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In vitro antibacterial efficacy of a blackcurrant oil shampoo/conditioner against *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

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In recent years the increase in reports of antibiotic resistance has made the introduction of new therapeutic strategies necessary. In the dog, the use of topical antiseptics for superficial pyoderma has proven of equivalent efficacy compared to systemic antibiotics. In particular, in canine surface pyoderma topical antiseptics have often shown equal efficacy compared to antibiotic treatments. Aim of this study was to test the *in vitro* antibacterial efficacy of a shampoo/conditioner containing blackcurrant seed oil, piroctone olamine, 18-beta-glycyrrhetic acid and ceramides. Fifty (50) bacterial strains were selected from dogs with superficial pyoderma, consisting of: 10 *Staphylococcus pseudintermedius*, 10 *Staphylococcus aureus*, 10 methicillin-resistant *Staphylococcus pseudintermedius*, 10 *Escherichia coli* and 10 *Pseudomonas aeruginosa* strains.
A concentration of 10^6 CFU/mL of selected bacteria was used for serial dilutions in a 96-well sterile plate; the wells were filled with 100ml of the tested shampoo (Ribes Pet Shampoo® Ultra, NBF Lines, Milan) and dilutions were incremented from 1:2 to 1:256. After incubation for 30 minutes at 37°C an aliquot of 10ml was taken from each well, seeded on Tryptone Soya agar with 5% sheep blood and incubated for 24 hours to evaluate the MIC. The product showed bactericidal activity against all strains up to and including the 1:16 dilution. At 1:34 dilution the first bacterial growth was shown for 4 MRSP, 4 *P. aeruginosa*, 2 *S. pseudintermedius* and 2 *E. Coli* strains; at 1:64 dilution for 6 *S. pseudintermedius*, 6 *S. aureus*, 4 MRSP, *P. aeruginosa* and *E. Coli* strains; at 1:128 dilution for 4 *E. coli*, 2 MRSP, *S. pseudintermedius*, *S. aureus* and *P. aeruginosa* strains. Finally, at 1:256 dilution growth was shown for only 2 strains of *S. aureus*.

Key words: Shampoo-therapy, blackcurrant seed oil, bactericidal activity, antibiotic-resistance.

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INTRODUCTION

The new veterinary medicines guidelines for the treatment of superficial or localized pyoderma recommend, as a first step, the use of shampoos and/or sprays formulated with antiseptics[1-2]. In view of the increasing incidence of antimicrobial resistance, especially of methicillin resistance in staphylococci, such as with *Staphylococcus pseudintermedius* (MRSP) and *Staphylococcus aureus* (MRSA), a different therapeutic approach has recently been promoted. The current approach favours the use of antiseptic-containing products, such as chlorhexidine at different concentrations, as a replacement for antibiotics that have often proved ineffective in *in vitro* tests[3]. Consequently, in both human and veterinary medicine, in the presence of superficial infections topical therapies are now increasingly popular. However, little is known about the specific antimicrobial activity of natural products which obviously differ from antiseptics similar to or belonging to the same class of chlorhexidine.

The study assesses the *in vitro* bactericidal activity of a blackcurrant seed oil shampoo/conditioner against bacteria causing pyoderma.

To counteract the development of antibiotic resistance various therapeutic approaches, especially in human medicine, have started to reconsider the therapeutic role of natural remedies such as honey essences[4-5], preparations with silver nanoparticles combined with medical plants[6] and alcoholic extracts of oregano[7-8]. These natural ingredients have been studied, either individually or in combination, but only few studies have focused on the *in vitro* antimicrobial activity of veterinary shampoos[6-9] against the main bacteria isolated from the skin of dogs with superficial pyoderma[1,2]. Aim of this study is therefore to describe the *in vitro* antibacterial activity of a commercial dermatological shampoo/conditioner containing blackcurrant seed oil, 18-beta glycyrrhetic acid, piroctone olamine and ceramides against different bacterial strains isolated from the skin of dogs with recurrent pyoderma.

