Original article

Effects of an enzyme agent containing mutanase and dextranase for treatment of biofilms in bacteria- and yeast-infected canine otitis externa

M. Fujimura

Fujimura Animal Allergy Hospital, Aomatanihigashi 5-10-26, Minou-city, Osaka 562-0022, Japan

Abstract

The purpose of this study was to evaluate in detail both the *in vivo* and *in vitro* efficacy of the enzyme agents, ZYMOX® Plus Otic (ZYMOX-P), in the treatment of canine otitis externa (OE). Eight dogs with a diagnosis of non-seasonal severe chronic OE were recruited for the study. ZYMOX-P was administered for 2-4 weeks. The Otitis Index Score (OTIS3) and bacteria or yeast colony growth were measured. Also, minimum biofilm (BF) formation inhibition concentration (MBIC) and BF bactericidal concentration (BBC) were measured *in vitro*. OTIS3 showed a statistically significant reduction after treatment (88.2%, p<0.001; pre-treatment = 11.0 ± 0.9; post-treatment = 1.3 ± 0.4, mean ± SEM). The individual OTIS scores, erythema, edema, erosions/ulcerations, exudate and pruritus showed significant reduction (85.7%, 95.7%, 83.3%, 80.0%, and 89.3%, respectively). Microscopic examination revealed the presence of BF exopolysaccharide in all 8 ear samples when stained with alcian blue. Seven of the 8 dogs (87.5%) showed a reduction in colony growth. ZYMOX-P was effective at 34-fold and 16-fold dilutions on MBIC and BBC, respectively. These findings indicate that ZYMOX-P has efficacy against BF-related infection and is beneficial when used for the management of canine OE.

Key words: biofilm, canine otitis externa, enzyme agents, drug-resistant bacteria

Introduction

Biofilms (BF) are bacterial communities encased within an extracellular matrix composed of exopolysaccharides, proteins, lipids, DNA, and ions (Stone VN et al. 2017). These structures are ubiquitous in nature and represent a major health concern because they are a cause of persistent infections and are estimated to account for 80% of all bacteria related infections (Stone et al. 2017). BF provide the bacteria with protection from external stresses and decrease their susceptibility to antimicrobial therapy and immune clearance, making treatment extremely difficult (Davies 2003). Otitis externa (OE) and oral plaque in dogs are two main types of BF-related problems of interest in veterinary medicine (Davies 2003, Chan et al. 2019). OE is mainly

Correspondence to: M. Fujimura, e-mail: hope3413@gmail.com

caused by *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* (Chan et al. 2019), while oral plaque is caused by *Staphylococcus mutans* (Forssten et al. 2010).

The development of drug-resistant bacteria is a major problem as the presence of BF may play an important role in the resistance of otic pathogens to antimicrobial agents (Chan et al. 2019). Identifying disinfectant agents that prevent the establishment of BF can be an effective strategy against disease (Stone et al. 2017).

In previous studies of OE, *in vitro* experiments were performed to assess the inhibitory effects of ear wash chelating agents N-acetylcysteine (NAC) and Tris--EDTA. The purpose of this study was to evaluate the clinical efficacy of ZYMOX® Plus Otic (pkb JAPAN Inc., Osaka, Japan) which contains mutanase and dextranase against BF-associated bacteria- and yeast-infections in canine OE.

Materials and Methods

Animals

Eight dogs from the Fujimura Animal Allergy Hospital (Osaka, Japan), with a diagnosis of non-seasonal severe chronic OE, were recruited in this study. The selection criterion for this study was chronic recurrent otitis. The breed, age, side of the ear with OE, underlying diseases related to otitis, presence or absence of BF, and isolated bacterial strains were recorded and are shown in Table 1. The study was conducted within an 8-month period with the owners' informed consent. There was no required ethical approval for this study as only prior approved medications were used in the study with the consent of the dog owners.

