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Characterization of Antimicrobial Substances Produced by *Enterococcus faecalis* MRR 10-3, Isolated from the Uropygial Gland of the Hoopoe (*Upupa epops*)

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The uropygial gland (preen gland) is a holocrine secretory gland situated at the base of the tail in birds which produces a hydrophobic fatty secretion. In certain birds, such as the hoopoe, *Upupa epops*, the composition of this secretion is influenced by both seasonal and sexual factors, becoming darker and more malodorous in females and in their nestlings during the nesting phase. The secretion is spread throughout the plumage when the bird preens itself, leaving its feathers flexible and waterproof. It is also thought to play a role in defending the bird against predators and parasites. We have isolated from the uropygial secretion of a nestling a bacterium that grows in monospecific culture which we have identified unambiguously by phenotypic and genotypic means as *Enterococcus faecalis*. The strain in question produces antibacterial substances that are active against all gram-positive bacteria assayed and also against some gram-negative strains. Its peptide nature identifies it as a bacteriocin within the group known as enterocins. Two peptides were purified to homogeneity (MR10A and MR10B), and matrix-assisted laser desorption ionization–time of flight (mass spectrometry) analysis showed masses of 5201.58 and 5207.7 Da, respectively. Amino acid sequencing of both peptides revealed high similarity with enterocin L50A and L50B (L. M. Cintas, P. Casaus, H. Holo, P. E. Hernández, I. F. Nes, and L. S. Håvarstein, J. Bacteriol. 180:1988–1994, 1998). PCR amplification of total DNA from strain MRR10-3 with primers for the L50A/B structural genes and sequencing of the amplified fragment revealed almost identical sequences, except for a single conservative change in residue 38 (Glu→Asp) in MR10A and two changes in residues 9 (Thr→Ala) and 15 (Leu→Phe) in MR10B. This is the first time that the production of bacteriocins by a bacterium isolated from the uropygial gland has been described. The production of these broad-spectrum antibacterial substances by an enterococcal strain living in the uropygial gland may be important to the hygiene of the nest and thus to the health of the eggs and chicks.

The uropygial gland (UG), also known as the preen gland, is a holocrine secretory gland situated at the base of the tail in birds. It produces lipids and waxy sebum that coat the bill and are transferred to the plumage during preening. Although it is believed that the main function of this secretion is to waterproof the feathers and make them flexible (21), it is also reported to have antimycotic (3) and antibacterial (29) properties. In some species, the chemical compositions of the secretions appear to differ between sexes and also change according to the season (24, 26), and thus, it has been suggested that the function of this gland may vary between sexes and at different times of the year. During the nesting phase of the hoopoe (*Upupa epops*), the UG secretions of the female and nestlings turn from white to brown and become more viscous and malodorous than that of the male (9). This special secretion has been interpreted as acting mainly as a repellent to predators (9, 27), an interpretation which has recently received experimental support from a closely related species, the red-billed woodhoopoe (*Phoeniculus purpureus*) (6). Nevertheless, hoopoes are hole-nesting species that reuse the same hole for several years and are thus under strong selection pressure from parasites during the nesting phase. While the male enters the nest only rarely during reproduction, the female incubates the eggs and stays inside the nest for the whole day for up to 5 to 6 days after the eggs hatch (9). It is possible, therefore, that the differences between the UG secretions of males and those of females and nestlings may be related to a wider risk of pathogenic infection of the birds inside the nest hole, and in fact, antimicrobial activity has recently been demonstrated in some of the chemical components of the secretion of the red-billed woodhoopoe (6). Curiously, a novel, atypical species of *Enterococcus*, *Enterococcus phoeniculicola*, has been found to grow in monocultures in the UG of this bird (22) and may be responsible for some of the properties of its secretions.

Enterococci are facultatively anaerobic, gram-positive, catalase-negative cocci belonging to the lactic-acid bacteria (LAB) group. The main habitat of enterococci is the intestines of warm-blooded animals (17), but because of their ability to survive heat treatment and adverse environmental conditions, they can be found in many foods of animal origin, such as milk, cheese, and fermented sausages (14).

