Identification of a Novel Two-Peptide Lantibiotic from *Vagococcus fluvialis*

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Prof. Dzung B Diep
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Reviewer comments:

Reviewer #1 (Comments for the Author):

The authors report on a novel two-component bacteriocin isolated from a species that was not previously reported as a bacteriocin-producer, although there was some evidence indicating the possible presence of bacteriocin-encoding genes in a recent report (doi: 10.1093/g3journal/jkaa034). The bacteriocin has a good chance to serve as a candidate for control of MDR enterococci and other health-challenging microorganisms (including Gram-negative bacteria, considering that some of the two-component bacteriocins are inhibiting them when combined with synergistically-acting antibiotics). Moreover, the species is considered by investigators as a potential probiotic for animals of agricultural importance. Therefore, the study is well-justified.
The authors used adequate methods to address the study's objective. These methods are sufficiently described in the manuscript's Materials and Methods chapter. The reviewer has just a few minor comments/suggestions which are penciled in the manuscript for the authors' convenience (see attached).

Reviewer #2 (Comments for the Author):

This is well planned, performed and written paper. Authors reports on new bacteriocins, produced by the strain belong to species that was never before reported as bacteriocinogenic. Some corrections needs to be taken into account by the authors.

Will be nice if authors can test more VRE strains in order to validate the suggested activity against different antibiotic resistance strains.

Authors have some repetitions in Results and Discussion sections. Please, try to be more focus and do not repeat the information. Moreover, in Results section try to be more focus on results and do not discuss the results. This needs to be dedicated to the discussion section.

Authors have preliminary test showing that BHI was optimal media for bacteriocin production? 60% ammonium sulphate was chosen based on preliminary ammonium sulphate % optimisation or was based on the literature? Please, consider to change the text and to be more informative.

Any reference for the method described under Ln 477-482?

Please, reference list needs additional attention. Please, pay attention to the use of italics, page numbers and volumes of the journals.

Reviewer #3 (Public repository details (Required)):

The DNA sequence of vagococcin T gene cluster is provided, but perhaps the authors should also provide the fully sequenced genome of the strains.

Reviewer #3 (Comments for the Author):

The paper submitted by Rosenbergová et al., describes the identification and characterization of a new two-peptide lantibiotic called vagococcin T. According to the authors this bacteriocin is the first one described in the genus Vagococcus and displays a range of activity against Gram-positive bacteria (except S.aureus) with notable activity against pathogens as E. faecium. From the point of view of the sequence, vagococcin T presents differences in sequence and structure with respect to other known two-peptide lantibiotics, especially the β-peptide. From the point of view of the action mechanism, the authors have shown a pore-forming activity and also they have characterized resistant mutants against the bacteriocin.

The paper is well written and the experiments are properly designed. However, some points should be addressed.

1) Line 90, please provide the data as supplementary information. The MIC for the antibiotics after the disk diffusion test and the rep-PCR.

2) Line 111. How did you identify the strain as Vagococcus fluvialis?

3) About the vagococcin gene cluster, can the author provide a figure comparing the organization of the different gene clusters of the known two-peptide lantibiotics? In figure 1, vcnG is indicated but is not in the gene cluster.

4) In Table 3, is it possible to provide the data as mg/L? Do you have any hypothesis about why S. aureus is resistant?

5) In figure 3, the tubes in which activity was observed ad the peaks from the HPLC do not match. Is it ok?

6) Considering that this bacteriocin is the first described with this topology and that this work is focused on the characterization of this new bacteriocin, in figure 5 the proposed dehydration profile and ring formation patron should be confirmed.

7) The strain full genome should be also deposited.

Staff Comments:

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Identification of a Novel Two-Peptide Lantibiotic from Vagococcus fluvialis

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Keywords: bacteriocin, Vagococcus, lantibiotic, antimicrobial, vagococcin T, pore formation

Abstract

Infections caused by multi-resistant pathogens have become a major problem in both human and veterinary medicine. Due to the declining efficacy of many antibiotics, new antimicrobials are needed. A promising alternative or addition to antibiotics are bacteriocins; antimicrobial peptides of bacterial origin with activity against many pathogens including antibiotic-resistant strains. From a sample of fermented maize, we isolated a Vagococcus fluvialis strain producing a bacteriocin with antimicrobial activity against multi-resistant Enterococcus faecium. Whole-genome sequencing revealed a novel two-peptide lantibiotic. Production of the lantibiotic by the isolate was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, which revealed distinct peaks at 4009.4 m/z and 3181.7 m/z in separate fractions from reversed-phase chromatography. The combination of the two peptides resulted in a 1200-fold increase in potency, confirming the two-peptide nature of the bacteriocin, named vagococcin T. The bacteriocin was demonstrated to kill sensitive cells by the formation of pores in the cell membrane, and its inhibition spectrum covers most Gram-positive bacteria, including multi-resistant pathogens. To our knowledge, this is the first bacteriocin characterized from Vagococcus.

Importance

Enterococci are common commensals in the intestines of humans and animals, but in recent years, they have been identified as one of the major causes of hospital-acquired infections due to their ability to quickly acquire virulence and antibiotic resistance determinants. Many hospital isolates are multi-resistant thereby making current therapeutic options critically limited. Novel antimicrobials or alternative therapeutic approaches are needed to overcome this global problem. Bacteriocins, natural ribosomally synthesized peptides produced by bacteria to eliminate other bacterial species living in a competitive environment, provide such an alternative. In this work, we purified and characterized a novel two-peptide lantibiotic produced by Vagococcus fluvialis isolated from fermented maize. The novel lantibiotic showed a broad inhibition spectrum of Gram-positive strains, including vancomycin-resistant Enterococcus faecium, demonstrating its therapeutic potential.
Introduction

Enterococci such as *E. faecium* and *E. faecalis* are regular commensals of human and animal intestines (1, 2). However, in recent years enterococci have become a concern in both human and veterinary medicine as they have emerged as some of the most prevalent nosocomial pathogens (3, 4). In addition to their ability to effectively acquire, harbor, and distribute antimicrobial resistance (AMR) determinants, enterococci are robust and able to survive on non-biotic surfaces for prolonged periods (5, 6). There is increasing evidence that overuse of antibiotics is a primary selection pressure for the acquisition and dissemination of antibiotic resistance in bacteria (7). To reduce the dissemination of AMR and to combat resistant bacteria, alternatives to antibiotics are needed. One such promising alternative is bacteriocins – natural proteinaceous compounds produced by bacteria with antimicrobial activity mostly against closely related species including pathogenic and antibiotic-resistant strains.

Small bacteriocins (less than 10 kDa) are classified based on their biosynthesis: post-translationally modified bacteriocins belong to class I while unmodified bacteriocins are members of class II (8, 9). Lanthipeptides, which belong to class I, are characterized by thioether linkages formed between cysteines and dehydrated serine and threonine residues to yield lanthionine and methyllanthionine, respectively (10). The organization of the ring structures then recognizes a specific target on sensitive cells such as lipid II, which is the docking molecule for most lantibiotics (11). The bacteriocin producer must protect itself from the lethal action of its own bacteriocin. For lantibiotics, self-immunity is achieved by the production of immunity proteins commonly named LanI and/or LanFE(G) (12, 13). The proteins LanFE(G) compose a specialized ABC-transporter that mediates the efflux of mature lanthipeptides from the cell, while LanI is thought to protect the producer extracellularly against the secreted lanthipeptide (12).

