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Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation

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SUMMARY

Among potential agents that might damage bird feathers are certain microorganisms which secrete enzymes that digest keratin, as is the case of the ubiquitous bacterium *Bacillus licheniformis*, present in both the feathers and skin of wild birds. It is therefore a good candidate for testing the effects of bird defences against feather-degrading microorganisms. One of these defences is the oil secreted by the uropygial gland, which birds use to protect their feathers against parasites. In previous studies we have shown how *Enterococcus faecalis* strains isolated from nestling hoopoes exert antagonistic effects against *B. licheniformis*, mediated by the production of bacteriocins. Consequently we hypothesized that this enterococcus and the bacteriocins it engenders might act as a defence against feather-degrading microorganisms in hoopoes. We investigated this hypothesis in a series of laboratory experiments and evaluated the extent to which the keratinolytic effects caused by *B. licheniformis* were reduced by the *E. faecalis* MRR10-3 strain, isolated from hoopoes, and its bacteriocins. In different treatments, feathers or pure keratin was incubated with *B. licheniformis*, *B. licheniformis* together with *E. faecalis* MRR10-3, and *B. licheniformis* together with the bacteriocins produced by *E. faecalis* MRR10-3. Our results were in accordance with the predicted effects on hoopoe feathers. There was a significant decrease both in pure keratin loss and in feather degradation in the presence of the symbiotic bacterium or its bacteriocin. These results suggest that by preening their feathers hoopoes benefit from their symbiotic relationship with bacteriocin-producing enterococci, which constitute a chemical defence against feather degradation.

Key words: *Bacillus licheniformis*, *Enterococcus faecalis*, feather degradation, symbiosis, *Upupa epops*, uropygial gland.

INTRODUCTION

Pressure from parasites has caused selection for a great variety of defence mechanisms in their hosts, including the immune system and behavioural traits that reduce the likelihood of infection and/or its negative effects (Price, 1980; Wakelin, 1996; Poulin, 1998). One defensive mechanism in birds that is particularly important in preventing feather degradation by pathogens consists of self cleaning, by using the secretion of the uropygial gland when preening (Jacob and Ziswiler, 1982). The many functions performed by a bird's plumage (apart from flight) include protection from external temperature (Ruben and Jones, 2000) and ultraviolet radiation (Wolf and Walsberg, 2000), but plumage also plays a role in sexual selection (Bennett et al., 1994). Feather degradation is detrimental to the reproductive success of birds (Pap et al., 2005). Plumage can be damaged by ectoparasites such as lice (Clayton, 1991; Kose and Møller, 1999) or microorganisms (e.g. Muza et al., 2000; Decostere et al., 2003) and so any mechanisms that reduce degradation would be of great selective advantage.

The protein keratin accounts for 90% of a feather's composition, and confers high mechanical stability and resistance to decomposition because the molecules are tightly packed and cross-linked to one another by cysteine bridges (Parry and North, 1998; Sangali and Brandelli, 2000). Feather-consuming organisms are therefore able either to digest keratin through the secretion of keratinases (Muza et al., 2000) or otherwise grow directly on feathers (Lucas et al., 2003; Grande et al., 2004) and affect the bird's fitness by, for instance, altering sexually selected colours (Shawkey et al., 2009).

It is known from studies in poultry (Gupta and Ramnani, 2006) that *Bacillus licheniformis* (Williams et al., 1990), *Streptomyces* spp. (Szábo et al., 2000), *Kocuria rosea* (Vidal et al., 2000) and *Microbacterium* spp. (Thys et al., 2004), among others, exert strong keratinolytic activity. One of the most frequent microorganisms isolated from feathers in the wild is *B. licheniformis* (Lucas et al., 2005; Whitaker et al., 2005). This bacterium adheres to the barbules and secretes keratinase, an enzyme that hydrolyses the keratin matrix of feathers. Under laboratory conditions *B. licheniformis* can completely degrade feathers in 24 h (Ramnani et al., 2005). Therefore, *B. licheniformis* is a good candidate for studying the effects of microorganisms on plumage quality and the efficiency of birds' defensive mechanisms against infection and the growth of feather-degrading bacteria.