Bacteriocins are usually defined as antimicrobial peptides

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that are synthesized ribosomally by bacteria and secreted extracellularly and that act against closely related species without affecting the producer strain (20). Nevertheless, many LAB bacteriocins, particularly those produced by enterococci (enterocins), are characterized by their broad range of activity against many gram-positive bacteria (18). In fact, these antimicrobial peptides, together with organic acids, are the main LAB substances responsible for the prevention of food spoilage (18, 19). The production of bacteriocins has been recorded in all major lines of prokaryotes (5, 20, 30) and may therefore play an important role in maintaining bacterial community structures (5, 28). There are several possible roles for these antibacterial peptides in the microbial ecosystem: elimination of competitors, increasing the chances of invasion and colonization of an already occupied niche, and defense against invasion by other bacteria and advancing neighboring cells. They may also play a part in quorum-sensing mechanisms (28).

The aims of this study were to identify the enterococcal strain MRR 10-3, isolated from the UG secretion of a nestling hoopoe, and to characterize the spectrum of activity, structure, and genetics of the antimicrobial substance produced by this strain.

TABLE 1. Antimicrobial activities of MR10 enterocins concentrated by CM-25 chromatography and assayed by drop test

Indicator strain	Sensitivity[a]	Reference or source[b]
E. faecalis S-47	++	15
E. faecalis JH2-2	++	31
E. faecium QF22	++	LC
E. faecium 34	++	LC
E. faecalis A-48-32	−	23
Lactococcus lactis LM2301	++	32
Listeria innocua 4030	++	CECT
L. monocytogenes 4032	++	CECT
S. aureus 240	+	CECT
Micrococcus luteus 241	++	CECT
Bacillus cereus LWL1	++	LC
B. licheniformis D13	++	LC
B. licheniformis 491	++	CECT
Salmonella choleraesuis 443	−	CECT
Klebsiella sp.	−	LC
E. coli 774	−	CECT
E. coli U-9	±	LC

[a] Bacteriocin activity is expressed as the size of inhibition zones: −, no inhibition; ±, turbid inhibition zone; +, <10 mm; ++, ≥10 mm.
[b] LC, laboratory collection; CECT, Spanish Type Culture Collection.

MATERIALS AND METHODS

Bacterial strain and culture media. The enterococcal strains used in this study and their sources are listed in Table 1. The strain *Enterococcus faecalis* MRR 10-3 was isolated in the course of an investigation into bacterial presence in the uropygial gland of the hoopoe (*Upupa epops*) from the UG secretion of a nestling during the 2004 breeding season in Guadix (Granada, southeastern Spain). The secretion was obtained with a micropipette directly from the UG of a live bird. To minimize the potential for contamination, the feathers around the natural opening of the gland were separated prior to sampling. Strain MRR 10-3 was selected among a wider sample of isolates because it produced an antimicrobial substance with a broad spectrum of activity. *E. faecalis* S-47 was used as an indicator strain in assays of antimicrobial activity (15). Other bacterial strains used to establish the inhibitory spectrum are listed in Table 1. All strains were grown in brain-heart infusion broth (BHI) (Scharlau, Barcelona, Spain) at 37°C for 18 h. Solid media were prepared by adding 1.7% agar to BHI medium. When necessary, to avoid the interference of any inhibitory effect from organic acids in the detection of antimicrobial substances, BHI agar was dissolved in 0.1 M sodium phosphate buffer, pH 7.2. Enterococci were routinely stored at 4°C and maintained as stocks at −80°C in 40% glycerol.

Identification of strain MRR 10-3. Strain MRR 10-3 was initially characterized phenotypically as described elsewhere (11). Biochemical means (API ID 20 Strep System, BioMérieux, Marcy l'Etoile, France) were used to identify the strain to the species level.

