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MORPHOFUNCTIONAL CHARACTERISTICS AND TREATMENT METHODS OF OTITIS IN SMALL DOMESTIC ANIMALS

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На правах рукописи

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МОРФОФУНКЦИОНАЛЬНАЯ ХАРАКТЕРИСТИКА И СПОСОБЫ ЛЕЧЕНИЯ ОТИТОВ У МЕЛКИХ ДОМАШНИХ ЖИВОТНЫХ

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CONTENTS LIST

ABBREVIATIONS LIST

AD - atopic dermatitis

AI - adhesion index

BE - buccal epitheliocytes

BS - buffered saline

BY - basidiomycetous yeast

CFU - colony forming unit

CLSI - Clinical and Laboratory Standards Institute

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FAR - Farnesol

M - Malassezia

MO - otitis aggravated by Malassezia

MP - Malassezia pachydermatis

MPA - meat-peptone agar

MPB - meat-peptone broth

PI - phagocytic index

PN - phagocytic number

SDA - Sabouraud dextrose agar

SDB - Sabouraud dextrose broth

SS - saline solution

YLF - yeast-like fungi

AP - amphotericin B

CC - clotrimazole

FU - fluconazole

IT – intraconazole

KT - ketoconazole

MC - miconazole

NS - nystatin

VOR - voriconazole

MIC – minimum inhibitory concentration

INTRODUCTION

Relevance of the problem. In dermatitis and otitis externa in dogs, the yeast fungus *Malassezia pachydermatis* (M. pachydermatis, MP) is often isolated. It has been proven that in case of immune deficiency of the organism, *Malassezia* (M) can be an etiological factor not only in skin but also in systemic pathology (Kiss et al. 1996; Bond et al. 2010). This pathology is of particular importance in veterinary medicine. The problem of malacesiosis in dogs currently occupies one of the leading places in the structure of morbidity in these animals (Ershov P.P., 2008; Pustovit E.A., Pimenov N.V. et al., 2024; Crespo M.J. et al. 2002; Hobi S. et al. 2022). According to a number of authors, an increase in the frequency of M lesions in dogs is noted annually in the world. First of all, the skin and its derivatives are affected, and the nervous, endocrine, excretory, and other systems of the body are involved in the pathological process for the second time. Very variable in its clinical manifestation, malacesiosis often passes under other diagnoses. Traditional methods of drug therapy for such animals are ineffective in terms of achieving complete remission, so the search for new drugs that allow effective therapy is especially important (Buommino et al. 2016; Bajwa et al. 2023). Despite the fact that modern antimycotic therapy is quite effective against acute infection, the treatment of chronic recurrent Malassezia otitis (MO) is not always effective (Brito et al. 2007; Weiler et al. 2013; Theelen et al. 2017). Thus, when systemic antimycotics (drugs from the azole group) are prescribed, the cure rate in the acute phase of MO is usually 80% (Li et al. 2020); while relapses of the disease are observed within 6 months in 20-22% of dogs (Guillot et al. 1999; Ilahi et al. 2018).

According to Duarte, E.R. et al. 2002, a significant recurrence rate of MO forces doctors to prolong the treatment regimen or increase the dose of antimycotic. According to Gupta, A.K. et al. 2000, prolongation of the treatment period can only shift the probability of recurrence of malacesiosis in time, and increasing the dose of the drug leads to additional side effects. The problem of anti-relapse therapy is further complicated by the fact that with the widespread use of drugs from the azole

group, M resistance to them may develop (Morris et al. 2004; Lyskova et al. 2007; Cordeiro et al. 2022).

In this regard, there is a need to find alternative ways of etiotropic therapy of MO. A possible promising direction of such a search is the study of herbal remedies that affect basidiomycetous yeast (BY). In recent years, interest in the treatment of M. pachydermatis infections with natural plant products has considerably raised due to the growing resistance to antifungals (Bismarck et al. 2020; Ebani and Mancianti 2020). Investigation of new antifungal agents for their impact on virulence is an important tool for exploring novel antifungal targets leading to improved therapeutic regimens. Plant essential oils and their active compounds deserve attention in this regard (El-Baz et al. 2021). Several articles have dealt with the antifungal effect of plant essential oils against M. pachydermatis (Khosravi et al. 2016; Váczi et al. 2018), but there are scanty data on the antifungal efficacy of their components, nor on their inhibitory activity against M. pachydermatis virulence factors, such as EPL production. In general, plant EOs are complex mixtures of natural compounds, and are well-known for their antiseptic and medicinal properties (analgesic, sedative, anti-inflammatory, spasmolytic, local anaesthetic, anti-carcinogenic, antibacterial, antifungal and antiviral) (Raut and Karuppayil 2014; Nazzaro et al. 2017). Most essential oils are composed of terpenes, terpenoids, and other aromatic and aliphatic constituents with low molecular weights. Few articles on M. pachydermatis, point to the antifungal or antibiofilm effect of EO components such as thymol, carvacrol and eugenol (Aiemsaard et al. 2019; Schlemmer et al. 2019; Sim et al. 2019). Based on the foregoing, it is especially important to study the effect of Farnesol on the virulence of M, which can serve as a basis for creating an alternative drug to existing antimycotics (Кудинова С.А., Луцай В.И. с соавт. 2021).