Lantibiotics are further subdivided into at least two types based on the differences in modification enzymes (14). Type I lanthipeptides, of which nisin is the founding member, use two separate enzymes for the dehydration (LanB) and cyclization (LanC) steps that produce the (methyl)lanthionine rings. Type II employs a single bifunctional enzyme (LanM) that catalyzes both steps (10, 14). LanM modification enzymes usually carry out the modification of two-peptide lantibiotics, which each consists of two different peptides exhibiting considerable synergy when combined but having little or no activity when assessed individually (15). The most studied two-peptide lantibiotic lacticin 3147 has potent activity against numerous pathogenic Gram-positive species, including vancomycin-resistant enterococci (VRE) (16). Lacticin 3147 also attenuates the growth of *Staphylococcus aureus* in a murine infection model and disrupts *Streptococcus mutans* biofilms, demonstrating the clinical potential of lantibiotics (17, 18).

*Vagococcus fluvialis* belongs to a genus of motile lactic acid bacteria most closely related to *Enterococcus* and *Carnobacterium*, and was first described as a phylogenetically distinct genus in 1989 (19, 20). Not much is known about *V. fluvialis*, most characterized isolates originated from the wounds of animals (pigs, horses, cattle) and from human clinical cases (20, 21). However, the species has also been isolated from the urine of healthy cattle and described as a potential probiotic in fish (22, 23). In this work, we describe the discovery
and characterization of a novel two-peptide lantibiotic produced by Vagococcus fluvialis. The bacteriocin was active against most Gram-positive strains tested, including animal and human pathogens such as multi-drug resistant E. faecium and mastitis-associated Streptococcus uberis (24). The bacteriocin gene cluster had an atypical organization and included what resembles a quorum-sensing system. To our knowledge, this is the first bacteriocin characterized from Vagococcus. We believe that this bacteriocin could serve an important role as a therapeutic in the future.

Results

Screening for bacteriocin producers against Enterococcus faecium

E. faecium LMG 20705 is a multidrug-resistant opportunistic pathogen. The resistance pattern was determined by AMRFinderPlus (Table S1) and the disc diffusion test according to EUCAST (data not shown) (25, 26). The strain was shown to be resistant to vancomycin, ampicillin and streptomycin, all of which are first-line therapeutics for enterococcal infections (27). In addition, the strain exhibited resistance to quinupristin/dalfopristin – a mixture of streptogramin B and A used for the treatment of serious VRE-related infections (28).

A total of 40 different samples of fermented fruits and vegetables were screened for the presence of bacteriocin producers that could inhibit the growth of E. faecium LMG 20705. From all samples, 17 colonies exhibited a distinct inhibition zone indicative of antimicrobial production. Repetitive element PCR (rep-PCR) was performed to examine the genetic similarity of these isolates. Nine unique DNA band profiles were observed after gel electrophoresis (data not shown). One representative from each group was selected for whole-genome sequencing to identify novel bacteriocin genes. The genomes were analyzed for bacteriocins by BAGEL4 and antiSMASH (29, 30). The analysis revealed that all but two isolates had genes for previously characterized bacteriocins known to be active against enterococci; subtilosin A (31), ericin S (32), enterolysin A (33), and NKR-5-3B (34). One of the two isolates with a potentially novel bacteriocin was a strain isolated from fermented maize, the genome of this isolate contained a gene cluster with an organization similar to two-peptide lantibiotic gene clusters. The best database hit for the predicted bacteriocin was the lantibiotic flavecin from Ruminococcus flavefaciens (35) with only 45% identity, suggesting that the isolate, identified as Vagococcus fluvialis, likely produced a novel two-peptide lantibiotic.

Genome analysis and identification of the vagococcin T gene cluster

The search for putative bacteriocin genes by antiSMASH resulted in the identification of a type II lantibiotic gene cluster (Fig. 1). Two bacteriocin genes vcnA1 and vcnA2 were identified and predicted to represent the a (vcnA1) and b (vcnA2) peptides of a two-peptide lantibiotic hereafter named vagococcin T. Located downstream of each of the vcnA1 and vcnA2 genes are genes encoding lantibiotic biosynthesis proteins vcnM1 and vcnM2, respectively. Both gene products, VcnM1 and VcnM2, showed sequence similarity with MrsM, the modification enzyme for the lantibiotic mersacidin (36). The predicted function of all proteins encoded in the vcn gene cluster is listed in Table 2.
Figure 1 Gene organization of the vagococcin T cluster in *V. fluvialis*. Bifunctional modification enzyme genes (green) are located downstream of lantibiotic precursor genes (blue). Lantibiotic transporter gene with leader removal function (gray) is located downstream of *vcnM1* and upstream from *vcnI*, encoding a potential immunity protein (yellow). Other genes involved in bacteriocin immunity are located at the beginning of the cluster. A group of genes resembling a quorum-sensing system (red) is located at the end of the cluster.

The *vcnT* gene is located downstream of *vcnM1* and encodes a C39 peptidase that shows 45% identity with MrsT, the mersacidin transport enzyme which cleaves the leader after the GG/GA motif – a typical cleavage site for many bacteriocin leaders (37). A GG-motif is indeed present in both VcnA1 and VcnA2 prepeptides (Fig. 2A). The mature peptides showed the highest homology to flavecin FlvA1a and FlvA2b peptides (42% and 46%, respectively) (35). Sequence alignment of Vcn Tα with other lantibiotic α-peptides (Fig. 2B) showed that Vcn Tα contain the same CTxTxE conserved motif believed to be essential for lipid II docking (38). Similarly, the conserved sequence (CPTxxCx/sxxC) typical for all β-peptides was found in Vcn Tβ (Fig. 2B).
Figure 2 Predicted amino acid sequences of vagococcin T prepeptides (A). Leader sequences are underlined and separated from the mature peptides by a space. Multiple sequence alignment of α- and β-peptides of known two-peptide lantibiotics: flavecin (Flv; P0DQM1, P0DGL4), haloduracin (Hal), lacticin 3147 (Ltn; O87236, O87237), lichenicidin (Lch; P86475, P86476), plantaricin W (Plw; D2KR94, Q9AF68), and staphylococcin C55 (Sac; Q9S4D3, Q9S4D2) (B). The sequence alignment was performed using T-Coffee and colored with BoxShade; black and gray shading corresponds to identical and similar amino acids, respectively.

The types of immunity genes present in lantibiotic gene clusters vary, and the encoded immunity proteins often show little sequence identity with each other (39). Two genes of the LanFE(G) immunity system are present in the vcn cluster, vcnF and vcnE, located at the start of the operon. VcnF showed 47% identity with the ATP-binding domain NisF of the NisFEG transporter, and contained the conserved sequences for both Walker A and B motifs (40). The last four genes in the cluster resembled an analog to the Fsr quorum-sensing system of E. faecalis; this type of quorum-sensing system has not previously been identified in other lantibiotic clusters (41). The product of the first ORF, designated vcnR, showed 39% identity to the response regulator (RR) FsrA (see Table 2). An FsrB homologue is encoded by the gene designated vcnQ2 with 36% identity (Q for quorum). The third component, a sensor histidine protein kinase (HPK) encoded by vcnK showed 35% identity with FsrC. Search for small reading frames that could encode the pheromone component of the quorum-sensing
system revealed a small ORF between vcnQ2 and vcnK. The product of this ORF gave no hits to any known peptides by BLAST search, however, sequence alignment showed 37% identity with FsrD, the gelatinase biosynthesis-activating pheromone (GBAP) prepeptide (42). It is therefore possible that the processed product of vcnQ1 is a pheromone.