For genotypic identification, total DNA was extracted from bacterial cultures grown to stationary phase using the AquaPure Genomic DNA isolation kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions and was used as a template for genus- or species-specific PCR amplification. Molecular identification of strain MRR 10-3 was carried out first by genus-specific PCR, using the primers Entero 1 and Entero 2 (10), which are expected to amplify a 404-bp fragment from the 16S rRNA gene (nucleotides 606 to 1029) from the genus *Enterococcus*. The amplification procedures in the assays for the identification of *Enterococcus* species were performed using primers specific for *E. faecalis* and *Enterococcus faecium*, as described by Dutka-Malen et al. (12), through the amplification of intragenic fragments of the species-specific genes encoding D-Ala–D-Ala ligases (*ddl* genes). Amplification reactions were carried out in an iCycler 170-8720 (Bio-Rad, Hercules, CA). *E. faecium* QF22 and *E. faecalis* JH2-2 were used as positive controls. The V3 variable region of the 16S rRNA gene was also amplified using the primers WO1_for and WO12_rev (25), purified with a Perfectprep Gel Cleanup kit (Eppendorf, Hamburg, Germany), and sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction automated sequencer (Perkin-Elmer, Foster City, CA). Homologies were searched for in the BLASTN database (National Center for Biotechnology Information) using BLAST (2).

Genetic characterization of the bacteriocin produced by strain MRR 10-3. Specific PCR amplifications were carried out using total DNA from strain MRR 10-3 as a template and primer-specific oligonucleotides from the 5′- and 3′-terminal regions of the structural genes of the different enterocins, as previously described by Achemchem et al. (1). Homologies were searched for in the BLASTN database (National Center for Biotechnology Information) using BLAST (2).

Inhibitory-activity assays. To assay the inhibitory activities of liquid samples throughout the purification process and to study the physicochemical properties of inhibitory substances, the agar well diffusion method (16), using 8-mm (outer diameter) stainless steel cylinders, was employed. The activities of serial dilutions of samples were determined and expressed, in arbitrary (AU) units per ml, as the inverse of the lowest sample dilution able to produce a visible inhibition on a lawn of the indicator strain. The specific activities of samples were expressed as AU bacteriocin/mg of protein.

To establish the inhibitory spectrum of the antimicrobial substance produced by strain MRR 10-3, the drop-plating technique was used. The appearance of a clear inhibition zone around the fractions (10 μl) assayed indicated the sensitivity of the indicator strain.

Bacteriocin production and purification. The influence of the growth medium on the production of inhibitory activity was studied by culturing *E. faecalis* MRR 10-3 at 37°C in different media: BHI (Scharlau), M-17 (Scharlau), GM-17 (M-17 with 1% glucose), and complex medium (CM) (16). Samples were extracted from cultures at regular intervals and tested for inhibitory activity by the agar well diffusion method.

Inhibitory substances were purified from batches of CM broth inoculated with an overnight culture of strain MRR 10-3 (4% vol/vol) and incubated at 37°C for 10 h. Bacteriocin was recovered by ion-exchange chromatography on carboxymethyl-Sephadex CM-25 and then by hydrophobic interaction and reversed-phase chromatography using a column filled with C_{18} (Waters Corporation, Milford, MA). Active fractions were purified by reversed-phase high-performance liquid chromatography on a C_{18} column (WAT036975; Waters) (1, 16). The protein concentrations of active samples were determined by the Bradford method (4).

Molecular masses and N-terminal sequences were characterized in the Plataforma de Proteómica of the Serveis Cientificotècnics of the Universitat de Barcelona (Barcelona, Spain). Briefly, the molecular mass of the purified bacteriocin was determined either by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)/TOF (mass spectrometry) (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA) or electrospray ionization-tandem mass spectrometry (Q-TOF Global; Micromass-Waters).

The amino acid sequences of the N termini were analyzed via Edman degradation using a Procise cLC 492 sequencer (Applied Biosystems). Materials, reagents, and standards were from ABI. The sequencing programs (operating steps) were based on the manufacturer's recommendations.

Stability against heat, pH, and enzymes. For assays of stability against heat, pH, and enzymes, aliquots of semipurified bacteriocin from CM-25 were used. In all cases, after being treated, samples were tested against *E. faecalis* S-47 by the well technique, and the remaining antimicrobial activity was calculated in comparison to an untreated control.

The chemical nature of the antimicrobial substance produced by strain MRR 10-3 was investigated by determining its sensitivities to different enzymes. Bacteriocin samples in phosphate buffer (50 mM, pH 7.2) were mixed with equal volumes of catalase (2 mg/ml), trypsin (5 mg/ml), and proteases from *Streptomyces griseus* (5 mg/ml) and *Aspergillus oryzae* (5 mg/ml) before being incubated for 1 h at 37°C.