Uropygial gland secretions usually include chemicals that are active against the growth of some Gram-positive bacteria (Jacob et al., 1997; Shawkey et al., 2003). Although these chemicals are probably produced mainly by the gland itself, bacteria living in the uropygial gland might also produce antimicrobial substances that inhibit the growth of feather-degrading bacteria. This is likely to be true in the case of the hoopoe (*Upupa epops*), which harbours enterococci that produce bacteriocin-like inhibitor substances in its uropygial gland (Martin-Platero et al., 2006; Soler et al., 2008). Specifically, the bacterium *E. faecalis* MRR10-3, found in the uropygial gland secretion of a nestling hoopoe, produces at least

two different bacteriocins with broad antimicrobial activity, including one active against *B. licheniformis* (Martin-Platero et al., 2006). Given that the relationship with symbiotic bacteria in the uropygial gland appears only during the nesting phase of females and offspring (Soler et al., 2008), that the females only sporadically leave the nest during this phase and that the probability of infection is considerably greater in hole nests (Møller and Erritzøe, 1996), the benefits of preventing keratinolytic activity by microorganisms would be significant for nestlings and incubating or brooding females.

The aim of our work was to test, through a series of laboratory experiments, the possible role of symbiotic bacteria and their bacteriocins in the prevention of feather degradation. We recorded the degradation of both hoopoe feathers and pure keratin by *B. licheniformis* under different treatments, including some in the added presence of strain MRR10-3 of *E. faecalis*, isolated from uropygial gland secretions of hoopoes, and some in the presence of the bacteriocins that it produces (Martín-Platero et al., 2006). There was a decrease in feather and keratin degradation when they were incubated with the *Bacillus* in the presence of the antagonistic *E. faecalis* or its bacteriocins, compared with when they were incubated with the *Bacillus* alone.

MATERIALS AND METHODS

Experimental procedure

We conducted two different experiments. The first consisted of measuring feather degradation after exposure to different experimental conditions (see below), whilst the second experiment was performed with pure keratin instead of feathers, under the same conditions, to compare the degradation effect on the structure of the protein itself with that found in whole feathers. Similar results would indicate that no other feather compounds, such as fat or wax, play any important part in the prevention of feather degradation indirectly by inducing *Enterococcus* bacteriocin production or even by the retention of bacteriocin molecules, owing to their hydrophobic character.

The bacteria were cultured in a 0.9% saline solution (SS, 0.9% sodium chloride in distilled water) containing either UV-sterilized feathers or pure sterile keratin. The solution was autoclaved in test tubes for 15 min at 121°C before the relevant components required for each treatment were added. Briefly, in treatment 1 (T1), to estimate the degradation effect of the keratinolytic bacterium upon feathers and keratin we added a culture of *B. licheniformis* (see below) to each of the substrate solutions. In treatment 2 (T2), to estimate the hypothetical effect of the symbiotic bacterium on the degradation by *B. licheniformis* of feathers and keratin we added a culture of *E. faecalis* MRR10-3 to a tube containing *B. licheniformis* and the relevant substrate solution. Treatment 3 (T3) was similar to T2 but in this case purified bacteriocin was added instead of *E. faecalis*. Thus, the differences in degradation between T1 and T2 and between T1 and T3 would indicate, respectively, the effect of the symbiotic bacterium and its bacteriocins in preventing feather degradation by *B. licheniformis*.

To check any possible effects of the experimental procedure we included several control treatments. Firstly, as controls to check any possible direct harmful effect of *E. faecalis* or the purified bacteriocin upon the keratin or feathers, we also added a culture of *E. faecalis* (control 1, C1) or purified bacteriocin (C2) to the substrate solution in the absence of *B. licheniformis*. Given that feathers and keratin may also be degraded in time by abiotic factors, in a third control group (C3) we incubated the substrate solution with neither bacteria nor bacteriocin. Finally, to rule out the possible effect of agitation of the substrate solution and to ascertain a reliable baseline degradation for the feathers, we used another control (C4), with no additions or incubation, merely keeping it in the fridge (4°C) from sterilization until the end of the experiment. In all cases we estimated the degree of feather or keratin degradation (see below) at the end of the incubation time.

In the experiment with feathers, each test tube contained a total of 3 ml of mixture, at a volume that ensured the whole feather was covered and therefore in contact with the incubation medium, composed as follows. T1: 2.9 ml SS plus 0.1 ml of *B. licheniformis* culture. T2: 2.8 ml SS plus 0.1 ml *B. licheniformis* culture and 0.1 ml *E. faecalis* culture. T3: 2.8 ml SS plus 0.1 ml *B. licheniformis* culture and 0.1 ml MR10 bacteriocin. C1: 2.9 ml plus 0.1 ml *E. faecalis* culture. C2: 2.9 ml plus 0.1 ml MR10 bacteriocin. C3: 3 ml SS. C4: 4°C treatment.