Another small ORF located between vcnT and vcnR also showed no sequence homology to known proteins by BLAST-search, however, the gene product had similar size, charge, and hydrophobicity as known lantibiotic immunity proteins. LanI proteins of comparable physicochemical properties include EcII, PepI, and LasI, the LanI immunity proteins for epididin 280, pep5, and lactocin S (39). The ORF located between vcnT and vcnR was therefore named vcnI and is further discussed in the Discussion section below.

| Gene product | Putative function                          | Homologue and sequence identity            |
|--------------|-------------------------------------------|--------------------------------------------|
| VcnF         | Bacteriocin immunity                      | NisF, 47% (AAC43327.1)                     |
| VcnE         | Bacteriocin immunity                      | MrsE, 22% (CAB60257.1)                     |
| VcnA2        | Vagococcin T ß-peptide                    | FlvA2b, 46% (P0DQL4.1)                     |
| VcnM2        | VcnA2 dehydratase and cyclase             | MrsM, 44% (CAB60261.1)                     |
| VcnA1        | Vagococcin T α-peptide                    | FlvA1a, 42% (P0DQM1.1)                     |
| VcnM1        | VcnA1 dehydratase and cyclase             | MrsM, 48% (CAB60261.1)                     |
| VcnT         | Bacteriocin maturation and export         | MrsT, 45% (KAF1340276.1)                   |
| VcnI         | Bacteriocin immunity                      | -                                          |
| VcnR         | Response regulator                        | FsrA, 39% (EIA6660097.1)                   |
| VcnQ2        | Pheromone maturation/export               | FsrB, 35% (EGO8521395.1)                   |
| VcnQ1        | Pheromone/signaling molecule prepeptide   | FsrD, 37% (CDK37795.1)                     |
| VcnK         | Protein histidine kinase                  | FsrC, 35% (EIP8082021.1)                   |

Because of the novelty of vagococcin T, the antimicrobial produced by the isolate of V. fluvialis was chosen for further characterization. Cell-free supernatants from the isolate contained an antimicrobial substance which was heat-stable and sensitive to proteinase K (data not shown), properties expected for bacteriocins, like vagococcin T (43).

**Purification of bacteriocin**

Purification of the predicted two-peptide lantibiotic produced by *V. fluvialis* was achieved with a three-step purification scheme consisting of ammonium sulfate precipitation, cation-exchange chromatography and reverse-phase chromatography (RPC) (44). During the RPC elution, two peaks corresponding to 29% and 36% of isopropanol were observed in the elution profile (Fig. 3). Collected fractions were assayed against *E. faecium* LMG 20705; low antimicrobial activity of 400 BU/ml was found only in the second peak (fractions 26 to 30), which would be expected due to the separation of the two peptides into separate fractions (45). To test this notion, fractions 21 to 24 were individually combined with fractions 26 to 30 in 1:1 (v/v) ratio to find any combination of fractions exhibiting synergy (Fig. S1). Indeed, the
highest synergy was observed between fractions 23 and 28 which in combination had antimicrobial activity of 51 200 BU/ml, representing a 1200-fold increase in activity with yield of 128% (Table 2).

![Figure 3](image_url) Reversed-phase chromatography elution profile of the sample obtained from cation-exchange chromatography. All collected fractions exhibited relatively low bacteriocin activity against *E. faecium* LMG 20705, but two fractions showed a significant increase in potency when assayed together (1:1 [v/v]), indicating the presence of a two-peptide bacteriocin. The inhibition of *E. faecium* LMG 20705 from individual and combined fractions is pictured in the top right corner.

**Table 2** Bacteriocin purification.

| Sample                           | Volume (mL) | Activity (BU/mL) | Total activity (BU) | Yield (%) |
|----------------------------------|-------------|------------------|---------------------|-----------|
| Supernatant                      | 1,000       | 80               | 80,000              | 100       |
| Ammonium sulfate precipitate     | 150         | 320              | 48,000              | 60        |
| Cation-exchange chromatography   | 100         | 160              | 16,000              | 20        |
| Reversed-phase chromatography    | 2           | 51,200           | 102,400             | 128       |

With purified bacteriocin, the biological activity of vagococcin T against number of bacteria was determined using the spot-on-lawn assay (Table 3). Lantibiotics are known to be very potent against Gram-positive bacteria but with limited activity against Gram-negative bacteria as observed for nisin (46), lichenicidin (47) or thusin (48). In addition to showing potent antimicrobial activity against the indicator *E. faecium* LMG 20705, Vagococcin T displayed a broad inhibition spectrum including all Gram-positive strains tested except for
Staphylococcus aureus. The Gram-negative strains Escherichia coli and Salmonella typhimurium were not inhibited, which is expected for the lipid II-targeting type A lantibiotics.

### Table 3 Inhibition spectrum of reversed-phase chromatography-purified vagococcin T.

| Indicator strain               | Sensitivity |
|-------------------------------|-------------|
| Bacillus cereus LMGT 2805     | ++          |
| Bacillus cereus LMGT 2731     | +           |
| Enterococcus faecalis LMGT 2333 | ++        |
| Enterococcus faecalis LMGT 3331 | ++        |
| Enterococcus faecium LMGT 2772 | ++        |
| Enterococcus faecium LMGT 3104 | ++        |
| Lactobacillus curvatus LMGT 2353 | +++      |
| Lactobacillus plantarum LMGT 2352 | ++        |
| Lactococcus garvieae LMGT 3390 | ++           |
| Lactococcus lactis LMGT 2081  | ++          |
| Listeria innocua LMGT 2710    | +++         |
| Listeria monocytogenes LMGT 2604 | ++        |
| Listeria monocytogenes LMGT 2650 | +          |
| Pediococcus acidilactici LMGT 2002 | +++      |
| Streptococcus dysgalactiae LMGT 3890 | +          |
| Streptococcus thermophilus LMGT 3555 | +++      |
| Streptococcus uberis LMGT 3912  | ++          |
| Streptococcus haemolyticus LMGT 4133 | +          |
| Staphylococcus aureus LMGT 3242 | –            |
| Salmonella typhimurium B1377 | –             |
| Escherichia coli TG1          | –             |

*a*Laboratory of Microbial Gene Technology (LMGT), Norwegian University of Life Sciences, Ås, Norway  
*b*Inhibition zone diameter (mm): (+) 5-9; (++) 10-14; (+++) >15, (−) no inhibition

Molecular Mass and Bacteriocin Identification

Given the synergism of fraction 23 on fraction 28, these fractions were analyzed further using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. The acquired spectra revealed the presence of only one distinct peak in each fraction. A peak at 4009.5 m/z can be seen in fraction 23 (Fig. 4A), which correlated well with the mass predicted for one of the two peptides by antiSMASH (30) (assuming 1 unmodified Ser/Thr). The peak in fraction 28 (Fig. 4B) at 3181.7 m/z, however, differed from the prediction by 70.1 Da (3111.6 Da, assuming 2 unmodified Ser/Thr). The reasoning behind this difference is given in the Discussion section below. A schematic representation of all post-translational modifications to Vcn Tα and Vcn Tβ consistent with the measured masses is shown in Figure 5. These results confirm that the antimicrobial activity produced by V. fluvialis was indeed caused by the predicted two-peptide lantibiotic vagococcin T.
**Figure 4** MALDI-TOF mass spectrometry analysis of fractions 23 (A) and 28 (B) from reversed-phase chromatography. The peaks at 4009.40 m/z and 3181.69 m/z represent Vcn Tα and Vcn Tβ peptides, respectively.