Otitis Index Scores

OE was assessed and the severity as well as response to treatment, were recorded using the otitis index scores with a scale of 0 to 3 (OTIS3). Erythema, edema/swelling, erosions/ulcerations, exudate, and pruritus were assessed as individual scores (Nuttall T et al. 2014). ZYMOX-P is a sticky solution that is easily applied to the ear canal. The dosage was independent of the intensity of the lesion. Rather, the dosage was dependent on the size of the dog. Small dogs (less than 10 kg) were given a dosage of 1.3 ml, medium breed dogs (10-25 kg) were given a dosage of 2.0 ml, and large breed dogs (over 25 kg) were given a dosage of 2.6 ml. OTIS3 for all the dogs in the study (n=8) was recorded twice, before treatment (pre) and 2-4 weeks after treatment (post) as shown in Table 1. Patient No. 5 (shih tzu) had OE in both ears with comparable OTIS3. Therefore, we were able to treat each ear individually and compare the effects of ZYMOX-P to ethylenediamine-tetraacetic acid (EDTA) and chlorhexidine combination solution.

Alcian blue staining for BF detenction

Alcian Blue Stain Solution pH 2.5 for Cytology (Mutou Pure Chemical Co., Tokyo, Japan) was used to detect BF (Holá et al. 2004) as directed by the manufacturer.

Identification of isolated pathogen strains and confirmation of colony growth

Bacterial harvests were isolated with a seed swab and then cultured and identified at Doubutsu Kensa, Inc. and Hoken Kagaku, Inc. (Osaka, Japan). The swabs were applied to sheep blood agar medium and BTB agar medium to culture bacteria. CHROMagar medium was used for yeast. MALDI Biotyper was used to identify bacteria and yeast species (Bruker Japan KK, Kanagawa, Japan; Schulthess et al. 2014). Minimum inhibitory concentrations (MICs) were determined from the Clinical and Laboratory Standards Institute (CLSI)-based micro-liquid diluted drug-sensitive panel. Automated measurements were made using a Beckman Coulter Microscan (Beckman Coulter, Inc., Brea, CA, USA; Wu et al. 2016). Colony score was measured as follows: negative or zero (0) when no colony formation was observed; colony growth in less than one third of the medium plate (1+); colonies covering one-third to two-thirds of the plate (2+); colonies occupying more than two-thirds of the medium (3+); colony formation extending to the entire surface of the medium (4+).

Determination of minimum BF formation inhibition concentration (MBIC) and BF bactericidal concentration (BBC)

The experiment was carried out at Dojindo Laboratories (Kumamoto, Japan). MBIC of ZYMOX-P was assessed using a biofilm formation assay kit. Suspension of *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900 (1.0×10^6 CFU/mL) was prepared for BF production. *P. aeruginosa* was used as a standard because it consistently forms stable BF. The enzyme was prepared in 2-fold serial dilutions and mixed with an equal volume of bacterial suspension (1:1) and the mixture was then dispensed into the wells of a 96-well microplate. The plate was then fitted with a 96-peg lid and incubated for 24 h to allow formation of biofilm

Table 1. Assessment of OE and identification of pathogen species.

No.	Breed	Age (years)	Underlying diseases	Left or right ear	Pathogen species identified	Treatment	OTIS3		Colony scores	
							Pre	Post	Pre	Post
1	Maltese	10	ALD	R	*Corynebacterium sp.*	○	15	3	1+	0
2	Shih tzu	11	CAD	R	*Staphylococcus pseudintermedius*	○	11	2	1+	0
3	Yorkshire terrier	14	None	L	*Pseudomonas aeruginosa*	○	12	0	3+	0
4	Shiba inu	9	CAD	R	MRSP	○	8	2	2+	0
5	Shih tzu	3	CAD	L	*Corynebacterium sp*	○	10	1	4+	0
					CNS	○			3+	1+
				R	*Corynebacterium sp.*	●	10	4	4+	2+
					CNS	●			3+	1+
6	Crossbreed	8	ALD	L	*Malassezia sp.*	○	10	0	1+	0
					Pseudomonas aeruginosa	○			2+	0
					CNS	○			2+	0
7	Shiba inu	11	CAD	R	MRS	○	14	1	1+	0
8	Standard poodle		FA	L	*Malassezia sp.*	○	10	1	1+	0
					CNS	○			1+	0

CAD: canine atopic dermatitis; ALD: Atopic like dermatitis; FA: Food allergy.
MRSP: Methicillin resistance Staphylococcus pseudintermedius; CNS: Coagulase Negative Staphylococcus. Underlined are multidrug-resistant bacteria.
○: ZYMOX-P; ●: EDTA; OTIS3: Otitis Index Total Scores with scale of 0-3.