To test pH stability, the samples were adjusted to selected pH values by adding different buffer solutions—orthophosphoric acid-NaOH (0.2 M, pH 4.6) for pH 4.5, sodium phosphate (0.2 M, pH 7) for pH 7, and glycine-NaOH (0.1 M, pH 9) for pH 9—and were then incubated at room temperature for 1 h before being neutralized. Subsequently, all samples (including a control) were adjusted to the same final volume with distilled water.

To determine heat stability, bacteriocin samples were heated to 60°C for 30 min, 80°C for 10 min, and 100°C for 5 min and then cooled to room temperature.

Nucleotide sequence accession number. The GenBank accession number for the MR10A and MR10B genes is DQ366596.

RESULTS AND DISCUSSION

Identification of the bacteriocinogenic strain MRR 10-3. The bacteriocinogenic strain MRR 10-3 was isolated during an earlier investigation carried out on the microbial communities in the secretion of the uropygial gland of the hoopoe. A preliminary study of its antagonistic properties carried out by the lawn-spotting technique revealed that this strain inhibited *Enterococcus faecalis* S-47, *E. faecalis* A-48-32, *Listeria monocytogenes*, and *Bacillus licheniformis* D-13. It should be emphasized that by conventional culture, this was the only bacterial strain found in the uropygial secretion of the bird. Strain MRR 10-3 was assigned to the genus *Enterococcus* according to the following phenotypic characteristics: gram-positive catalase-negative coccus, capable of growing on Kenner fecal-agar selective medium, in an alkaline medium (pH 9.6) and in the presence of 6.5% NaCl, and also by 2-3-5 triphenyl-tetrazolium chloride reduction and esculin hydrolysis. According to the reaction profile obtained using the API Rapid ID 20 Strep identification system, strain MRR 10-3 was assigned, although quite ambiguously, to the species *E. faecalis* (55.6% similarity). Thus, we resorted to molecular methods to verify the identity of the strain. The PCR results obtained by first using the genus-specific primers Entero 1 and Entero 2, which amplify a 404-bp fragment of rRNA from *Enterococcus*, and afterwards two different primer pairs specific for *E. faecium* and *E. faecalis*, which amplify the 940 bp corresponding to the intragenic fragments of the species-specific genes encoding D-Ala–D-Ala ligases (*ddl* genes) (12), strongly suggest that MRR 10-3 belongs to the species *E. faecalis* (data not shown). Additionally, amplifying and sequencing a 700-bp fragment of the small 16S rRNA gene subunit, including the V3 region, with primers WO1_for and WO12_rev (25) resulted in a sequence that was 100% identical to those of various *E. faecalis* strains, including the reference *E. faecalis* V583. Thus, strain MRR 10-3 was identified unambiguously as belonging to the species *E. faecalis*.

Physical-chemical characterization of enterocins MR10. To discover the chemical nature of the inhibitory substance produced by strain MRR 10-3, a study of the stabilities of active semipurified samples from CM-25 was carried out in the presence of proteolytic enzymes and catalase and changes in heat and pH. The results showed that the inhibitory substance was highly stable up to 100°C and from pH 4.6 to 9. Nevertheless, it completely lost its activity when treated with trypsin and proteases, though not with catalase, pointing to its peptide nature (data not shown). Thus, we named this substance bacteriocin, or enterocin, MR10.

TABLE 2. Purification of enterocins produced by *E. faecalis* MMR 10-3

Purification step	Total activity (AU)	Total protein (mg)	Sp act (AU/mg)	Yield (%)	Purification (*n*-fold)
Culture broth	100,000	30.00	3,333	100	1
Cation exchange	56,050	21.25	2,638	56.05	0.79
Reversed phase	303,650	22.74	13,354	300.03	4.01