The degree of development. A whole galaxy of researchers studied malasseziosis in animals (Manoyan M.G., Ershov P.P., et al. 2006; Aiemsaard J. et al. 2019; Schlemmer K.B. et al. 2019; Sim J.X.F. et al. 2019; Puigdemont A. et al. 2021; Vercelli C. et al. 2021; Angiolella L. et al. 2023; Scheler J. et al. 2024), who

studied its spread, causes, pathogenesis, and treatment regimens. In recent years, there has been interest in the treatment of M. pachydermatis with natural herbal products has increased significantly due to the growing resistance to antifungal drugs (Bismarck D. et al. 2020; Ebani V.V., Mancianti F. 2020). According to Duarte, E.R. et al. 2002, a significant recurrence rate of otitis media forces doctors to prolong the treatment regimen or increase the dose of the antimycotic. According to Gupta, A.K. et al. 2000, prolonging the treatment period can only shift the likelihood of recurrence of malasseziosis over time, and increasing the dose of the drug leads to additional side effects. The problem of anti-relapse therapy is further complicated by the fact that with the widespread use of drugs from the azole group, resistance to them may develop (Morris D.O. et al. 2004; Lyskova P. et al. 2007; Cordeiro B. et al. 2022). Therefore, the research of new antifungal drugs, the improvement of therapeutic regimens and their effect on the virulence of adductors are relevant in veterinary medicine.

Purpose and objectives of the study. The aim of the study: to evaluate the potentiating effect of the herbal preparation Farnesol in relation to modern antifungal agents in the complex treatment of acute Malassezia otitis in an in vivo model.

To achieve the intended goal, we were set the following tasks:

1. To study the properties of Malassezia pachydermatis strains accompanying bacterial flora from clinically ill dogs and cats with Malassezia otitis;

2. To study the ability of Farnesol to destroy M. pachydermatis biofilms in vitro and enhance the effect of antifungal agents;

3. To evaluate the effectiveness of in vivo models with M. pachydermatis infection;

4. To determine the effect of Farnesol on the treatment of Malassezia otitis in rabbits

5. To prove the potentiating effect of Farnesol on antimicrobial agents in the model of Malassezia otitis.

Scientific novelty. For the first time, a correlation was proven between the resistance of Malassezia pachydermatis strains to modern antifungal agents and the ability to form biofilms. For this purpose, the strongest biofilm producers (optical density above 0.4) and the most resistant strains to antifungal agents (resistance was observed to 7 of 8 drugs) were selected. An analysis of the species diversity of microorganisms in malassezia otitis was carried out: in dogs. M. pachydermatis was more often isolated with staphylococci and streptococci, and in cats - with enterobacteria and staphylococci. For the first time, the maximum values of the adhesion index of M. pachydermatis (8.28±0.62) and the adhesion coefficient (70.62±4.91%) to the buccal epithelium of dogs were established. For the first time, the maximum values of the phagocytic index of M. pachydermatis (83.1±2.7%) and phagocytic number (9.4±1.1) to alveolar macrophages in rats were established. An original model of acute Malassezia otitis in rabbits was proposed. The therapeutic and anti-relapse effect of Farnesol was proven in in vivo experiments. Effective concentrations of Farnesol (12.5-200 μM/ml) were determined, leading to a 55-71% decrease in M. pachydermatis biofilms. It was proven that Farnesol in a dose of 25-200 μM/ml enhances the effect of antimycotics (Amphotericin B, Nystatin, Voriconazole and Ketoconazole) or reactivates a drug that has ceased to act on the pathogen (Clotrimazole). It was found that adding Farnesol to the treatment regimen for fungal otitis in dogs resulted in a decrease in hyperemia, itching, edema, and purulent exudate on days 5-7 of treatment, and complete clinical recovery of animals occurred by day 10-14 of therapy. At the same time, an increase in hemoglobin by 1.22 times, a decrease in the number of leukocytes by 1.30 times were recorded, against the background of a decrease in eosinophils by 1.42 times and band neutrophils by 1.41 times, in the experimental group after treatment, when compared with the control.

Practical significance. An effective and very easy-to-use technology for modeling Malassezia pachydermatis in laboratory animals has been developed. The proposed treatment regimen for Malassezia otitis makes it possible to achieve visible

clinical improvement that exceeds the healing rate of modern commercial drugs in veterinary medicine. The drug farnesol of natural origin can be either a primary or an additional therapeutic agent intended for the treatment of Malassezia otitis in dogs and cats and the prevention of its relapses. The inclusion of Farnesol in the treatment regimen can be considered as an alternative to the use of antimycotics in case of development of resistance to them.

In the course of the work, a working collection of Malassezia pachydermatis strains was collected, which will be used in further studies of the Department of Veterinary Medicine to study antagonistic relationships between microorganisms of different species.