on the pegs. Subsequently, the plates were processed according to the manufacturer's instructions and the amount of biofilm was quantified using the crystal violet method. The absorbance at 590 nm was measured using a microplate reader (PerkinElmer Japan Co., Ltd. Yokohama, Japan). BBC of ZYMOX-P was assessed using a biofilm viability assay kit (Dojindo Laboratories, Kumamoto, Japan). BF was produced as described above. After incubation, the plates were processed according to the manufacturer's instructions and BBC of the enzyme prepared in the 2-fold serial dilutions were quantified using the water soluble tetrazolium salts (WST) method (Tsukatani et al. 2020). Briefly, WST-8 {2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl) 5-(2,4-disulfophenyl)-2H-tetrazlium} assay can quantify live cells and determine cell proliferation. 200 ul of LB washing solution from the experiment was added into each well of a 96-well plate. 10 ul of WST solution (Dojindo, Kumamoto, Japan) was then added into each well and incubated for 2 h at 37°C and the absorbance at 450 nm was measured (Zhu et al. 2022).

Statistical analysis

Statistical analysis was performed using the Welch's t-test and statistical significance was defined as p<0.05.

Results

Clinical observations and isolation of pathogens

Eight dogs ranging in age from 3 to 14 years with OE symptoms were examined and assessed. The summary of the clinical data is shown in Table 1. OE-related underlying diseases were diagnosed as canine atopic dermatitis (n=4), atopic-like dermatitis (n=2), and food allergy (n=1); one dog displayed no underlying disease. Four dogs were colonized in the right ear, 3 dogs in the left ear and 1 dog in both ears. The dogs were found to be colonized by 14 different bacteria and yeast strains. The dogs were infected with 3 strains of *Corynebacterium* sp., 2 strains of *Pseudomonas aeruginosa*, and 7 strains of *Staphylococcus* sp. Upon further investigation, *Staphylococcus* sp. was confirmed to be one strain of *S. pseudintermedius*, 2 strains of methicillin-resistant *S. pseudintermedius* (MRSP), and 4 strains of coagulase-negative *Staphylococcus* (CNS, S. epidermidis). Out of the 12 isolated bacterial strains, 7 (58.3%) were antibiotic multidrug-resistant (Table 1, underlined). We also tested for specific drug resistance against ampicillin, gentamicin, minocycline, chloramphenicol, fosfomycin, oxacillin, enrofloxacin, colistin, cephalosporin, sulfamethoxazole, sulfamethoxazole trimethoprim and clindamycin and the data are shown in Table 2. In all, 6 dogs out of 8 were infected with drug resistant bacteria. Two species of *Malassezia* were isolated and identified by mass spectrometry (*M. pachydermatis* and *M. globosa*).

Table 2. Antibiotic resistat bacteria profile.