In the experiments with keratin, 24 mg per tube of sterile keratin-azure (Sigma-Aldrich, Madrid, Spain) was used as the substrate instead of feathers. Furthermore, in this case tubes from each treatment received a total volume of 6 ml of mixture to allow the estimation of keratin degradation at three different times whilst maintaining the correct proportions between the different components of the mixture.

Each treatment was replicated three times with a feather from a different hoopoe. After establishing the different experimental conditions, the substrates (feather or keratin) were introduced under sterile conditions into the tubes containing the experimental solutions. The tubes were homogenized by vigorous agitation and then incubated at 28°C under continuous agitation at 180 r.p.m. Although the optimum temperature for keratinolytic activity in *B. licheniformis* is around 40°C (Suntornsuk and Suntornsuk, 2003) we incubated them at a lower temperature to simulate more closely the natural conditions to which bird feathers are exposed inside the nest hole.

In the experiments with feathers, degradation was estimated after 7 days incubation, whilst the keratin experiments lasted 16 days because in previous tests carried out to decide the optimum incubation time we found that this was the time needed to detect degradation by *B. licheniformis* under our laboratory conditions. Nevertheless, in the keratin experiments we also took samples (500 µl) after 2 and 5 days to be centrifuged and analysed by spectrophotometry (see below for details) to check for any previously unperceived degradation.

Sampling and preparation of feathers

Feathers were obtained from one male and two female adult hoopoes *Upupa epops* L. captured in mist-nets at the beginning of the breeding season of 2006 at around 1000 m.a.s.l. in the 'Hoya de Guadix', in southeast Spain. Eight breast feathers from each individual were removed. To avoid differences in degradation due to melanin (Goldstein et al., 2004) we selected feathers of approximately the same size and pale-brown colour, which to our eyes were neither broken nor degraded in any way. The breast has been described as one of the parts of a bird's body that undergoes most preening activity (Van Liere et al., 1991) and therefore forms the natural setting for the hypothetical battle between *B. licheniformis* and symbionts from the hoopoe's uropygial gland.

The purpose of our experiment was to estimate the interference with keratin degradation in the natural scenario where the two bacteria coexist, i.e. the feather. We did not consider individual characteristics, such as identity, sex, or age, of the birds from which individual feathers were obtained as this would require an unacceptably complicated experimental design. Rather, we used two

different media, hoopoe feathers and the main component of the feathers, keratin, and tested the effects of the symbiotic bacteria and its bacteriocins upon the degradation of these media by the feather-degrading bacterium *B. licheniformis*. Similar results with feathers and pure keratin would allow us to discuss the functionality of the symbiotic bacteria under natural conditions.

All the feathers were collected on the same day and the experiments were performed 1 week later in the laboratory. After collection, the feathers were kept in Petri dishes in the dark at 4°C until the experiments were begun. Just before the experiments started the feathers were sterilized by exposure on both sides to UV radiation for 20 min.

Preparation of bacterial cultures and bacteriocin samples

As the feather-degrading bacterial strain we used *B. licheniformis* D13 from our own Department of Microbiology collection. Although *B. licheniformis* is quite a diverse feather-degrading bacterium (see Burtt and Ichida, 1999; Lucas et al., 2003), its keratinolytic activity is a generalized trait of the species and therefore does not vary between different strains (see Brandelli, 2008). Thus we used the well-known strain D13 in our experimental tests. As the bacteriocin-producing bacterium we used *E. faecalis* MRR10-3, isolated from the uropygial gland of a nestling hoopoe. The antagonistic activity of this strain against *B. licheniformis* and other bacterial species has previously been tested (Martin-Platero et al., 2006). Both D13 and MRR10-3 bacteria were inoculated on tryptone soy broth (Scharlau, Barcelona, Spain) and incubated for 12 h before use in the experiments. The *E. faecalis* culture was incubated at 37°C without agitation and *B. licheniformis* was incubated at 28°C under agitation to prevent the typical clumping of this species when grown in a liquid medium.