Provisions submitted for defence.

1. The most aggressive clinical strain of M. pachydermatis Cd23 was selected for its ability to form biofilms, resistance to antifungal drugs, ability to adhere to epithelial cells and be phagocytized by macrophages.
2. Malassezia otitis is reproduced in a laboratory rabbit model by infecting the auricle with an aggressive strain of yeast-like fungi without prior treatment with antibiotics and estradiol.
3. The therapeutic effect of Farnesol in combination with modern antifungal drugs was demonstrated in a laboratory model of Malassezia otitis, consisting in rapid ear sanitation within a month and restoration of rabbit blood counts to normal.
4. The therapeutic effect of Farnesol was established due to partial death of the yeast-like fungi population as a result of their lysis and a decrease in the ability to form biofilms.
5. Farnesol has been shown to enhance the action of antifungal agents, which may serve as an alternative to the search for new drugs.

Degree of reliability and testing of research results. The reliability of the results of the research, the validity of the main provisions of the work, conclusions and proposals are justified by a sufficient number of animals in the

experimental groups, the study of Russian and, mainly, foreign literature on the research topic, clinical, morphological, microbiological, biochemical data were obtained using modern methods on certified equipment with subsequent statistical processing and analysis of the results obtained.

The dissertation materials were reported and discussed at the meetings of the Department of Veterinary Medicine of the ATI RUDN (2021-2023), the XV International scientific and practical conference of young scientists “Innovative processes in agriculture” RUDN (April 2023); XXV All-Russian Student Scientific and Practical Conference of Nizhnevartovsk State University, Nizhnevartovsk (April 2023); International Scientific Student Conference (ISSC-2023) Novosibirsk (April 2023); VII All-Russian Congress on Medical Microbiology, Clinical Mycology and Immunology - XXVI Kashkin Readings. St. Petersburg (June 2023).

Publications. The main provisions of the dissertation work are presented in 7 scientific papers, 2 of which are in peer-reviewed publications recommended by the Higher Attestation Commission list, and 2 in journals indexed in the Web of Science and Scopus databases.

Structure and scope of the dissertation. The main content of the work is presented on 125 pages, the manuscript consists of an introduction, a literature review, the main content of the work, including materials and methods, the results of one’s own research, analysis and discussion of research results, as well as a conclusion and a list of references. The list of references includes 157 titles. The work is illustrated with 34 tables and 24 drawings.

CHAPTER 1. LITERATURE REVIEW

The basidiomycetous yeast (BY) *Malassezia pachydermatis* is a commensal but also the most isolated pathogenic yeast in Veterinary medicine (Blake et al. 2017; Bajwa et al. 2023). The mechanisms of the transition of MP from a commensal to a pathogen are not fully understood. Generally, treatment of M-associated diseases is based on topical application of an antifungal drug combined with antibiotics to control bacterial infection and glucocorticoids to reduce inflammation (Honnavar et al. 2016; Puigdemont et al. 2021; Sachivkina et al. 2022).

Inflammatory processes of the middle ear, complicated by a fungal infection, are a frequently diagnosed pathology. According to literature data, otitis externa account for 20% of all diseases encountered in veterinary practice. It has also been found that otitis externa of the outer ear in dogs and cats are five times more common than in other animal species (Patterson et al. 2002; Juntachai et al. 2009; Ebani et al. 2020b).

Currently, there is a tendency to increase the incidence of opportunistic mycoses in animals. Dermatomycoses (microsporia, trichophytia) are replaced by diseases caused by opportunistic fungi. One of these are YLF of the genera *Candida* and BY *Malassezia* (Miron et al. 2014; Sachivkina et al. 2022a).

Although fungi of the genus M are the most common etiological agents of infectious animal otitis externa, it must be borne in mind that these diseases can also be caused by other types of fungi and yeast (Gue´ho et al. 2011; Nardoni et al. 2017; Yang et al. 2023). This indicates the need for qualified species identification of micriorganism isolated from animals suspected of having M infection.

The pathogenic properties of BY of the genus M and their clinical role in infectious diseases of animals are still topics of discussion. The factors due to which the transition of a microorganism from a non-pathogenic to a clearly pathogenic form capable of causing a disease has not been fully clarified. There is no consensus on whether M infections can be considered as an independent disease, or whether they are only an aggravating factor against the background of other pathologies (Rosser et al. 2004; Bensignor et al. 2006; Sim et al. 2019).

In veterinary mycology, the clinical role of BY M in animal skin diseases has been established relatively recently. Intensive research in recent decades has made a significant contribution to the understanding of the pathogenesis of M infections. Currently, most researchers share the opinion that the pathogenicity of fungi of the genus M is "opportunistic" in nature, i.e. BY is able to exhibit pathogenic properties only against the background of certain predisposing factors. So, in favorable conditions (increased sebum secretion and humidity, violation of the epidermal barrier), they actively multiply, the yeast form of the fungus turns into mycelial, M is introduced into the epidermis, showing pathogenic properties. However, according to another theory, the transformation from the yeast phase to the mycelial phase is not due to the special pathogenicity of the latter, but is only a consequence of lipid metabolism disorders in the host body. This is based on the fact that the MP species is not able to transform into a mycelial form (Álvarez-Pérez et al. 2014; Wu et al. 2015; Lee et al. 2022).