**Figure 5** Proposed biosynthetic scheme for vagococcin T α- and β-peptides. The structures of Vcn Tα and Vcn Tβ were deduced from the known structures of other two-peptide lantibiotics. Lanthionine rings (Ala-S-Ala) are formed between didehydroalanine (Dha), derived from...
serine (green), and cysteine residues (blue); methylanthionine (Abu-S-Ala) rings are formed between didehydrobutyrine (Dhb), derived from threonine (orange), and cysteine residues.

**Pore-forming nature of vagococcin T**

To assess whether vagococcin T is a pore former, propidium iodide (PI) assay was conducted. PI is a membrane impermeant dye which increases its fluorescence efficiency/quantum yield when bound to double-stranded DNA (49). After exposing the indicator strain to the known pore-forming lantibiotics nisin A and nisin Z in the presence of extracellular PI, an increase in emission was detected (Fig. 6). Similar results were also obtained for vagococcin T, implying that vagococcin T has a similar mode of action involving pore-formation. The negative control micrococcin P1, a bacteriocin which kills cells by inhibition of protein synthesis (50), caused little or no increase in fluorescence as it does not form pores.

**Figure 6** Bacteriocin-induced pore formation assay. Propidium iodide fluorescence intensity over time in the presence of *E. faecium* and the antimicrobials vagococcin T (Vcn T), nisin A (Nis A), nisin Z (Nis Z) and micrococcin P1 (Mic P1). An increase in emission is observed for the pore-forming nisin A and nisin Z. Micrococcin P1, a non-pore-forming bacteriocin, was used as a negative control.

To further corroborate our results that vagococcin T is membrane-active, the indicator cells exposed to vagococcin T were examined by scanning electron microscopy (SEM). Clear differences were observed for bacteriocin-treated compared to untreated cells (Fig. 7). Treated cells appeared collapsed/shriveled, suggesting loss of turgor pressure. Irregular dark spots were visible on some cells, possibly indicating pores or damage to the cell envelope. In addition, an extracellular matrix-like material was visible only with the treated cells. In comparison, the cell surface of untreated cells was smooth without ruptures or signs of cell damage.
Figure 7 Scanning electron microscopy (SEM) showing the effect of vagococcin T on *E. faecium* cells (30,000x magnification). Cells incubated without vagococcin T showed no visible cell damage (A), while the vagococcin T treated had shriveled appearance following a 2-hour incubation with 10x MIC of vagococcin T (B). Signs of cell damage and lysis is indicated by red arrows.

**Stress response involved in resistance to vagococcin T**

Resistant colonies of *E. faecium* LMG 20705 were occasionally visible within the inhibition zones of vagococcin T. The increased tolerance to vagococcin T of four randomly selected spontaneous mutants was tested and showed a 64- to 256-fold increase in MIC (minimum inhibitory concentration) compared to the wild-type (Table 4). The frequency of resistant mutants was estimated to be $8.7 \times 10^{-7}$ based on plating techniques. Whole-genome sequencing was performed on the four mutants to identify the possible mechanism for the increased tolerance to vagococcin T. Three of the four mutants had mutations in *liaF* (M1-M3), two with non-conservative missense mutations (Ile108Asn, Trp141Ser) and one with a frameshift from amino acid position 9 (Val9fs, M2). Several mutations were found in various genes of mutant M4, none of which could be directly linked to the increased tolerance to vagococcin T, see Table 4. *liaF* encodes a negative regulator of LiaRS, a two-component regulatory system involved in cell envelope stress response induced by lipid II-interacting antimicrobials (51). We examined the cross-resistance of *liaF* mutants to other membrane-active bacteriocins – nisin A and garvicin KS (44). As expected, both nisin A and garvicin KS showed reduced bioactivity (4- to 32-fold) toward the mutants compared to the wild-type strain.
Table 4 Mutations identified in E. faecium LMG 20705 spontaneous mutants with increased tolerance to vagococcin T.

| E. faecium mutant | Fold increase of MIC<sup>a</sup> | Mutation<sup>b</sup> | Protein | Reference |
|-------------------|-------------------------------|---------------------|---------|-----------|
|                   | Vcn T | Nis A | Gar KS |                 |           |
| M1                | 256   | 16   | 4      | c. 323T>A; p. Ile108Asn | stress regulator protein LiaF | WP_002328613.1 |
| M2                | 256   | 32   | 4      | c. 24dupT; p. Val9fs | stress regulator protein LiaF | WP_002328613.1 |
| M3                | 256   | 32   | 8      | c. 422G>C; p. Trp141Ser | stress regulator protein LiaF | WP_002328613.1 |
| M4                | 64    | 8    | 4      | c. 605T>A; p. Val202Glu | aldose 1-epimerase | WP_002328285.1 |
|                   |       |      |        | c. 514G>A; p. Gly172Arg | metal-dependent hydrolase | WP_002287133.1 |
|                   |       |      |        | c. 187A>G; p. Ile63Val | hypothetical protein | WP_100970561.1 |
|                   |       |      |        | c. 277A>T; p. Thr93Ser | Mg2+ cation transporter (CorA family protein) | WP_002318987.1 |

<sup>a</sup>Vcn T – vagococcin T, Nis A – nisin A, Gar KS – garvicin KS

<sup>b</sup>c – coding DNA, p – protein, > – substitution, dup – duplication, fs – frameshift

Discussion

Bacteriocins are a promising alternative to traditional antibiotics, as they display activity against antibiotic-resistant pathogens and have many desirable properties for the control of microorganisms. They are often produced by probiotic species with GRAS (generally regarded as safe) status, have high potency, and low toxicity (52). In addition, bacteriocins are arguably more easily amenable to biotechnological manipulation as they are defined by structural genes. Given the high potency and potential clinical applications of bacteriocins, we sought to find new bacteriocins with possible therapeutic use. To this end, we screened for bacteriocin producers in fermented fruits and vegetables that inhibited the growth of the indicator strain, a multi-drug resistant E. faecium. From a sample of fermented maize, we successfully isolated a strain of V. fluvialis producing a two-peptide lantibiotic named vagococcin T.