Bacteriocins were purified by ion-exchange chromatography on carboxymethyl-Sephadex CM-25 from cultures of *E. faecalis* MRR10-3 in complex medium broth (Galvez et al., 1986) according to a method described previously (Martin-Platero et al., 2006).

Degradation measurements

Three independent replicates of all treatments were made for statistical analyses.

Feathers

After incubation the feathers were washed in distilled water to remove free-living bacteria and medium and immediately prepared (including C4) for observation under a scanning electron microscope (SEM). They were fixed with 2.5% glutaraldehyde in 0.1 mol l^{-1} sodium cacodilate buffer (pH 7.4) for 2 h at 4°C. Any remaining fixative was eliminated by washing with the same buffer and then feathers were postfixed with 1% osmium tetroxide for 1 h at room temperature. The samples were washed with distilled water, dehydrated by immersion in a graded series of ethanol and dried in a Polaron CPD 7501 dryer (Quorum Technologies, Newhaven, East Sussex, UK). They were then coated with a layer of gold by perpendicular ionic bombing in a Polaron Unit SEM Coating E5000 for examination under a Gemini 1530 scanning electron microscope (Leo, Overkogen, Germany). Three pictures of different parts of each feather were taken at ×50 magnification.

The level of degradation was estimated visually by five different people uninvolved in the experiments. They were provided with a degradation index based on the progressive narrowing and shortening of the barbules. Degradation starts from the distal part, from whence it continues until the barbule is completely destroyed (Fig. 1). Thus, the index comprises six different levels (from 0 to 5) defined as: 0, if the barbules are intact (no degradation); 1, when just the tips of the barbules are degraded; 2, when approximately half of the barbules are degraded; 3, if more than half are degraded; 4, if there are still some vestigial barbules; and 5, if they have disappeared or are close to disappearing (as shown in Fig. 2). The observers were unaware of picture identity, i.e. from which feather or experimental treatments they were taken. Thus, we were able to calculate the repeatability not only of observer estimations but also of different feathers. The evaluation was made by using the one-way ANOVA (Senar, 1999), for which *P*-values were calculated according to different repeatability indices.

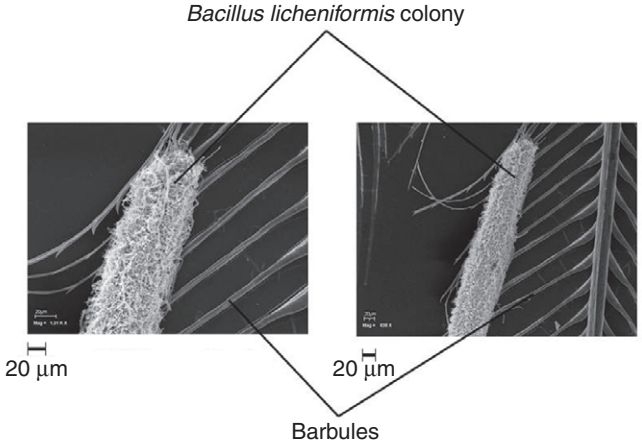

Fig. 1. Scanning electron microscope (SEM) microphotographs in which the narrowing effect of a *Bacillus licheniformis* D13 colony upon the barbules can be seen. A detail of a *B. licheniformis* colony growing on the feather is shown. It can be seen that after the keratin is digested the feather tips are thinner and even disappear.

Keratin

After 2, 5 and 16 days incubation, the test tubes were centrifuged at 17,000 ***g*** for 5 min to remove cells and any unsolubilized, non-hydrolysed keratin, and keratin degradation was then estimated by measuring the liberation of azo dye spectrophotometrically at 595 nm (Santos et al., 1996). Blue colouring of the supernatant indicates the presence of peptides deriving from the hydrolysis of keratin and so the absorbance at 595 nm of the supernatants was directly related to the quantity of keratin degraded.

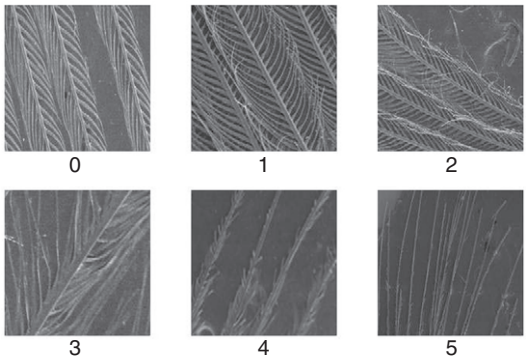

Fig. 2. SEM microphotographs showing the different levels used for the visual estimation of feather degradation. Numbers indicate the levels used by observers to estimate feather degradation, from 0 (no degradation) to 5 (barbules completely degraded).