The development of the pathological process in *Malasseziosis* is associated with a multiple increase in the population of microorganisms in the lesion. The population of BY in sick animals increases by 100 – 10,000 times. Moreover, an increase in the number of cells of the genus M is noted not only on the surface of the skin, but also on the mucous membranes of the nasal cavity, vulva and prepuce, i.e. the factors predisposing to this are systemic (Cafarchia et al. 2007; Tee et al. 2019; Ebani et al. 2020a).

On the one hand, the primary factor for increasing the BY population is a violation of the physical, chemical and immunological mechanisms of host defense, which normally limit the fungal colonization of the skin (Liu et al. 2007; Dizotti et al. 2007; Jain et al. 2022). On the other hand, communication of microorganisms within the population, carried out by means of "signaling molecules", plays a key role in starting the pathological process. It is assumed that when a population reaches a certain number, a "sense of quorum" arises in it, which serves as a starting signal for the activation of pathogenicity factors and, as a consequence, leads to the

development of an infectious process (Hossain et al. 2007; Sachivkina et al. 2021; Vercelli et al. 2021).

The mechanism of intrapopulation communication of microorganisms is associated with such a phenomenon as the formation of biofilms – supra-organizational structures that provide protective and trophic functions. Biofilms are differentiated communities of microorganisms formed by a single microbial agent or a mixture of fungal and bacterial species. Biofilms are attached to biotic or abiotic surfaces, and their structure contributes to the innate physical and chemical resistance of microorganisms (Cafarchia et al. 2005; Lee et al. 2019; Bismarck et al. 2020). It is known that the ability to form biofilms is one of the pathogenicity factors of the *Candida* genus (Sachivkina et al. 2019). And in 2007 it was found that BY of the genus M are also capable of forming biofilms on the surface of various substrates (Kaneko et al. 2007).

Researchers have found that symbiotic relationships of BY and skin-dwelling bacteria (in particular, *Staphylococci*) play an important role in the pathogenesis of the disease. Staphylococci also produce lipase, which disrupts the secretory function of the skin and creates favorable conditions for the growth of both organisms, while such conditions are unfavorable for other competitive microorganisms (Lee et al. 2020; Gómez-García et al. 2022). In addition to *Staphylococci*, other types of bacteria and microscopic fungi can play a role in the pathogenesis of the disease. So, from dogs with external otitis caused by MP were also isolated bacterias such as: *Staphylococcus spp., Pseudomonas spp., Proteus spp., Streptococcus spp.,* as well as fungi – *Candida spp*. and *Aspergillus spp* (Lenchenko et al. 2020; Kumari et al. 2022; Sachivkina et al. 2022b).

Significant virulence factors of BY fungi of the genus M are hydrolytic enzymes that cause the invasion of the fungus into host tissues. Lipolytic enzymes are able to hydrolyze skin secretion lipids to free fatty acids. In turn, free fatty acids inhibit the growth of other microorganisms, increasing the competitiveness of BY (Ortiz et al. 2013; Puig et al. 2017; Park et al. 2021).

Recently, in the scientific literature, close attention has been paid to the study of herbal medicines, the possibility of their use for the treatment of infectious (fungal and bacterial) diseases (Ahmad et al. 2011; Chan et al. 2018; Sachivkina et al. 2021). According to the literature and our own research, Farnesol ($C_{15}H_{26}O$) - Far has proven its antimycotic efficacy *in vivo* and *in vitro* models (Jabra-Rizk et al. 2006; Sachivkina et al. 2021a; Alkhanjaf et al. 2022). Chemically, Far is an acyclic sesquiterpene alcohol, it is a thermally stable molecule. It is not exposed to extreme pH values, which is especially important in the development of YLF infection. Far as a quorum-sensing (QS) molecule participates in the regulation of various physiological processes in unicellular fungi, including filamentation, biofilm formation, drug susceptibility and apoptosis. This compound is produced by many microorganisms, and is also contained in various essential oils of plants, for example, in the flowers of *Tilia europaea* (Sachivkina et al. 2020; Sachivkina et al. 2021b). Despite a large number of scientific articles and our own studies proving the antibiotic effect of Far against *Candida spp.*, the effect of this molecule in relation to BY of the genus M has not yet been studied.

Today, in veterinary medicine, only a few doctors recommend phytopreparations for the treatment of otitis externa and dermatitis, and there is still relatively little data on their effectiveness in the scientific literature, especially in Russian. Therefore, the development of targeted therapy using alternative means can become one of the directions in solving global problems of infectious animal diseases, as well as increased resistance of microorganisms (Khosravi et al. 2016; Aiemsaard et al. 2019; Schlemmer et al. 2019). Essential oils of herbal origin such as tea tree oil, lime oil, rosemary oil, have gained global importance in dermatology. These oils are rich in aromatic secondary metabolites, especially terpenes and phenolic components that impart substantial antimicrobial properties and resisting biofilm production (Weseler et al. 2002; Nocera et al. 2020; Bohmova et al. 2019; Angiolella et al. 2023).