To our knowledge, vagococcin T was first bacteriocin identified from the genus Vagococcus. The two bacteriocin genes, vcnA1 and vcnA2, are separated by a vcnM gene, which is an unusual arrangement – two-peptide bacteriocin genes are most often located adjacent to each other in tandem. Because of the low sequence similarity of the two vagococcin T prepeptides (20% sequence identity), each of the two vcnM gene products are likely dedicated to modifying its cognate bacteriocin peptide. Upstream of the bacteriocin genes in the same operon is the gene pair vcnFE encoding an ABC-transporter which likely has a dual role, in export of the bacteriocins peptides and immunity; a property which is common for other lantibiotics including nisin, mersacidin and lacticin 3147 (39). At the end of the vcn cluster is an operon encoding proteins with homology to the Fsr quorum-sensing system from E. faecalis. In the Fsr system, the FsrD prepeptide is exported and processed by FsrB into a small 11 amino acid cyclic peptide pheromone. A membrane-bound sensor HPK FsrC (VcnK) then responds to the pheromone and activates the intracellular RR FsrA (VcnR) (30). VcnQ2 and VcnQ1 show 35% and 37% sequence identity with FsrB and FsrD, respectively (Table 1). The majority of circular peptide pheromones have been reported to
form a thiolactone linkage between the C-terminal amino acid (methionine, phenylalanine, or leucine) and a cysteine located three or four residues from the N-terminal cleavage site (53). However, the peptide processed from FsrD contains a lactone linkage between the C-terminal methionine and the hydroxyl group of a serine residue (42). In addition, an autoinducing peptide containing a lactone ring between the C-terminal phenylalanine and a serine residue has been identified in *S. intermedia* (54). VcnQ1 may be processed similarly, forming a lactone linkage between serine and the C-terminal phenylalanine. Interestingly, the closest homologue to VcnQ1 was found to be an unannotated *orf* (159 nt) in the locus of the circular bacteriocin enterocin NKR-5-3B (Ent53B) produced by the strain *E. faecium* NKR-5-3 (GenBank Accession: LC068607) (55). The *orf* is arranged similarly to vcnQ1 between genes encoding an HPK and a FsrB-like protein (*orf5* and *orf6*). The predicted mature product of this *orf* contains an 11 amino acid sequence showing 73% identity (100% similarity) to the putative VcnQ1-derived pheromone. *E. faecium* NKR-5-3 produces multiple bacteriocins; enterocins NKR-5-3A, B, C, D, and Z (Ent53A, Ent53B, Ent53C, Ent53D, and Ent53Z) (56). An inducing peptide Ent53D has been shown to regulate the transcription of the aforementioned bacteriocins except for NKR-5-3B (56). A derivative of the unannotated *orf* in *E. faecium* NKR-5-3 genome may be involved in the regulation of NKR-5-3B. However, it is presently not known if VcnQRK constitutes a functional quorum-sensing system in *V. fluvialis*; characterization of the *vcn* regulatory system is beyond the scope of the present study.

The production of vagococcin T by the *V. fluvialis* isolate was confirmed by bacteriocin purification and MALDI-TOF MS. Vagococcin T was purified from the cell-free supernatant using a common purification scheme for bacteriocins involving ammonium sulfate precipitation followed by cation-exchange- and reversed-phase chromatography. The elution profile from reversed-phase chromatography showed two distinct peaks, indicating the presence of a two-peptide bacteriocin. Indeed, when assayed individually, only fraction 28 exhibited some antimicrobial activity (400 BU/ml) against the indicator strain. However, when all combinations of fractions were assayed (fractions 20 to 30), a significant increase in potency (51 200 BU/ml) was observed for the combination of fractions 23 and 28. Despite not corresponding to the two peaks in the elution profile, the high synergy observed for the combination was strong evidence of a two-peptide bacteriocin.

Mass determination of each fraction revealed a single distinct peak at 4009.4 m/z and 3181.69 m/z for fractions 23 and 28, respectively. Analysis of *V. fluvialis* genome by the RiPP mining tool antiSMASH (57) identified a lanthipeptide gene cluster encoding two putative lanthipeptide precursors. In addition to predicting lanthipeptide genes, antiSMASH predicts the leader cleavage site, dehydrations, crosslinks, and the expected masses. The mass predicted for Vcn Tα (4010.6 Da), assuming one unmodified serine or threonine, corresponded well with the measured value of 4009.4 m/z. However, the mass predicted for Vcn Tβ (3111.6 Da) was approximately 71 Da lower than the mass obtained by MALDI-TOF MS. The reason for this discrepancy is likely caused by inaccurate leader peptide prediction. The predicted Vcn Tα leader peptide is a typical double-glycine-type leader with a GG| cleavage site, while the Vcn Tβ leader cleavage site was predicted to be (G)GA|. The predicted mass of Vcn Tβ with the addition of alanine is 3181.5 Da which is consistent with the measured mass of 3181.67 m/z. The close correspondence between the measured and the
theoretical masses provides strong evidence that the purified bacteriocin vagococcin T is the
gene product of vcnA1 and vcnA2. Predicted structures of Vcn Tα and Vcn Tβ peptides are
consistent with the structures of other two-peptide lantibiotics (Fig. 5).

The α-peptide of most two-component lantibiotics employs lipid II as a docking
molecule to exert its antimicrobial activity (58, 59). A lipid II-binding motif was found in Vcn
Tα (see Fig. 2B), suggesting a lipid II-dependent mode of action of vagococcin T. It is
believed that the b-peptide of lipid II-targeting two-component lantibiotics binds to the
complex formed between lipid II and the α-peptide, which then leads to pore formation. The
predicted mode of action involving pore formation was consistent with SEM showing E.
faecium with a shriveled appearance, lysed cells, and cell debris following the exposure to
vagococcin T (see Fig. 7). The extracellular matrix-like material is likely consisting of cell
debris cross-linked by the fixing agent. The pore formation property is further supported by
the fact that Vcn T showed a comparable pore-forming ability to nisin A, a known pore-
forming lantibiotic (60, 61).

For many lantibiotics, the type of immunity system appears to correlate with the mode
of action of the lantibiotic (12, 13). It is believed that producers of pore-forming lantibiotics
require both the Lanl and LanFe(G) components for immunity (13, 62). However, no Lanl
component was immediately apparent in the vcn cluster, despite the evident pore-forming
mode of action of vagococcin T (see Fig. 6). On further analysis, a small ORF was found
downstream of vcnT, encoding a predicted transmembrane, cationic, 50 amino acid protein
(charge 5 at pH 7). The protein sequence shows no homology to known proteins but shares
similar properties with PepI, EciI, and LasJ (LanI component of Pep5, epicidin, and lactocin
S, respectively), all predicted transmembrane proteins, 57-69 amino acids in length with a
charge of 4-6 (at pH 7). Due to this similarity, we believe this ORF to be involved in
lantibiotic immunity and is thus named vcnI.

Upon challenging the E. faecium indicator strain to the bacteriocin we observed
resistant cells with a frequency of 8.7 x 10^-7. Three randomly selected isolates with the
highest tolerance to vagococcin T all had mutations in liaF, a negative regulator (repressor) of
the LiaRS cell envelope stress response system (lipid II-interacting antibiotics response
regulator and sensor). Previous studies have shown that membrane-active antimicrobials
decouple the repression by LiaF, allowing the HPK LiaS and its cognate RR LiaR to trigger
genes involved in resistance (63). The effect of genetic disruption of liaF is likely similar to
the decoupling of LiaF-mediated repression. Orthologs of the Lia system exist in most
Firmicutes, and all systems investigated so far regulate the expression of genes that protect
the cell against perturbations in the cell envelope (51). In Bacillus subtilis, the LiaFSR system
is one of the primary response systems against lipid II-interacting antibiotics such as
vancomycin and bacitracin (64) but is also induced by cationic antimicrobial peptides, organic
solvents, and detergents (65–67). The genes regulated by the Lia system vary between
species; in Staphylococcus aureus the LiaRS homolog (VraSR) upregulates genes encoding
penicillin-binding proteins and proteins involved in teichoic acid synthesis, chaperones, and
membrane lipid biosynthesis, that together confer resistance to beta-lactam antibiotics (68–
71). Even though the LiaFSR regulon in enterococci remains unknown, the LiaFSR system
has been implicated in resistance to daptomycin and antimicrobial peptides due to the
redistribution of cardiolipin microdomains away from the division septum (72, 73). All liaF
mutants displayed low-level cross-resistance to nisin A, another lipid II-interacting lantibiotic (Table 4). These results confirm the role of LiaFSR in mediating resistance to vagococcin T which further supports the lipid II-mediated mode of action of the bacteriocin.