Bacteriocin production, purification, molecular-mass determination, and partial amino acid sequence. Bacteriocin production was studied in different liquid media: BHI, M-17, GM-17, and CM. Major amounts of inhibitory activity were detected in GM-17 and CM after 8 and 12 h of growth, respectively. Thus, for purification purposes, 1 liter of 10-h CM culture (with a total of 100,000 AU) was used due to its optimum production qualities and minimal interference with the components of the growth medium. Inhibitory activities from culture broths were recovered by cation-exchange chromatography on carboxymethyl-Sephadex CM-25 and by hydrophobic interaction chromatography on C_{18}, following the procedure described above. The purification procedure details are summarized in Table 2. It is noteworthy that after cation-exchange on CM-25, 80,000 AU was not retained in this weak cationic matrix; thus, with the 56,050 AU that was adsorbed and subsequently eluted, we were able to calculate an increase in activity of 36,050 AU compared to the total 100,000 AU in the initial culture. This increase could be attributed to cell surface enterocin adsorption due to its cationic charge. In this way, enterocin adsorbed onto cells may well have been eliminated with them during the centrifugation step before activity was determined, thus leading to an underestimation of total enterocin production. Because the cation-exchange was done with a crude culture without removing the cells, the portion of bacteriocin adsorbed onto cells may have been interchanged by resin union and so increased the total amount of inhibitory activity detected. One other notable result was that despite our use of a high gel/supernatant ratio (1:10), a considerable part of the activity (80,000 AU) was not retained in the cationic matrix. Thus, we cannot rule out the possibility that another different, noncationic bacteriocin was present.

During the second purification step, inhibitory activity coming from CM-25 exchange was concentrated by hydrophobic interaction chromatography into C_{18}. Once again, the total activity recovered from C_{18} after this step was higher (three times) than that introduced. This remarkable increase in activity might be a result of some type of intermolecular cooperation when the bacteriocin was concentrated or of some conformational change in its structure brought about by the organic solvents used in the hydrophobic interaction chromatography.

Subsequent purification of the active fractions by reversed-phase high-performance liquid chromatography showed the

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MR10A : MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIDWIKKHI : 44
L50A  : MGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIEWIKKHI : 44

MR10B : MGAIAKLVAKFGWPFIKKFYKQIMQFIGQGWTIDQIEKWLKRH- : 43
L50B  : MGAIAKLVTKFGWPLIKKFYKQIMQFIGQGWTIDQIEKWLKRH- : 43
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FIG. 1. Deduced amino acid sequences of enterocins MR10A and MR10B obtained from the translation of the ADN fragment amplified using specific oligonucleotides for enterocin L50A and L50B structural genes as primers. The respective alignments with L50A and L50B enterocins are also shown.

existence of two distinct peaks, 1 and 2, with antimicrobial activities that showed a synergistic antibacterial action (data not shown). This cooperative behavior may also explain the progressive increase in the total bacteriocin AU observed throughout the purification process, which would be favored by the concentration (molecular approximation) of both molecules. The mass spectrometry of these fractions analyzed by MALDI-TOF showed molecular weights of 5201.58 (enterocin MR10A) and 5207.5 (enterocin MR10B) for peaks 2 and 1, respectively. When the N-terminal amino acid sequencing of the two fractions was carried out by automated Edman degradation, identical sequences (MGAIAKLVAK) were obtained for both of them on the first 10 cycles. A comparison of this partial sequence with those of other known bacteriocins held in protein databases showed that it was 90% similar to that of enterocin L50B (differing in the threonine residue at position 9, which had been permuted by alanine) and identical to that of L50A (7, 13). Enterocins L50A/B are bacteriocins belonging to class II of the LAB bacteriocins and are composed of two hydrophobic/cation peptides that act synergistically when combined, especially with some indicator strains (7, 13).

Antimicrobial spectrum of enterocin MR10. The antibacterial activities of enterocin MRR 10-3 samples concentrated by CM-25 chromatography (MR10A plus MR10B) are shown in Table 1. MR10 was active against all gram-positive bacteria assayed, including *Staphylococcus aureus* and two strains of *Bacillus licheniformis*, and was also slightly active against *Escherichia coli* U-9. When bacteriocin samples from CM-25 exchange were subsequently concentrated by C_{18} chromatography, a clear increase in activity against this gram-negative bacterium was observed. These active samples coming from CM-25 showed no activity against the bacteriocin AS-48 producer *E. faecalis* A-48-32. This result is particularly noteworthy because *E. faecalis* strain A-48-32 was susceptible when challenged by the MRR 10-3 strain by the spots on the lawn assay (data not shown), thus indicating that it may produce other noncationic bacteriocins that are not retained in the CM-25 matrix.