Ecology of *Malassezia* in dogs and cats.

Culture studies clearly show that MP is the predominant cutaneous BY in both healthy dogs and dogs with M dermatitis or otitis media (Nardoni et al. 2008; Cabañes et al. 2014; Triana et al. 2015). MP is also most important in cats, but other M species are more commonly found in this host (Bond et al. 1995; Bond et al. 2020). Several investigators have investigated M colonization in different anatomical regions of different breeds of healthy adult dogs (Guillot et al. 1996; Nardoni et al. 2008; Neves et al. 2018). The overall conclusion from these studies is that the perioral region and interdigital skin are frequently (up to 80%) colonized by MP in healthy dogs of different breeds, while yeasts are less frequently (<25%) detected on the skin of dogs, armpit, groin and back. Feline skin can be colonized by several M. species. Although MP remains the most common, as in dogs, lipid dependent species isolated from cats include *M. sympodialis, M. globosa, M. furfur, M. nana*, and *M. slooffiae* (Cafarchia et al. 2005; Jain et al. 2017). *M. nana* is the most common lipid-dependent species in cats, especially in the ear canal, and this animal host appears to be dominated by a specific *M. nana* genotype (Bajwa et al. 2023). *M. slooffiae* has been predominantly, but not exclusively, isolated from the ungual folds of cats (Carrillo-Muñoz et al. 2013; Bond et al. 2020). Recently, methods based on PCR have enabled better characterization of the complex microbial communities found on the skin of animals and allowed the detection of M species that would otherwise be missed using culture methods (Gaitanis et al. 2009; Puig et al. 2019). Theelen et al. reported that skin mycobiota in dogs was influenced by various factors, including environmental exposure, cohabitation with other pets, and skin health (Theelen et al. 2018). Surprisingly, M yeasts were not the most abundant fungal organisms on healthy canine skin. In addition, these authors were unable to detect any significant differences in relative M levels between healthy and allergic dogs. In cats, metagenomic analysis has shown that the skin is inhabited by bacterial communities that are different for each body site (Celis-Ramirez et al. 2017; Rudenko et al. 2021), while fungal communities appear to be more unique at the individual level (Chen et al. 2005). When samples were collected from healthy

and allergic cats, the most common fungal sequences were identified as environmental filamentous contaminants rather than M-yeast, which was identified in 30% and 21% of samples from healthy and allergic cats but was rarely more than 1% of the relative abundance of fungi (Bond et al. 1996). The aims of a recent study by Gaitanis et al. were to assess how genotype and environment can influence the bacterial and fungal microbiota of cat skin (Gaitanis et al. 2009). They demonstrated with PCR that *M. stricta* and *M. globosa* were the most common M species. Sequences corresponding to *M. slooffiae, M. furfur, M. nana, M. pachydermatis, M. dermatis, M sympodialis, M. japonica, M. obtusa*, and *M. yamatoensis*. M numbers varied significantly between cat breeds, with Devon Rex having the highest abundance. No significant difference was found in the abundance of any M species between different cat breeds or when comparing domestic and outdoor animals. Taken together, these studies demonstrate a significant discrepancy between culture and molecular studies in determining the M species component in the skin mycobiota in dogs and cats.

BY *Malassezia* cause various immune reactions in the macroorganism, and the immunogenic properties are largely due to the lipid layer of these fungi. They interact with various immunocompetent cells, including antigen-presenting dendritic cells, macrophages, eosinophils, neutrophils, stimulating the synthesis of cytokines and chemokines of various functional groups. It has been established that M can both stimulate and inhibit the synthesis of pro-inflammatory cytokines (IL-1, IL-6, TNF) (Di Cerbo et al. 2016; Ebani et al. 2017; Sell et al. 2022). As a result, the inflammatory response in MO can vary from almost imperceptible to pronounced.

Clinical presentations of *Malassezia* infection in dogs and cats.

Factors contributing to MO can be of the most diverse nature. As noted by Sim et al. (Sim et al. 2019), the skin, especially in the auditory canal, is a fragile multicomponent system, and even small changes in the skin microclimate can upset the delicate balance in the secretory mechanism and microflora composition, which, in turn, can lead to opportunistic infections.