No	Isolates	MPIPC	ABPC	CEX	CLDM	MINO	CP	ST	ERFX	GM	CL	FOX
1	MRSP	R	R	R	R	S	R		R	R	I	R
2	MRSP	R	R	R	R	S	N	S	R	R	N	S
3	Corynebacterium sp.	R	R	R	S	S	S		R	I		S
4	Corynebacterium sp.	N	R	R	S	S	I	R	S	I		R
5	Corynebacterium sp.	N	R	R	S	S	I	R	S	I		R
6	P. aeruginosa	N	N	N	N	N	N	N	R	S	S	I
7	P. aeruginosa	N	N	N	N	N	N	N	N	S	S	S
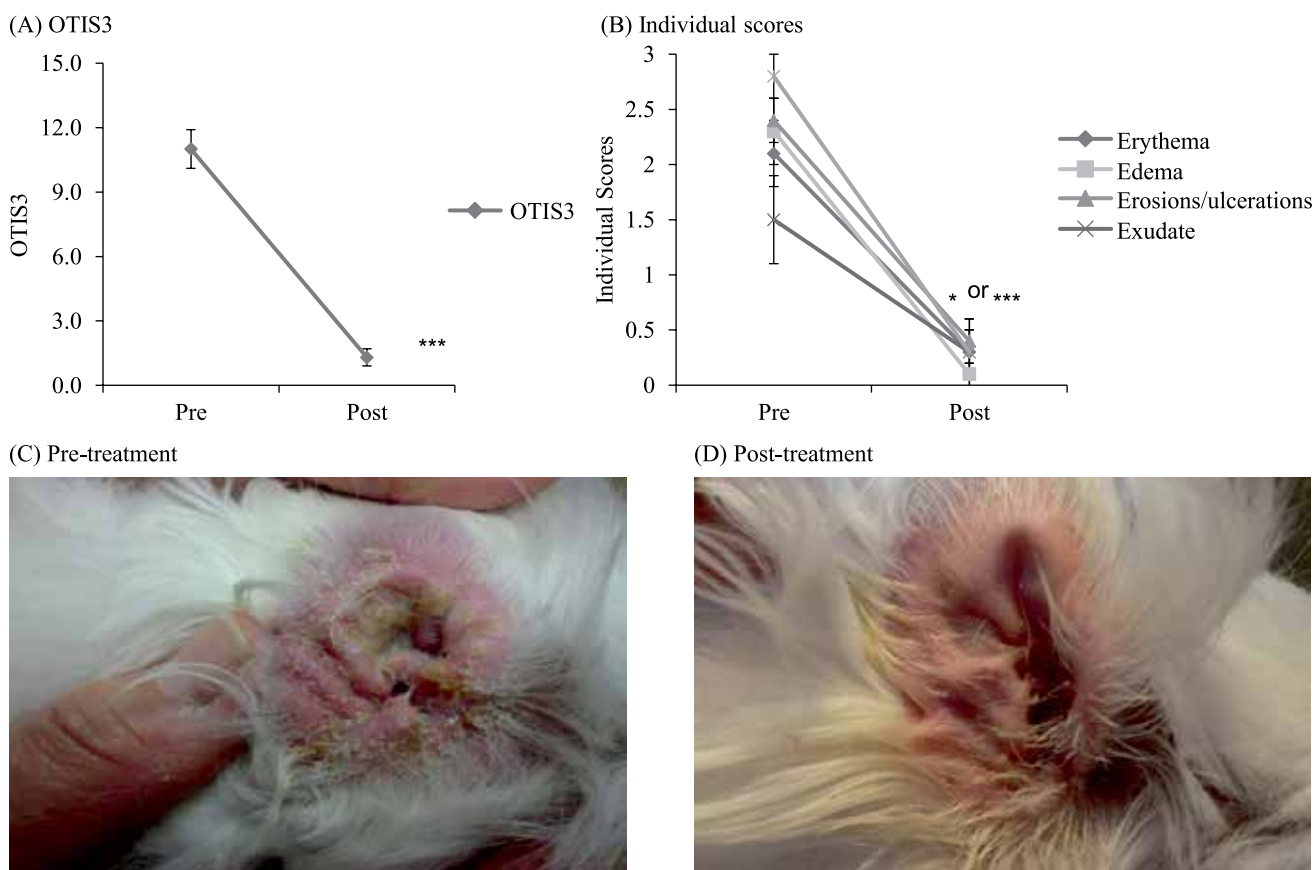
Fig. 1. Evaluation of canine otitis externa after treatment with ZYMOX-P. Dogs were assessed before (Pre) or after treatment (Post) and evaluated using (A) OTIS3 and individual symptoms (B). Photograph of a representative dog (Dog No.1) is shown before (C) and after (D) treatment. * p<0.05, *** p<0.001.

Evaluation of treatment outcomes using OTIS3

The dogs were assessed and assigned OTIS3 before treatment. ZYMOX-P, which contains the enzymes mutanase and dextranase, was used as a treatment against OE. After 2-4 weeks, OTIS3 was determined again and compared to pre-treatment scores. There was a statistically significant reduction of 88.2% (p<0.001) in the average OTIS3 from pre=11.0±0.9 to post=1.3±0.4 (Fig. 1A). Individual scores showed dramatic reductions – erythema (85.7%), edema (95.7%), erosions/ulcerations (83.3%), exudate (80.0%) and pruritus (89.3%). These decreases were statistically significant (exudate: p<0.05; others: p<0.001; Fig. 1B). A photograph of a representative dog (Dog No.1) is shown before and after treatment with ZYMOX-P (Fig. 1C and 1D).