Genetic characterization of enterocins MR10A and MR10B. Plasmid analysis revealed that the original *E. faecalis* MRR 10-3 strain did not contain any plasmids (results not shown), so these genes must be harbored in the chromosome. PCR amplification was carried out to search for the structural genes responsible for MRR 10-3 enterocins. Given the similarity of the enterocins L50A/L50B to those produced by strain MRR 10-3 of *E. faecalis*, PCR amplifications were made using primers from these structural genes (7, 13) and total genomic DNA as a template. In this way, both structural genes were detected (data not shown).

The PCR product obtained was purified and sequenced (Fig. 1) and corresponded to a DNA fragment which could be translated into two peptides that were homologous to enterocins L50A and L50B, although with a conservative change in residue 38 (Glu→Asp) in MR10A and two changes in residues 9 (Thr→Ala) and 15 (Leu→Phe) in MR10B. The theoretical molecular weights of peptides MR10A and MR10B, as deduced from their presumptive genes, are 5,175.32 and 5,181.25, respectively, which differs from the weights assigned by MALDI-TOF spectrometry to these peptides (5,201.5 and 5,207.8, respectively). Differences between the theoretical weights and the empirical weights found by the MALDI-TOF method have been encountered in all the enterocins similar to L50A/B (1, 8), but this may be ascribed to oxidation in the methionine residues or other alterations in the molecules produced during the purification process.

The amino acid compositions of both the MR10A and MR10B peptides point to their being cationic, with each having eight basic residues. In fact, the predicted isoeletric points for MR10A and MR10B are 10.02 and 10.23, respectively. Thus, the high proportion of hydrophobic polar-uncharged amino acids (21 hydrophobic residues plus 4 polar-uncharged residues in MR10A and 19 hydrophobic residues plus 4 polar-uncharged residues in MR10B) indicates the hydrophobic nature of these peptides. The hydrophobic nature of both MR10A/B, if they are indeed generated in the UG, would allow them to use the waxy UG secretion as a vehicle, thus being spread with the lipids throughout the plumage of the bird and onto the surface of the egg. It is also possible that the bacteriocins might be produced in situ in the feathers and on the eggs by strain MRR 10-3, which itself would have been spread with the secretion.

We also investigated the presence in strain MRR 10-3 of the other enterocin genes described in *E. faecalis* and *E. faecium* by PCR amplification. For this purpose, we used specific primers (1) for the structural genes of enterocins AS-48, EJ97, bac31, 1071A, and 1071B from *E. faecalis* and enterocins A, B, P, and Q from *E. faecium*. Positive and negative controls were carried out. The results were negative for all the enterocins assayed.

Concluding remarks. The results presented here on the physical-chemical characterizations, activity spectra, amino acid sequences, and molecular masses indicate that enterocins MR10A and MR10B from *E. faecalis* MRR 10-3 are variants of enterocins L50A and L50B. Nevertheless, several traits render the molecules described here different from the others: first,

enterocins L50A and L50B show no activity against *E. coli*; second, L50A and L50B are plasmid encoded (7, 13), whereas the MR10 enterocin genes are harbored in the chromosome; and third, the L50A and L50B producer strains belong to the species *E. faecium*, while enterocins MR10A and MR10B are produced by *E. faecalis*. However, the most interesting trait of enterocin MR10 is undoubtedly the source of the producer strain, the uropygial gland of a nestling hoopoe. This strain was isolated from the UG secretion during the period of reproduction, when it is dark and pungent. Until now, the suggested defensive properties against pathogens provided by uropygial secretions have been related to the antibacterial and antimycotic properties of acids and alcohols contained in its waxes, chemical products that are all apparently produced by the birds themselves. The bacteriocins produced by enterococci could widen the range of antimicrobial uropygial substances and help the hoopoe to defend itself against diseases, such as feather degradation produced by *B. licheniformis*.

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