The affected skin is usually erythematous, often with a greasy brownish-black coating that entangles the lower part of the hair; intertriginous zones are often involved (Kiss et al. 1996; Corona et al. 2021). Itching, which varies from minimal to severe, is usually the dominant symptom. Hyperpigmentation, lichenification, malodor, traumatic alopecia, and otitis externa often co-occur. In otitis externa, ear canal discharge is usually ceruminous and rarely purulent, and the inflammation usually extends to the pinnae. Cases of M present with erythema and swelling of the claw folds, waxy or crusted brown exudate, reddish-brown discoloration of the nails, and may coexist with broader hairy pododermatitis. The occasional onset of frantic facial itching in dogs with variable, sometimes mild chin/perioral erythema may be misdiagnosed as a neurological disease (Cafarchia et al. 2004; Hoes et al. 2022). The signs of M dermatitis may mimic or exacerbate the symptoms of canine AD. Signs of comorbidities may be obvious at first, although they usually show up best after the secondary M infection has cleared up. M-dermatitis can present in cats with an allergic skin disease phenotype, idiopathic facial dermatitis, feline acne, and serious internal diseases such as feline paraneoplastic alopecia and thymoma-associated exfoliative dermatitis (Rosser et al. 2004). Customer expectations should be managed appropriately; residual skin involvement usually persists despite successful antifungal therapy.

Diagnostic approach *Malassezia* in the veterinary.

A recent clinical consensus guidance document presents a detailed diagnostic algorithm for use in the veterinary clinic (Anokhina et al. 2009; Cabañes et al. 2016; Marrero et al. 2017). The importance of investigating and correcting comorbid skin conditions and other predisposing factors, if possible, cannot be overemphasized if a chronic or relapsing course is to be prevented. Cytology using swabs of lesions rolled onto a glass slide is usually best limited to use in the ear canal, as the shedding of scales and yeasts from the skin is lower than with tape strips and dry scrapings (Patterson et al. 2002; Marrero et al. 2017). In a recent randomized, blinded, prospective study of 30 dogs with otitis externa, cytological samples obtained with

a conventional cotton swab contained comparable numbers of yeasts and bacteria, but fewer inflammatory cells, compared with samples prepared by aspiration of material from the otitis externa ear canal with soft rubber tube (Swinney et al. 2008). In an effort to improve the sensitivity of MP cytology in canine ear, Puig et al. developed a quantitative PCR method based on the amplification of a single copy of the ß-tubulin gene (Puig et al. 2019). The authors concluded that the results were accurate and showed better sensitivity compared with cytology; this technique may have useful applications in diagnosis and monitoring of therapy, as well as in pathogenesis research and therapeutic product development.

Treatment of *Malassezia* otitis.

The therapy of external MO is based on topical drugs. Since this disease is of an exclusively superficial nature, systemic drugs, as a rule, do not reach therapeutic concentrations in the ear secretion, which is why they cannot effectively act on the pathogen. Systemic therapy may be indicated only in cases of deep erosion or ulceration of the auditory canal, when otitis externa transforms into otitis media (Gupta et al. 2000; Pistelli et al. 2012; Nardoni et al. 2014). Most commercial preparations intended for the treatment of MO contain a combination of active substances - antibacterial, antifungal and anti-inflammatory action. The basis of the drug is of great importance - its composition affects both the activity of the active substances and their bioavailability. Some bases may be irritating, i.e., topical preparations must be well balanced in composition. The following antifungal agents may be used therein.

Nystatin. A polyene antimycotic that binds to fungal cell membrane sterols, disrupting cell permeability and causing osmotic destruction. NS is mainly used to treat candidiasis; however, some studies have also demonstrated its clinical efficacy in M infections (Vitanza et al. 2019; Cordeiro et al. 2022; Hoes et al. 2022). Usually, nystatin is available in the form of obturating ointments, which limits its use in exudative or ceruminous external otitis.

Azole antimycotics. Imidazoles (CC, MI, KT) and triazoles (IT, FU) have the same mechanism of antifungal action: impaired ergosterol synthesis of the fungal cell wall by inhibiting the cytochrome P450 system. The azoles effectiveness against M spp. is distributed as follows (Lyskova et al. 2007; Marrero et al. 2017): CC (comparable to NS in efficiency), MI (10 times more effective than NS), KT and IT. Strains of different geographical origin may vary in their sensitivity to certain azoles. CC and MI as part of a number of veterinary preparations are widely used for the treatment of MO in dogs and cats. In clinical conditions, M resistance to CC is observed more often than to MI. KT is available only in the form of medicines (tablets, creams). In some cases, veterinarians make their own 1-2% form for the treatment of otitis media, when other azoles are ineffective. IT is mainly used as a systemic preparation, but is also available in the form of a 1% solution, which can be used externally (Jabra-Rizk et al. 2006; Cordeiro et al. 2022).

Allylamines. Antimycotics of this series cause a violation of the biosynthesis of ergosterol by inhibiting squalene epoxidase. In vitro experiments have shown high activity of terbinafine against many species of the genus M, including MP (Chan et al. 2018; D'agostino et al. 2019; Čonková et al. 2022). However, terbinafine is inferior to IT and KT in terms of activity; while for half of the M. spp. it has shown low activity (Cafarchia et al. 2015; Sachivkina et al. 2019a).