Statistical procedure

All the analyses were made using Statistica 6.1 (StatSoft 2001) software and differences between treatments were explored using GLM (error type VI), in which the dependent variable was the degradation level for the substrate.

With the feathers, the treatment (fixed effect) and the observer (random effect) were the predictor variables explaining degradation. A non-significant interaction between treatment and observer would indicate that the effect of the treatment does not depend significantly upon observer identity. We also checked the effect of the hoopoe individual on the degradation level: given that the repeatability between observers was significant, we calculated the mean degradation for each picture, which was the dependent variable, and by using the treatment as the fixed factor and the individual as the random factor, we calculated the interaction between these two predictor variables. For the analyses of the effect of the treatment (between effect) upon keratin degradation *versus* incubation time, estimations at three different times (three estimations per sample at 2, 5 and 16 days) were taken to be 'within factor' in a repeated-measures ANOVA. *Post-hoc* comparisons (i.e. Tukey HSD test) were used to check for statistically significant differences between the various treatment groups.

RESULTS

Interference in feather degradation by *E. faecalis* MRR10-3 and bacteriocin MR10

We found significant repeatability of feather degradation estimates for different observers (F=7.90; d.f.=71, 285; P<0.001; R=77%) and feathers (F=8.82; d.f.=21, 335; P<0.001; R=85%), which validated our estimations.

Experimental treatments significantly affected the degree of feather degradation (effect of treatment, F=14.81; d.f.=6, 298; P<0.001), which was confirmed by all the observers (i.e. interaction between observer and treatment, F=0.76; d.f.=24, 298; P=0.79). The significant differences associated with experimental treatments were due to the high degradation of feathers incubated with *B. licheniformis* (T1 in Fig. 3). In accordance with this, *post-hoc* comparisons revealed that the level of degradation estimated for feathers incubated with *B. licheniformis* was significantly higher than that estimated for other groups (Tukey HSD *post-hoc* comparisons, all P<0.001; Fig. 3) and no significant differences were detected between the rest of the groups (P>0.4). Moreover, visual observation of the feathers after incubation (although with no quantification) revealed the existence of strongly adhering bacterial colonies in those incubated with the bacillus alone, but not in those that included the antagonistic enterococci or bacteriocins (M.R.-R., personal observation).

When calculating the influence of the individual hoopoe on the treatment effect, we found that the interaction between these two variables was not significant (F=0.22; d.f.=11, 47; P=0.99), while the treatment effect was (F=11; d.f.=6, 21.4; P<0.001). Therefore, the treatment effect was independent of the hoopoe individual.

Interference in keratin degradation by *E. faecalis* MRR10-3 and bacteriocin MR10

In experiments with purified keratin the highest degradation index once more occurred in the group treated with *B. licheniformis* alone, but these differences were appreciable only after 16 days incubation (repeated-measures ANOVA: effect of treatment, F=128.24; d.f.=5, 6; P<0.001; effect of time, F=593.3; d.f.=2, 12; P<0.001; interaction between R1 and treatment, F=108.06; d.f.=10, 12; P<0.001; Fig. 4). Degradation estimated for treatment with the bacillus alone was significantly different from that of treatment with the bacillus plus the enterococcus (Tukey HSD *post-hoc* comparisons P=0.04) and with the bacillus plus the bacteriocin (P<0.001), and also significantly different from the saline solution, bacteriocin and enterococcus controls (P<0.001 in all cases). Nevertheless, the treatment in which *E. faecalis* alone was added (C1) resulted in higher keratin degradation than other control groups (C1–C2 P=0.002, C1–C3 P=0.01), suggesting possible moderate keratinolytic activity on the part of *E. faecalis*.