Dog No. 5 (shih tzu) exhibited a very similar level of disease in both ears (OTIS3=10). Therefore, to test the efficacy of treatment, the dog was treated with ZYMOX-P in one ear (L=left ear) and EDTA alone in the other ear (R=right ear). Treatment and total scores were recorded daily for 5 consecutive days and scores were as follows: Day 1 (R=10 and L= 10); Day 2 (R=7 and L=4); Day 3 (R=6 and L=2); Day 4 (R=5 and L=1); Day 5 (R=4 and L=1). EDTA treatment showed only

(A) Colony scores
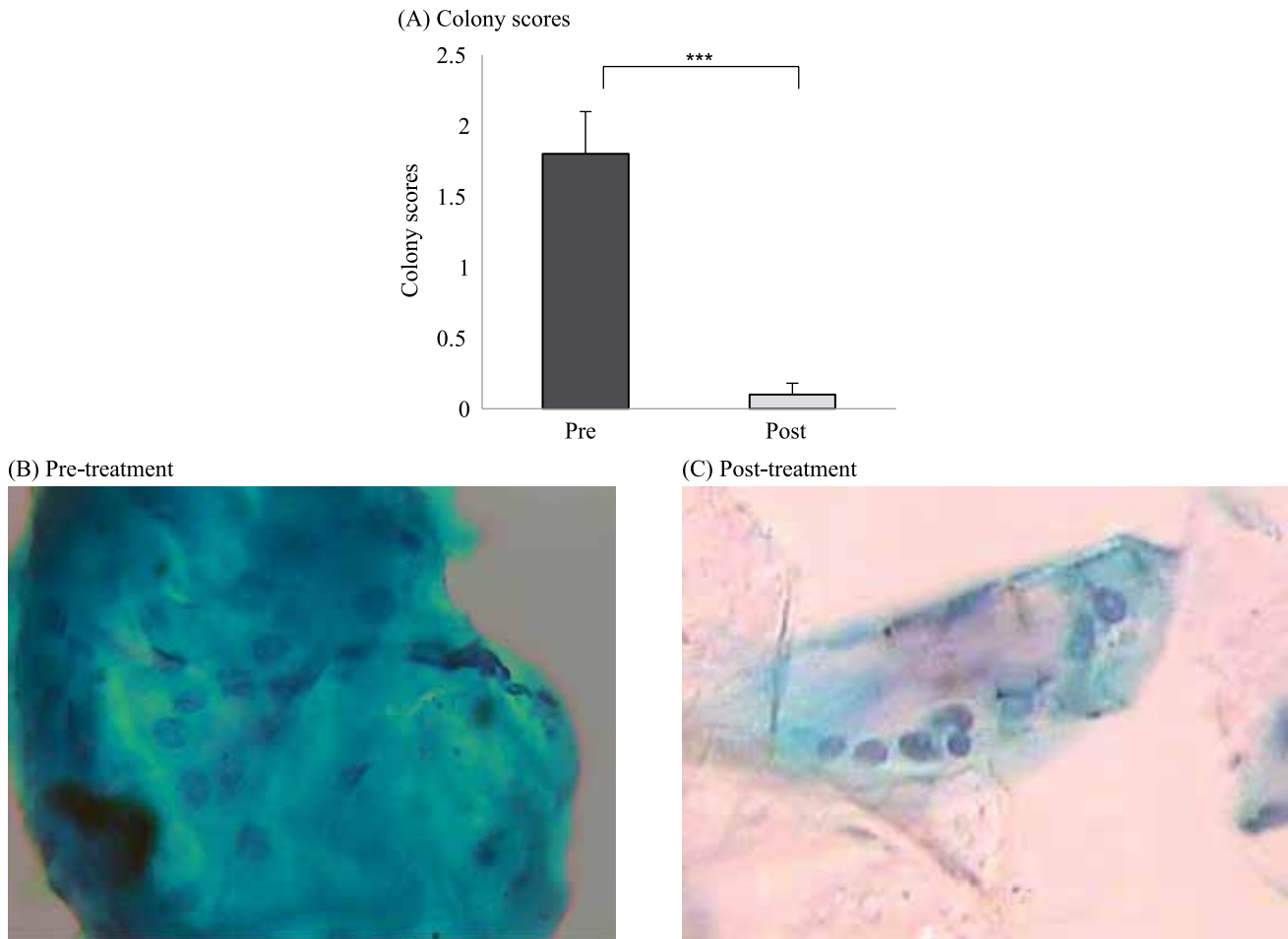

(B) Pre-treatment

(C) Post-treatment

Fig. 2. Colony scores and biofilm (BF) exopolysaccharide staining. (A) Colony growth of isolated pathogens was evaluated using colony scores before (Pre) and after (Post) treatment. *** p<0.001. Alcian blue staining of BF exopolysaccharide before (B) and after (C) treatment. A representative staining is shown. x100.

