated for 15 min with xylene and mounted  
1040 with coverslip using Eukitt mounting media (EMS, Hatfield, PA, USA). Finally, explants were left  
1041 overnight to dry before examination of tissue organization under the microscope (Leitz DMRB  
1042 microscope (Leica) equipped with Leica DFC 450C camera) (18).

1043

1044 **SI References**

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