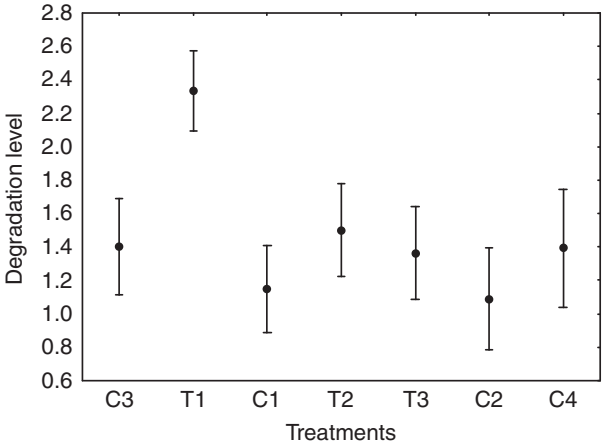

Fig. 3. Estimated degradation levels of feathers exposed to different treatments expressed as the mean values ± standard errors. T1: *B. licheniformis*; T2: *B. licheniformis* + *E. faecalis*; T3: *B. licheniformis* + bacteriocins; C1: *E. faecalis*; C2: bacteriocins; C3: control feathers with incubation; C4: control feathers without incubation.

DISCUSSION

Previous findings have shown that the symbiotic *E. faecalis* strains isolated from the hoopoe uropygial gland exert strong antagonistic activity against *B. licheniformis*, a keratin-degrading bacterium found amongst birds (Soler et al., 2008). Our past findings that one particular strain, MRR10-3, isolated from the hoopoe uropygial gland, produces two related bacteriocins active against this and other

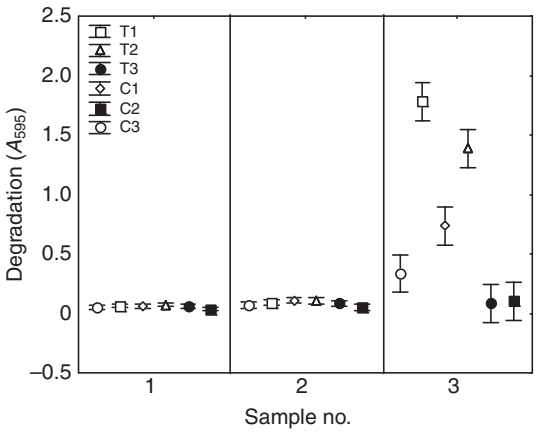

Fig. 4. Estimated degradation level of keratin exposed to different treatments expressed as the mean absorbance value at 595 nm (A_{595}) ± standard error, in which sample number 1 represents the 2nd day of the experiment, sample number 2 represents the 5th day, and sample number 3 represents the 16th day of incubation. T1: *B. licheniformis*; T2: *B. licheniformis* + *E. faecalis*; T3: *B. licheniformis* + bacteriocins; C1: *E. faecalis*; C2: bacteriocins; C3: control feathers with incubation.

potential bird pathogens (Martin-Platero et al., 2006) led us to suspect that this antagonistic effect may well be mediated by bacteriocins. Although it is generally assumed that bacteriocins confer ecological advantages upon producer bacteria by suppressing competitors, the effect of these antimicrobials in natural environments remains intriguing and far from clear (Riley and Chavan, 2007). The theoretical importance of the effects of bacteriocin production is even more interesting when bacteriocin-producing microorganisms exist in symbiosis (*sensu* De Bary, 1879) with macroorganisms, and consequently bacteriocin production might indirectly affect the fitness of the hosts. This is probably true of enteroccoci living in the uropygial gland of hoopoes, a symbiotic system in which the inhibition produced by the bacteriocin reduces the bacterial load on the eggshells and thus increases the probability of successful hatching (Soler et al., 2008).

We found that both feathers and pure keratin were degraded by *B. licheniformis* at a much slower rate when the symbiotic bacterium *E. faecalis* or its bacteriocin was brought into contact with the bacillus and the substrate (feathers or keratin). The differences detected in keratin degradation between the tubes containing *B. licheniformis* alone and those also containing *E. faecalis* isolated from hoopoe uropygial glands or its bacteriocin suggest that the enterococcus partially prevents the degradation of keratin by *B. licheniformis* and that it is completely inhibited by the isolated bacteriocin. These results indicate that the antagonistic activity of a bacterial species living in a hoopoe's uropygial gland against a generalist pathogenic, keratin-degrading bacterium widely found amongst birds is quite considerable. Furthermore, this detected inhibition of keratinase activity that may damage the feathers accords with the hypothesis that the beneficial effects of the symbiotic bacteria are mediated by the bacteriocin (Martín-Platero et al., 2006; Soler et al., 2008). Importantly, the results were quite similar whether we used a pure keratin or a natural feather substrate, which indicates that no other feather compound such as fat or wax influences the antagonistic activity detected to any significant extent. This observation is important since some authors have reported that the inhibitory effect of bacteriocins may differ greatly depending upon the peculiarities of the environment (Riley and Chavan, 2007), including the chemical composition of the environment and physical conditions of the medium (see Cleveland et al., 2001). For example, bacteriocins frequently fail to inhibit food-borne pathogens in their normal surroundings with the same efficacy as they do in laboratory cultures (Garriga et al., 2002; Muñoz et al., 2007).