MATERIALS AND METHODS

Bacterial characterization

Fifty (50) bacterial strains were selected from skin swabs of dogs with recurrent superficial pyoderma, consisting of: 10 *Staphylococcus pseudintermedius* (SP), 10 *Staphylococcus aureus* (SA), 10 Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP), 10 *Escherichia coli* (EC) and 10 *Pseudomonas aeruginosa* (PA) strains. The samples, seeded on Tryptone Soya Agar plates with 5% sheep blood, Mannitol Salt Agar and McConkey Agar (Oxoid®, Italy), were incubated for 24-48 hrs at 37°C before the final reading. Following phenotypic identification standards, the pure isolates were subjected to Gram staining, catalase and oxidase testing. Biochemical-enzymatic identification was performed using API® tunnels with the APIweb database (bioMérieux®, France) using an acceptance value ≥ 85% for species affinity. The staphylococci identified by the system as *Staphylococcus intermedius* are here referred to as *Staphylococcus pseudintermedius* (SP), as per the recent classification of the related group[10]. The tests used for the detection of the methicillin-resistant strains were: oxacillin diffusion test (ODD - 5 µg/mL), based on the Kirby-Bauer test method and in agreement with the indications of the Clinical Laboratory and Standard Institute[11], and seeding on a selective Brilliance MRSA2 Agar[12] (Oxoid®, Italy). Forty (40) bacterial strains, with the exception of MRSPs, showed variable characteristics of *in vitro* antibiotic sensitivity, with resistance to not more than two different classes of antibiotics; the staphylococcal population was therefore defined as methicillin-sensitive and the remaining strains as non-MDR (Multi-Drug Resistant[13]).

In vitro sensitivity tests

The serial *in-vitro* sensitivity test of the blackcurrant oil shampoo/conditioner (Ribes Pet Shampoo Ultra® NBF Lines, Milan) was performed using a plate microdilution method[14-15]. Pure bacterial strains were isolated and suspended in phosphate buffered saline (PBS; pH 7.2) up to a densitometer-measured 0.5 McFarland turbidity (DEN-1B, Biosan® Sia, Latvia), corresponding to a bacterial concentration of about 108 CFU/mL. The bacterial suspension, which was then further diluted 1:100 to obtain a final concentration of 106 CFU/mL, was then used for serial dilutions in a 96-well sterile plate containing 100 µl of the shampoo to be tested (Ribes Pet Shampoo Ultra®, NBF Lines, Milan), with stepwise concentrations from 1:2 to 1:256[4-5]; the positive control consisted of the bacterial suspension added to 100 µL of PBS without antiseptic. After 30 min. incubation at +37°C, an aliquot of 10 µl was taken from each well and seeded on Tryptone Soya Agar with 5% sheep's blood and then incubated for an additional 24 hours.

The *in vitro* evaluation of the product's bactericidal activity was performed on strains of methicillin sensitive and resistant *S. pseudintermedius* and of *S. aureus*, *E. coli* and *P. aeruginosa* isolated from the skin of dogs with superficial pyoderma.

Table 1 - Number of strains for each species at serial dilutions and relative CFUs						
DIL.	**MRSP**	**SP**	**SA**	**PA**	**EC**	**UFC/ml**
1:2	0	0	0	0	0	0
1:4	0	0	0	0	0	0
1:8	0	0	0	0	0	0
1:16	0	0	0	0	0	0
1:32	4	2	0	4	2	0-10
1:64	4	6	6	4	4	11-20
1:128	2	2	2	2	4	21-50
1:256	-	-	2	-	-	> 100
Total	10	10	10	10	10	

The plate colony-forming units (CFUs) were then visually counted, up to a maximum of 100, and then defined as "confluent" when too numerous to be counted individually. Finally, the minimum bactericidal concentration[16] (MBC) was determined, defined as the lowest concentration capable of inhibiting bacterial growth of at least 99.9% of the initial population[17] (no plate growth).

RESULTS

For all the 50 strains tested the product showed a complete bactericidal activity up to and including the fourth 1:16 dilution. The first bacterial growth was observed at 1:34 dilution, in particular for 4 strains of MRSP, 4 strains of *P. aeruginosa*, 2 strains of *S. pseudintermedius* and 2 strains of *E. coli*. At 1:64 dilution growth was observed for 6 strains of *S. pseudintermedius*, 6 of *S. aureus*, 4 of MRSP, *P. aeruginosa* and *E. coli*. At the subsequent 1:128 dilution for 4 strains of *E. coli*, 2 of MRSP, *S. pseudintermedius*, *S. aureus* and *P. aeruginosa*. At the last 1:256 dilution, growth was found for only 2 strains of *S. aureus* (Table 1). The detected CFUs were homogeneous among the different bacterial species and related to the dilutions made, with progressive growth until confluence at 1:256 dilution (table 1).

DISCUSSION

The blackcurrant oil shampoo/conditioner tested was shown to have *in vitro* bactericidal activity against the most common skin pathogens responsible for canine surface pyoderma. Previous research, carried out using the same methodology, had shown the *in vitro* antimicrobial properties of other skin and/or ear products[14-15], confirming the bactericidal effect of known local antiseptics such as gluconate chlorhexidine at different concentrations, Tris-EDTA, isopropyl alcohol and benzoyl peroxide, often in association with the low pH values of the disinfectant solution or with other components having antimicrobial activity. The shampoo-conditioner used in the study has been commercially available for some time; the label indications are for the shampoo-therapy of dogs with dermatitis, in view of its antibacterial, anti-inflammatory and skin-soothing and protective activity. In view of such indications, it was decided to verify the real *in vitro* bactericidal efficacy of the product against the most common bacteria responsible for canine superficial pyoderma.