a moderate decrease in OTIS3 while ZYMOX-P treatment showed a dramatic reduction.

Effect of treatment on pathogen growth

The effect of ZYMOX-P on pathogen growth was investigated. Pathogen isolates were cultured to determine whether treatment affected the growth of bacteria and yeast. 12 isolates were recovered from the 8 dogs after treatment. Of the 12, 11 isolates did not form colonies (91.7%). One isolate from the right ear of Dog No. 5 (shih tzu) had a colony score of 1+ (Table 1). In addition, a statistically significant reduction of 94.4% was observed in the colony score after ZYMOX-P treatment (pre = 1.8 ± 0.3; post = 0.1 ± 0.1; Fig. 2A). In contrast, 2 strains isolated from a dog treated with EDTA alone exhibited colony growth (1+ and 2+). Only Dog No. 8 (standard poodle) had ear wing lesions and a normal ear canal. From this dog, *Malassezia* sp. was isolated and the disappearance of the colonies was confirmed 24 h after treatment was administered.

The presence of BF was also investigated. Ear samples were stained with alcian blue, revealing the presence of BF exopolysaccharide in the infected dogs (Fig. 2). MBIC and BBC of ZYMOX-P were evaluated *in vitro*. For the determination of MBIC, we found that the 64-fold dilution resulted in inhibitory effects (Fig. 3A) while BBC was observed at a 16-fold dilution (Fig. 3B).

Discussion

Canine otitis externa is a common problem in veterinary medicine. Most of the chronic refractory canine OE is caused by underlying disease such as allergy (Jacobson 2002). Usually, *Corynebacterium sp., Staphylococcus sp.*, and *Pseudomonas aeruginosa* cause subsequent secondary infections in dog ears and need veterinary intervention (Chan et al. 2019, Bradley et al. 2020). Antibiotic resistance is another major challenge. A previous study found 25.9% (72/278) of *S. pseudintermedius* isolates were multidrug resistant
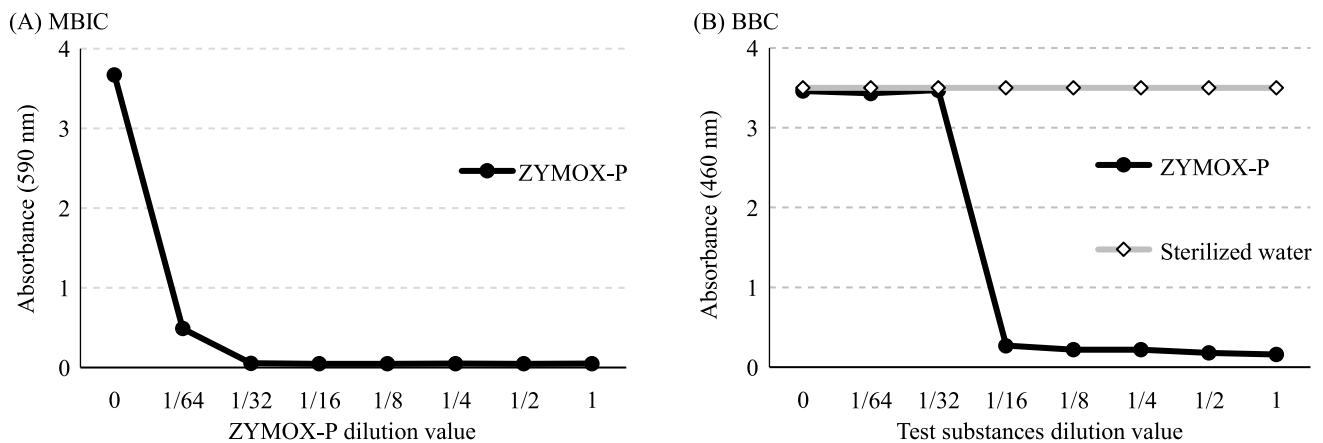
(A) MBIC

Fig. 3. The effects of ZYMOX-P on pathogens were evaluated *in vitro*. Minimum BF formation inhibition concentration (MBIC) in (A) and BF bactericidal concentration (BBC) in (B) are shown.