In our experimental approach feathers were immersed in a 0.9% saline solution together with *B. licheniformis* and in some treatments *E. faecalis* or the bacteriocin was also included in the solution. This solution did not represent the natural conditions in which the bacteriocin is supposed to act in hoopoe feathers and consequently we cannot rule out the possibility of different results if the experiment was performed under natural conditions. Although it was demonstrated antagonistic activity in laboratory cultures (Martín-Platero et al., 2006), confirmation of such effects in a more natural scenario is needed. There is, however, evidence to show antagonistic activity on the part of the bacteriocinogenic strain living in the uropygial gland and in the gland secretion by hoopoes in the natural nest (Soler et al., 2008). We have also found that *B. licheniformis* and *E. faecalis* bacteria coexist in hoopoe feathers (M.R.-R., unpublished data). Consequently, it would seem very likely that under natural conditions symbiotic *E. faecalis* strains and/or their metabolites coexist with *B. licheniformis* on hoopoe feathers and that our results from experiments conducted under controlled laboratory conditions could be extrapolated to what might be expected to occur in nature.

Bacillus licheniformis occurs naturally in the plumage of birds (Whitaker et al., 2005) and its digestion of β-keratin would harm the feathers of its hosts (Whitaker et al., 2005). It is known that keratinase production and the feather-degradation activity of bacilli can be inhibited by some antimicrobial substances that may exist in secretions from the uropygial gland (Jacob et al., 1997; Bandyopadhyay and Bhattacharyya, 1999). In this way, preening has often been proposed to have a beneficial effect on plumage maintenance not only by reducing the ectoparasite load (Møller, 1991; Rózsa, 1993) but also by inhibiting bacterial growth (Burtt and Ichida, 1999). It was recently found (Reneerkens et al., 2008) that preen waxes reduce *B. licheniformis* activity in feathers, although when different chemical compounds pertaining to uropygial gland secretions were tested no changes in bacillus growth were observed. Our results indicate, however, that the feather-protecting antimicrobial properties of the uropygial gland of hoopoes are, at least partially, mediated by its incumbent *E. faecalis*, and provide the first evidence for the inhibition of feather degradation mediated by a bacterium (or the bacteriocins it produces) living in the uropygial gland of a wild bird.

Because female hoopoes do not abandon the nest during the period of incubation and brooding, preening with this uropygial gland secretion may protect their feathers from harmful bacteria. For growing nestlings the antagonistic effects of symbiotic bacteria against feather decomposers would be of prime importance for developing a healthy plumage, which will determine their flight capability (Machmer et al., 1992; Barbosa et al., 2003). Filming inside the nests has revealed that both nestlings and females spread their brown secretion onto their plumage; in fact, during approximately 71 h of recording of incubating females there were about 79 plumage-preening events (M.M.-V., unpublished data).

Traits that reduce feather degradation would be of selective advantage. Given the high risk of parasitism (Møller and Erritzøe, 1996) and the large number and diversity of bacteria in these environments, some of which have the potential to become opportunistic pathogens (Pinowski et al., 1994; Burtt, 1999), this benefit would be particularly important for hole-nesting birds such as hoopoes, which do not regularly clean out their nests. In addition, it is known that the keratinolytic activity of bacilli increases considerably with high temperature and humidity (Burtt and Ichida, 1999; Cristol et al., 2005), which are typical environmental conditions in hole nests during the incubation and nestling period. Unhatched eggs, eggshells from hatched or broken eggs, dead chicks, faeces and remains of food usually accumulate in hoopoe nests (J.J.S., M.M.-V. and M.R.-R., personal observation). This organic material could theoretically be a focus of infection during the nesting period. Thus, it is likely that, in addition to *B. licheniformis*, the wide antimicrobial spectrum of the bacteriocin of uropygial gland symbiotic bacteria (Martin-Platero et al., 2006) provides hoopoes with protection against a considerable range of potentially pathogenic bacteria during the nesting phase of reproduction.

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