Antifungal drug susceptibility testing for *Malassezia.*

The increasing dominance of polyresistant strains of M spp. has forced scientists worldwide to consider new approaches for antimycotic drugs. A prerequisite is the safety and non-toxicity of new drugs for the animals (Miguel et al. 2010; Corona et al. 2021). Accumulation of MP on the animal skin surface can lead to subsequent invasion, which can cause lymphogenic and hematogenic spread of BY, generalization of the process and dissemination into internal organs (Sachivkina et al. 2010; Capoci et al. 2015; Theelen et al. 2018). Moreover, the induction of in vitro FU resistance in MP otitis or *M. furfur* fungemia or KT-resistant stains in dogs with otitis (Weiler et al. 2013; Chan et al. 2018), suggested the occurrence of drug

resistance phenomena in these yeast species (Patterson et al. 2002; Rosser et al. 2004). Antifungal susceptibility test methods have not yet been standardized, neither by CLSI or by EUCAST (Clinical and Laboratory Standards Institute (CLSI), 2020), resulting in the absence of clinical breakpoints for these BY species. A recent study showed that drug efflux pumps (EPMs) are involved as defense mechanisms to azole drugs in BY (Iatta et al. 2014; Ehemann et al. 2023). By using a broth microdilution chequerboard analysis, the in vitro efficacy of azoles in combination with EPMs was evaluated. The synergistic effect of FU and VOR was observed only in M strains with FU MIC more 128 μg/mL for *M. furfur*, FU MIC more 64 μg/mL for MP, and VOR MIC more 4 μg/mL in both Malassezia spp., suggesting that the above FU and VOR MIC values might be considered the cut-off to discriminate susceptible and resistant strains (Iatta et al. 2015; Vinciguerra et al. 2019). Finally, the in vitro susceptibility of M for echinocandins suggests that this genus is intrinsically resistant to these drugs. Indeed, MIC more 32 μg/mL were usually recorded for MP and *M. furfur* regardless of the employed CLSI protocol for testing drug efficacy (Iatta et al. 2014). For treatment of M-related infections, azoles, and the polyene antimycotics are frequently employed in animals. Topical antifungal agents (mainly azoles) are adequate for the management of localized skin lesions, while systemic IT or FU for severe skin diseases (Cafarchia et al. 2015). For MO infections, there are only recommendations to remove first ear contents before use antifungal drugs such as FU, AP, and/or VOR (Crespo et al. 2000; Shafiei et al. 2020; Čonková et al. 2022). AP is effective in the treatment of M systemic infections, but both FU and MC fail to prevent *Malassezia* fungemia. Usually, about 24 days of AP treatment might be useful for a positive outcome of M fungemia but the length of the treatment might be different depending on the M species (Bensignor et al. 2006; Weiler et al. 2013; Schlemmer et al. 2019).

Mammalian models of fungal infections

Predisposing factors: immunosuppression and ketoacidotic diabetes in laboratory animals are actively used to model fungal infections in mammals and other vertebrates.

Immunosuppression.

Hematologic malignancies themselves and their associated treatment, such as preparation for stem cell transplantation, are usually associated with severe immunosuppression. A decrease in the number of white blood cells (leukopenia) and especially a decrease in neutrophil granulocytes (neutropenia) makes such patients very susceptible to infections caused by bacterial and fungal pathogens (Seyedmousavi et al. 2018). Thus, leukopenia/neutropenia is a common risk factor for infectious diseases, and various protocols have been developed to induce them in animal models, for example, by using cytostatic drugs such as cyclophosphamide in mice or cytarabine in rabbits, which target replicating cells, including in the bone marrow (Gebremariam et al. 2015). These substances lead to a rapid drop in neutrophil numbers because the short half-life of these immune cells requires constant replenishment from the bone marrow. In contrast, resident immune cells such as tissue macrophages are not acutely affected by cytotoxic therapy, so commonly used protocols additionally use corticosteroids (eg, cortisone acetate) to impair the function of these cells. In this case, deep immunosuppression can usually be caused by using the above-mentioned drugs after 2-3-4 days, and after experimental infection, leukopenia can be maintained by repeated use of a cytostatic drug, if necessary (Lewis et al. 2011). Successful immunosuppression can be easily confirmed by determining the white blood cell count on blood smears or using hematology counters (Sondhi et al. 2000).

As an alternative to cytostatic therapy, monoclonal antibodies can deplete specific types of immune cells. Consequently, neutropenia can be temporarily induced in animals without necessarily affecting other immune cell populations (Han et al. 1997). This approach has been used to study the relative influence of distinct immune cell populations on fungal infections such as aspergillosis and

candidiasis (Stephens-Romero et al. 2005; Sachivkina et al. 2010) but has not yet been applied to malacesiosis models. Interestingly, the type of neutrophil depletion has been shown to influence the susceptibility of mice to aspergillosis (Stephens-Romero et al. 2005; Kwon-Chung et al. 2009). In contrast to cytostatics, the use of corticosteroids does not reduce the number of circulating neutrophils, but impairs their antifungal function (Gupta et al. 2000; Pistelli et al. 2012; Nardoni et al. 2014). As a consequence, animals receiving corticosteroid therapy became susceptible to infections by filamentous fungi, including Mucorales, but the pathogenesis differed from leukopenic animals. This has been well demonstrated in murine pulmonary aspergillosis, in which fulminant fungal growth, angioinvasion, and tissue necrosis lead to pathogenesis in leukopenic mice, while a pronounced influx of neutrophils leads to tissue destruction in corticosteroid-treated animals (Han et al. 1997; Roilides et al. 2012). In addition, fungal virulence factors that negatively affect immune cells play a role in infection of corticosteroid-treated animals, but not leukopenic animals (Stephens-Romero et al. 2005; Kwon-Chung et al. 2009; Roilides et al. 2012).