(Qekwana et al. 2017) although the study did not report the site of collection. Our investigation found that 7 out of 12 bacteria strains isolated from the ears were antibiotic-resistant (58.3%). It may be that pathogens that infect the ear may have a higher percentage of drug resistant strains. *Pseudomonas aeruginosa* which was isolated in this study may also contribute to the higher percentage of drug resistance.

There is much known about BF and its role in the formation of oral plaque in veterinary medicine (Peters 2012, Qekwana et al. 2017, Cunha et al. 2018). *Staphylococcus mutans* destroys teeth when BF water--insoluble glucans cause oral plaque formation (Bowen 2016). The water-insoluble glucan molecules in oral plaque are mainly composed of the polysaccharides, dextran and mutan. The enzymes mutanase and dextranase decompose these water-insoluble glucans to effectively remove plaque (Otsuka et al. 2015). ZYMOX-P is an improved version of the anti-plaque enzymes mutanase and dextranase for treatment of ear otitis. The aim of our study was to show detailed *in vitro* and *in vivo* efficacy of ZYMOX-P.

As hypothesized, we were able to confirm the formation of BF by alcian blue staining (Holá et al. 2004) and observed greenish blue staining of mucopolysaccharides from earwax smears (Fig. 2). Although most BF stains show a crisp light blue color in humans, our stains of dog OE were different in that the color was greenish blue. We believe this difference may be due to the amount of earwax present.

Our study found that ZYMOX-P destroyed BF and had a bactericidal effect against *Pseudomonas aeruginosa in vitro.* We were also able to show *in vivo* efficacy in one dog which had the same level of disease (same OTIS3) in both the right and left ears. We show that the effect of the enzyme *in vivo* was much stronger than that of the EDTA agent.

The next area of great interest *in vivo* would be to

investigate viable but non-culturable (VBNC) bacteria. Although such bacteria were visible in the dogs' earwax after treatment, we did not observe colony growth *in vitro*. After ZYMOX-P treatment, colony growth inhibition was observed in almost all dogs. In addition, 11 out of 12 pathogen strains, including both bacteria and yeast did not form any colonies on standard plate media in contrast to treatment with EDTA alone. In particular, the dog No. 8 showed no colony growth just one day after enzyme treatment, demonstrating the efficacy of ZYMOX-P. This may suggest that the enzymes cause the bacteria to go into a VBNC state and regulate OE. The growth of OE pathogens has been found to be influenced by a variety of factors such as the presence of antimicrobials, stressors such as starvation, exposure to temperatures outside the growth range, and exposure to chemical and thermal treatments. However, the microbes often recover after discontinuation of antibiotic treatment or environmental stressors (Klancnik et al. 2009, Ding et al. 2011, Su et al. 2013, Harms et al. 2016, Ayrapetyan et al. 2018).

ZYMOX-P contains lysozyme, lactoferrin, and lactoperoxidase enzymes in addition to mutanase and dextranase. Lysozyme (lysogenic enzyme) destroys the cell wall and lactoferrin binds to iron, which is a nutrient necessary for bacterial growth, leading to malnutrition of bacteria. Lactoperoxidase (oxidoreductase) blocks the transport pathways of glucose and amino acids that nourish cells (Carlsson et al. 1983, Li et al. 2019, Zarzosa-Moreno et al. 2020). These enzymes induce stress and make the environment unfavorable for the microorganisms and may cause pathogens to enter a VBNC state. If this hypothesis is correct, it is important to consider alternative new approaches rather than traditional bacterial culture identification and susceptibility-based antibiotic selection.

In conclusion, the results of this study show that the enzyme agent, Advanced Formula ZYMOX® Plus Otic

(which includes the enzymes mutanase and dextranase), is beneficial when used for the management of canine OE.

Acknowledgements

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