The appearance of vagococcin T-resistant colonies of *E. faecium* exemplifies the hardiness of enterococcal populations. Combination therapies will likely be needed to effectively control enterococcal populations in the future. Formulations combining bacteriocins with different modes of action have been developed and showed increased potency and broader inhibition spectrum with a very low frequency of resistance (74–76).

In summary, in this work, we describe the isolation and characterization of a new two-component lantibiotic vagococcin T showing a broad antimicrobial spectrum against Gram-positive species, including multidrug-resistant strains. Furthermore, we show that mutations in the *liaF* gene confer resistance to vagococcin T and other antimicrobials. This connection highlights LiaF and the stress response system as an appealing target for future drug development and combination therapies. Further work is required to establish the potential of vagococcin T as a therapeutic in human or veterinary medicine.
Materials and Methods

Bacterial strains and growth conditions

The indicator strain *E. faecium* LMG 20705 (FAIR-E 102) was obtained from the LMG collection (BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent Belgium). *E. faecium* LMG 20705 was grown in M17 broth supplemented with 0.5 % w/v glucose (GM17) and incubated at 37°C without shaking. All other bacterial strains were grown in brain heart infusion (BHI) broth at 30°C without shaking.

Screening for bacteriocin producers

A selection of 40 different fruit and vegetables were purchased from a local market (Oslo, Norway) and prepared as described previously (77). Samples were screened for bacteriocin producers using a multi-layer soft agar technique. Briefly, 10-fold serial dilutions of samples were prepared in sterile saline. An aliquot (10 ml) of each dilution was mixed with 5 ml of BHI soft-agar (0.7% w/v agar), plated on a BHI agar plate (1.5% w/v agar) and allowed to solidify. A second layer of BHI soft agar was poured on top, and the plates were incubated overnight at 30°C. Then, an overnight culture of the indicator strain was diluted 1:100 in 5 ml BHI soft agar and poured over the plate. After an additional overnight incubation at 30°C, colonies showing a clear zone of inhibition were re-streaked to obtain pure cultures. The pure culture was retested against the indicator strain before being stored in 20% glycerol at -80°C for later use.

DNA sequencing and repetitive element PCR fingerprinting

Genomic DNA was isolated and purified using a GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Saint-Louis, MO, USA) according to the provided protocol. The 16S rRNA gene was amplified using the universal primers 11F (5'-TAACACATGCAAGTCGAACG-3') and 4R (5'-ACGGGGCGGTGTGTRC-3'). The PCR product was purified using NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions and sent to Eurofins Genomics for Sanger sequencing. Repetitive element PCR (rep-PCR) fingerprinting was performed using ERIC1R (5’-ATGTAAGCTCCTGGGGATTCAC-3’), ERIC2 (5’-AAGTAAGTGAAGGGGGATTCAC-3’) and LL-repl (5’-TACAAACAAAACAAAAAC-3’) as previously described (78, 79).

Whole-genome sequencing was performed by BGI (Beijing Genomics Institute) using the DNBSeq sequencing platform (150 bp paired-end). Reads were error corrected and assembled using SPAdes v3.14.1 (80). The resulting contigs were submitted to antiSMASH and BAGEL4 for the identification of potential bacteriocin genes (29, 57).

Bacteriocin purification

The bacteriocin-producing strain was cultivated in 1 liter of BHI broth at 30°C for 24 hours. Cells were removed by centrifugation (10,000 g, 30 min, 4°C) and the bacteriocin was
precipitated from culture supernatant with ammonium sulfate (60% saturation, 4°C, overnight). The precipitate was harvested by centrifugation (15,000 g, 40 min, 4°C), redissolved in 700 ml of distilled water and adjusted to a pH of 3.5 with 1 M hydrochloric acid. The sample was applied to a Hi-Prep 16/10 SP-XL column (GE Healthcare, Chicago, IL, USA). Unbound material was washed from the column with 150 ml of 25 mM sodium citrate-phosphate buffer (pH 3.5). The bacteriocin was eluted with 100 ml of 0.5 M sodium chloride, eluate was then applied to a 1 mL Resource RPC column (GE Healthcare, Chicago, IL, USA) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech, Amersham, UK). The column was previously equilibrated with 0.1% v/v TFA and the bacteriocin was eluted from the column using a linear gradient (40 CV) of isopropanol containing 0.1% v/v TFA at 1 ml/min.

**Bacteriocin activity assays**

Bacteriocin activity was assayed in microtiter plates as previously described (81). An overnight culture of the indicator E. faecium LMG 20705 or mutants was diluted 50-fold in GM17 broth containing twofold dilutions of the sample to a total volume of 200 µl. The plate was incubated at 37°C for approximately 4 hours, after which the absorbance at 600 nm was measured using a SPECTROstar Nano plate reader (BMG Labtech, Ortenberg, Germany). Bacteriocin activity was expressed in bacteriocin units (BU) per ml – one BU is the amount of bacteriocin that inhibits the growth of the indicator strain by at least 50% in 200 ml of culture (81). Nisin A was prepared by thoroughly resuspending milk solids containing 2.5% nisin A in 0.05% acetic acid (N5764; Sigma, St. Louis, MO, USA) and discarding remaining solids by centrifugation. Micrococcin P1 was purified as previously described (77).

**Spot-on-lawn assay** was used to obtain the inhibition spectrum of purified vagococcin T. Vagococcin T solution was prepared by mixing fractions with the highest synergy in a 1:1 ratio. Fresh overnight cultures were diluted 1:100 in 5 ml of BHI soft-agar and poured onto a BHI agar plate. Once the layer solidified, 2 µl of vagococcin T solution was spotted on the lawn. The plates were incubated overnight at 30°C and the inhibition zones were measured.

**Propidium iodide assay**

The pore-forming mode of action of vagococcin T was investigated using the propidium iodide (PI) method. An overnight culture of the indicator was washed twice in phosphate-buffered saline (PBS) and adjusted to an OD<sub>600</sub> of 0.7 with PBS in the wells of a black microtiter plate containing 20 µM PI (final concentration) and vagococcin T. Fluorescence was measured at 5-min intervals for 2 hours using a FLUOstar OPTIMA reader (BMG LABTECH, Ortenberg, Germany) with excitation at 535 nm and emission at 617 nm.

**MALDI-TOF mass spectrometry**

MALDI-TOF MS was performed on an ultrafleXtreme mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in reflectron mode. The instrument was externally calibrated with peptide calibration standard II (Bruker Daltonics, Bremen, Germany) and positively charged ions in the range of 1000 to 6000 m/z were analyzed. RPC purified
fractions and matrix [a-cyano-4-hydroxycinnamic acid (HCCA)] were mixed in 1:1 ratio and applied on a Bruker MTP 384 steel target plate (Bruker Daltonics, Bremen, Germany) for analysis.