In recent years there has been a growing focus on antimicrobial topical products as a single or adjuvant therapy in the treatment of bacterial or superficial mycotic infections of dogs[18-19]. The emergency related to the increasing antibiotic resistance has favoured the development of new strategies and of new antimicrobial products. In human medicine the therapeutic role of natural phytotherapeutic or related products[4-5-6-7-8] has been investigated and their antimicrobial properties have been shown to be attributable to differing mechanisms. In the case of pasteurised extracts of different honey essences, the antimicrobial activity is apparently attributable to the production of hydrogen peroxide, to the low pH and to the high sugar content which makes them highly osmolarising agents[4]. Similarly, pasteurised extracts of wildflower honey have been tested against strains of MRSA and MSSA, with positive and encouraging results[5]. The *in vitro* efficacy of a shampoo containing silver nano-particles synthesized from *Aradica indica* (neem oil) in combination

Bacterial growth inhibition was observed in all tested bacterial strains up to and including the 1:16 dilution, even for methicillin-resistant strains.

The emergence of antibiotic resistance has favoured research in the field of topical therapies. The shampoo studied contains blackcurrant seed oil and piroctone olamine, constituents already known for their antiseptic properties.

with vegetable oil extracts from medical plants such as *Cymbopogon citratus*, *Cymbopogon martini* and *Eucalyptus globules* has shown a powerful antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger* and *Aspergillus fumigatus*[6]. The antibacterial and antioxidant properties of alcoholic extracts of *Origanum vulgare* have been attributed to the presence of phenolic isomers of carvacrol and thymol, the two main components of this essential oil. The high cytotoxic/antioxidant activity and the MIC of this substance have been proven via *in vitro* studies against *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*[7] and *Streptococcus pyogenes*[8].

With regard to the product under investigation, it has been shown – also in other studies - that blackcurrant seed oil, an agent already known in human dermatology for its antioxidant and anti-inflammatory properties, does have an *in vitro* antimicrobial activity[20-21]. Piroctone olamine has an important antiseptic action thanks to its intrinsic broad-spectrum antibacterial activity against Gram positive and Gram negative bacteria and fungi; 18 -glycyrrhetic acid, vegetable proteins, tocopherol and a complex of ceramides have instead an emollient and protective action for the skin barrier. The combination of such constituents exhibits an *in vitro* antibacterial effect which can cause bacterial death within 30 minutes of contact at dilutions four times greater than the original product. To date, it is not possible to determine whether the specific bactericidal activity is caused by a single constituent, compared to the others, or whether it is only the set of all the components together that determines this *in vitro* effect.

Although the study does provide new data which allow for a better understanding of the antimicrobial activity of shampoos for veterinary use[6] the study still presents some criticalities, i.e. the limitations related to it being an *in vitro* analysis, the limited number of strains tested and the inability to separate the constituents of the shampoo in order to assess their specific individual activity. Nonetheless, the blackcurrant oil shampoo can be considered as a local antiseptic, similarly to other shampoos characterized by the presence of chlorhexidine at different concentrations and/or of Tris-EDTA, whose bactericidal action has already been reported *in vitro*[15-20] and *in vivo*[22-23]. In the past, natural peptides and synthetic derivatives such as lactoferricin were also tested in order to prove their *in vitro* bactericidal activity and their possible use as an alternative in topical therapies[24-25]. In any case, the absence of bacterial growth up to a 1:32 dilution and the presence of only two strains of SA with growth >100 CFU/ml confirm a significant microbial load reduction, testifying in favour of the efficacy of the shampoo studied. In the future, a comparative study versus antiseptics such as chlorhexidine is envisaged, in order to confirm the shampoo's antimicrobial activity also *in vivo*.

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KEY POINTS

- Veterinary guidelines suggest the use of shampoo therapy for the treatment of superficial pyoderma in order to limit the use of antibiotics.
- The shampoo/conditioner tested against 50 bacterial strains showed *in vitro* bactericidal activity up to and including the 1:16 dilution.
- The methicillin-resistant staphylococci used (MRSP) proved sensitive to the shampoo being tested.
- As well as ear care products, the dermatological shampoo can also be used as a product with local antiseptic activity.
- The shampoo's various constituents have a synergistic bactericidal action.
- Additional studies are envisaged in order to confirm the product's *in vivo* bactericidal activity.

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