**Scanning electron microscopy**

The indicator strain was grown to mid-log phase (OD$_{600}$ ~ 0.6) and incubated with vagococcin T (10x MIC) for 2 hours at 37°C with gentle shaking. A culture with no bacteriocin added was used as a control. After incubation, cells were harvested by centrifugation (10,000g, 5 min), washed twice in PBS and resuspended in fixing solution (1.25% w/v glutaraldehyde, 2% w/v formaldehyde, PBS) for overnight incubation at 4°C. Fixed cells were then washed three times in PBS and allowed to sediment/attach onto poly-L-lysine coated glass coverslips at 4°C for 1 hour. Subsequently, attached cells were dehydrated with an increasing ethanol series (30, 50, 70, 90, 96% v/v) for 10 min each and finally washed four times in 100% ethanol. Cells were dried by critical-point drying using a CPD 030 critical point dryer (BAL-TEC, Los Angeles, CA, USA). Coverslips were sputter coated with palladium-gold using a Polaron Range sputter coater (Quorum Technologies, Lewes, UK). Microscopy was performed on an EVO50 EP scanning electron microscope (Zeiss, Oberkochen, Germany) at 20 kV and a probe current of 15 pA.

**Mutant analysis**

To characterize mutants of *E. faecium* LMG 20705 resistant to vagococcin T, a total of 20 plates were made as described for the spot-on-lawn assay. However, to avoid sequencing clones of the same mutant, the lawn on each plate was prepared from genetically independent cultures (inoculated with different single colonies). Colonies that were observed at or near the center of the inhibition zone from vagococcin T following overnight incubation were picked. Colonies from several agar plates were re-streaked to obtain pure cultures. The resistance to vagococcin T was confirmed and quantified by determining the bacteriocin activity towards the mutants compared to the wild type strain. Genomic DNA of mutant strains was isolated with GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Saint-Louis, MO, USA) according to manufacturer’s instructions and sent to Novogene (Novogene Bioinformatics Technology Co., Ltd, Beijing, China) for sequencing (NovaSeq 150 bp paired-end). Reads from the wild type was assembled using SPAdes v3.15.3 to obtain reference contigs. Snippy was used to identify variants by mapping the reads from mutant isolates to the reference contigs using the default settings (82).

**Accession number**

The DNA sequence of vagococcin T gene cluster was submitted to GenBank under the accession number OM959625.
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Figure S1 Fractions (1 µl) from reversed-phase chromatography corresponding to the first (21 to 24) and second peak (26 to 30) were spotted individually (to the left and above black bars) and in combination (1:1 v/v ratio) on a lawn of *E. faecium* LMG 20705. Fractions spotted individually produced no or only small/diffuse inhibition zones, some fractions produced large inhibition zones when spotted in combination with the largest zone produced by a combination of fractions 23 and 28.
Table S1 Antibiotic resistance of *Enterococcus faecium* LMG 20705.

| Antibiotic               | Gene<sup>a</sup> | Gene product                                      | Accession number |
|--------------------------|-------------------|---------------------------------------------------|------------------|
| ampicillin<sup>b</sup>   | -                 | -                                                 | -                |
| aminoglycoside           | aac(6’)-Ii        | aminoglycoside 6’-N-acetyltransferase              | WP_002293989.1   |
| clindamycin<sup>b</sup>  | lnuB              | lincosamide nucleotidyltransferase                | WP_002294514.1   |
| erythromycin<sup>b</sup> | ermB              | rRNA adenine N-6-methyltransferase                | WP_001038795.1   |
| kanamycin<sup>b</sup>    | aph(3’)-IIIa      | aminoglycoside O-phosphotransferase               | WP_001096887.1   |
| pleuromutilin            | eatA              | ABC-F type ribosomal protection protein           | WP_002296175.1   |
| spectinomycin            | ant(9)-Ia         | aminoglycoside nucleotidyltransferase             | WP_002294509.1   |
| streptogramin A<sup>b,*</sup> | lasE        | ABC-F type ribosomal protection protein           | WP_002294513.1   |
| streptogramin B<sup>b,*</sup> | msrC         | ABC-F type ribosomal protection protein           | WP_063854349.1   |
| streptomycin<sup>b</sup> | ant(6)-Ia        | aminoglycoside nucleotidyltransferase             | WP_001255866.1   |
| streptothricin           | sat4              | streptothricin N-acetyltransferase                | WP_000627290.1   |
| tetracycline<sup>b</sup> | tetL              | tetracycline efflux MFS transporter               | WP_002294500.1   |
|                         | tetM              | tetracycline resistance ribosomal protection protein | WP_063856394.1   |
| vancomycin<sup>b</sup>/teicoplanin<sup>b</sup> | vanA           | D-alanine-(R)-lactate ligase                      | WP_001079845.1   |
|                         | vanHA             | D-lactate dehydrogenase                          | WP_001059542.1   |
|                         | vanRA             | DNA-binding response regulator                    | WP_001280781.1   |
|                         | vanSA             | histidine kinase                                  | WP_002305818.1   |
|                         | vanXA             | D-Ala-D-Ala dipeptidase                           | WP_000402348.1   |
|                         | vanYA             | D-Ala-D-Ala carboxypeptidase                      | WP_001812592.1   |
|                         | vanZA             | glycopeptide resistance protein                   | WP_000516404.1   |

<sup>a</sup> Found in *E. faecium* LMG 20705 genome with AMRFinderPlus

<sup>b</sup> Tested and confirmed by disc diffusion method according to EUCAST

<sup>*</sup> Quinopristin/dalfopristin resistance
Reviewer comments:

Reviewer #1 (Comments for the Author):

The authors report on a novel two-component bacteriocin isolated from a species that was not previously reported as a bacteriocin-producer, although there was some evidence indicating the possible presence of bacteriocin-encoding genes in a recent report (doi: 10.1093/g3journal/jkaa034). The bacteriocin has a good chance to serve as a candidate for control of MDR enterococci and other health-challenging microorganisms (including Gram-negative bacteria, considering that some of the two-component bacteriocins are inhibiting them when combined with synergistically-acting antibiotics). Moreover, the species is considered by investigators as a potential probiotic for animals of agricultural importance. Therefore, the study is well-justified. The authors used adequate methods to address the study's objective. These methods are sufficiently described in the manuscript's Materials and Methods chapter. The reviewer has just a few minor comments/suggestions which are penciled in the manuscript for the authors' convenience (see attached).

Line 18: I am not very sure, but wouldn’t it be more correct to say that the sequencing revealed the genes for a putative two-peptide lantibiotic?

Our response: the suggested rephrasing has been made.

Line 81: Actually, in this paper the authors also reported on the presence of the genes coding for a bacteriocin in their isolate, although the bacteriocin synthesis etc. were not confirmed.

Our response: as the reviewer points out, putative bacteriocin genes have been found bioinformatically in genomes classified within the genus Vagococcus, for this reason we use the term characterized as opposed to discovered or found. Characterization implies a description of its property and/or quality.

Line 172: Here and elsewhere: please, consider giving the isolate its strain identity and mention the species name along with the strain designation throughout the manuscript. This is important for the future traceability.

Our response: the isolate has been giving a strain name Vagococcus fluvialis LMGT 4216 and submitted to our strain collection (LMGT). The full name is now used throughout the manuscript. In addition, both the sequencing reads and full assembly has been submitted to NCBI with the accession number PRJNA836177. The accession number has been included in the accession number section in the paper.

Line 241: Error bars on the graph? If error bars make the image too messy and these are the averages, please, add it in the figure legend.

Our response: this assay has now been repeated with the requested error bars included in the new figure (graph).

Line 331-336: This is purely a repetition of the results and as such does not belong to the Discussion chapter of the manuscript. Please, consider removing.
Our response: these lines have now been rephrased and shortened to be less repetitive of the results section.

Line 337: The use of the term “synergy” requires support with data on FIC or isobolograms. Perhaps, it would be more appropriate to say that the noticeably higher activity was observed in... etc.

Our response: although we disagree that using the term “synergy” requires quantitative data, the recommended change has been made.

Line 409-410: If the space allows, the authors may consider adding a few words on the possible broadening of the bacteriocin inhibition spectrum by including Gram-negatives, when used in combination with synergistically acting antimicrobials – see, for instance: doi: 10.1186/1471-2180-13-212

Our response: We are excited to explore these possibilities in future work, in the current manuscript the focus is on the discovery, isolation, and characterization of the bacteriocin.

Line 411: Data statistical analysis is missing – as applied for some assays (see comment in the manuscript).

Our response: the necessary statistical analysis/information for Figure 6 (propidium iodide assay) has been added to the materials and methods section for this assay.

Line 442: city, country

Our response: city and country has been added.

Line 447: Please, see the reviewer’s comment regarding the need for the strain designation.

Our response: the full name with strain designation is now used here.

Line 455: Please, check the journal’s requirements. For most journals, only the first time the manufacturer’s information should be delivered in full, and every next citation should contain only the name of the company.

Our response: the suggested change has

Line 469: Strictly speaking, the name of the company is Sigma-Aldrich.

Our response: the name of the company is now written in full.
Reviewer #2 (Comments for the Author):

This is well planned, performed and written paper. Authors reports on new bacteriocins, produced by the strain belong to species that was never before reported as bacteriocinogenic. Some corrections needs to be taken into account by the authors.

Will be nice if authors can test more VRE strains in order to validate the suggested activity against different antibiotic resistance strains.

Authors have some repetitions in Results and Discussion sections. Please, try to be more focus and do not repeat the information. Moreover, in Results section try to be more focus on results and do not discuss the results. This needs to be dedicated to the discussion section.

**Our response:** We believe it is clear from the text that no cross resistance is expected between bacteriocins like vagococcin T and antibiotics like vancomycin, due to their different target and mode of action. The focus of this work is on the discovery, isolation, and characterization of vagococcin T.

Authors have preliminary test showing that BHI was optimal media for bacteriocin production? 60% ammonium sulphate was chosen based on preliminary ammonium sulphate % optimisation or was based on the literature? Please, consider to change the text and to be more informative.

**Our response:** an explanation for this choice has been included in the discussion.

Any reference for the method described under Ln 477-482?

**Our response:** references to the publications this method has been adapted from is now added. Also changed the phrasing “the propidium iodide method” to “a propidium iodide method”, as the method used is adapted from more than one publication and not one specific described method.

Please, reference list needs additional attention. Please, pay attention to the use of italics, page numbers and volumes of the journals.

**Our response:** as the reviewer correctly points out, the reference list was not properly formatted. This is now fixed.

Reviewer #3 (Public repository details (Required)):

The DNA sequence of vagococcin T gene cluster is provided, but perhaps the authors should also provide the fully sequenced genome of the strains.

**Our response:** the full annotated genome and sequencing reads has been made public on NCBI, accession numbers are added to the manuscript.
The paper submitted by Rosenbergová et al., describes the identification and characterization of a new two-peptide lantibiotic called vagococcin T. According to the authors this bacteriocin is the first one described in the genus Vagococcus and displays a range of activity against Gram-positive bacteria (except S. aureus) with notable activity against pathogens as E. faecium. From the point of view of the sequence, vagococcin T presents differences in sequence and structure with respect to other known two-peptide lantibiotics, especially the β-peptide. From the point of view of the action mechanism, the authors have shown a pore-forming activity and also they have characterized resistant mutants against the bacteriocin.

The paper is well written and the experiments are properly designed. However, some points should be addressed.

1) Line 90, please provide the data as supplementary information. The MIC for the antibiotics after the disk diffusion test and the rep-PCR.

**Our response:** the antibiotic resistance status of the strain can be determined by either a MIC assay or a disc diffusion test, according to EUCAST, in our opinion doing both is redundant and adds no additional information. Also, Rep-PCR is a routine procedure which only aids in excluding clones/duplicates during screening and sequencing, the profiles provide no useful information to the reader as far as we can tell.

2) Line 111. How did you identify the strain as Vagococcus fluvialis?

**Our response:** the full annotated genome and sequencing reads has been made public on NCBI, accession numbers are added to the manuscript. NCBI Prokaryotic Genome Annotation Pipeline was used to annotate the assembly and to verify the taxonomy, the highest average nucleotide identity to type assemblies was with *Vagococcus fluvialis*.

3) About the vagococcin gene cluster, can the author provide a figure comparing the organization of the different gene clusters of the known two-peptide lantibiotics? In figure 1, vcnG is indicated but is not in the gene cluster.

**Our response:** A comparison of (two-peptide) lantibiotic clusters are presented in numerous publications and reviews, we feel that including clusters of many other lantibiotics would add clutter to the manuscript.

4) In Table 3, is it possible to provide the data as mg/L? Do you have any hypothesis about why S. aureus is resistant?

**Our response:** to our knowledge, there are no good and/or useful method of determining the concentration of lantibiotics without a standard. And in our experience, methods developed for proteins (Lowry Method, Bradford Assay, Qubit, NanoDrop/absorbance) are not well-suited for lantibiotics (e.g. nisin). Instead we have changed the Table text to include the volume of antimicrobial used. Regarding resistance to *S. aureus*, it is common that a bacteriocin is devoid of activity against certain species and genera, and the reason behind this is often obscure. Hence further investigation into this path requires
much extra work as well as being beyond the scope of the present study. We also feel that any hypothesis now would be pure speculation which we try to avoid.

5) In figure 3, the tubes in which activity was observed and the peaks from the HPLC do not match. Is it ok?

Our response: we mention this observation in the discussion, although the two peaks did not correspond with the bacteriocin peptides, they were present at the same stages of the elution in all purification runs. The nature behind the mismatch is not clear but we have identified the fractions containing the involved peptides, not only by bioactivity assay but by (MALDI-TOF) mass spectrometry which reveals their expected masses.

6) Considering that this bacteriocin is the first described with this topology and that this work is focused on the characterization of this new bacteriocin, in figure 5 the proposed dehydration profile and ring formation pattern should be confirmed.

Our response: we do not agree that this bacteriocin is the first described with this ring topology, the proposed ring formation is well-conserved, and the proposed structures are justifiable with the literature. In addition, the results presented in this work do not rely on the exact structure of the bacteriocin. A structure is proposed to correlate the gene products to the molecular weights determined by MALDI-TOF MS, which show a considerable agreement.

7) The strain full genome should be also deposited.

Our response: the full annotated genome and sequencing reads has been made public on NCBI, accession numbers are added to the manuscript.
May 24, 2022

Prof. Dzung B Diep
Norwegian University of Life Sciences
Laboratory of Microbial Gene Technology
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Re: Spectrum00954-22R1 (Identification of a Novel Two-Peptide Lantibiotic from Vagococcus fluvialis)

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