Diabetes.

Poorly controlled diabetes mellitus associated with ketoacidosis (diabetic ketoacidosis: DKA) is a risk factor for various infections, especially fungal infections (Al-Awar et al. 2016). Various models have been developed to study type 1 and type 2 diabetes in mice and rats: chemical ablation of insulin-producing cells; models with increased levels of spontaneous autoimmune diabetes; obese models (King et al. 2016; Schlemmer et al. 2019). For studies of fungal infections, of these three regimens, the first is most often used - chemical ablation of insulin-producing beta cells by using streptozotocin or alloxan. The advantage of this model is that it is independent of the genetic background and age of the animals. A single injection of streptozotocin is sufficient to induce DKA, but it is important to use the correct drug and dosage of the chemical to achieve stable results without causing mortality (Goyal et al. 2016; King et al. 2016). Because not all animals will develop ketoacidosis following induction, blood glucose levels should be measured to confirm hyperglycemia; Additionally, ketosis can be detected in urine samples (Wu

et al. 2015; Lee et al. 2022). It should be noted that some studies in mice have used combined streptozotocin-induced DKA and mild corticosteroid immunosuppression (King et al. 2016).

Regardless of the immunosuppression method, there are ways to prevent spontaneous infections in immunosuppressed animals by taking precautions. Depending on the cleanliness of the animal house, prophylactic antibiotic treatment may be included. It is suggested by Al-Awar et al. 2016 that uninfected, immunocompromised controls should be included as sentinel animals to detect spontaneous infections and evaluate the effects of immunosuppressive therapy on their overall health, clinical parameters, and immune responses, as well as potential side effects.

How to select host species, route of infection and predisposing factors?

Ideally, an infection model accurately mimics infection in the species of interest. However, most of the research over the past two decades has been done in mice. This could be ascribed to the generally moo fetched, quick era time, ease of dealing with and accessibility of research facilities for this species. Mice have the included advantage that different immunosuppression and diabetes treatment conventions are well built up, a expansive number of apparatuses are accessible for atomic investigation of have reactions, and hereditarily adjusted creatures can be utilized to address particular investigate questions (Weseler et al. 2002; Bohmova et al. 2019). Rabbits have moreover been utilized for inquire about, and due to their bigger estimate, they have a few common focal points: for case, rehashed inspecting can be done and the test estimate is bigger. Be that as it may, the impediment is the altogether higher support costs and space prerequisites (Nocera et al. 2020; Angiolella et al. 2023). Likewise, the route of infection determines the primary organ affected by the fungus and the type and impact of interactions with host cells. After pulmonary infection, the first steps of interaction occur with lung epithelial cells, and the lungs are not the main target after intravenous infection (Han et al. 1997). This must be taken into account when translating results from in vitro approaches to *in vivo* models.

Intravenous infection is also frequently used in studies assessing the effectiveness of therapeutic agents, especially antifungals. For these studies, it is important to be able to reliably induce severe infection. Moreover, antifungal drugs should ideally save patients from the most severe forms of the disease and in the absence of a host defense response. This can be modeled by intravenous inoculation into immunocompromised mice; if therapeutic interventions are effective in this model, they may also provide benefit in less severe cases of infection (Bismarck et al. 2020; Puigdemont et al. 2021; Vercelli et al. 2021).

Localized subcutaneous Rhizopus infection has been detected in diabetic rabbits and rats and in immunocompromised mice by subcutaneous injection of fungal spores (Moulari et al. 2007; Nardoni et al. 2017;). In addition, intradermal administration of different strains of Rhizomucor and Lichtheimia spp. may cause temporary lesions in immunocompetent mice (Sim et al. 2019a). In rabbits, subcutaneous infections have resulted in granuloma formation (Sim et al. 2019b). Interestingly, the fungi persist within these granulomas and the infection can be reactivated by inducing diabetic ketoacidosis (Goyal et al. 2016). Although these models may not reflect the more complex pathophysiological changes associated with traumatic skin injury and subsequent infections, they have the advantage of being technically simple and thus can be easily used for future research.

Malassezia **models as a way to understand host–microbe interactions.**

Little is known about the virulence properties and infection mechanisms of M spp., and the implementation of infection models may allow for the evaluation of the interaction of these yeasts with hosts, the virulence of different species or strains of a specific species, and antifungal activity. There are different types of suitable models in which virulence and infection can be studied, but it is critical to realize that the results obtained in each model provide partial answers, as was mentioned before. It is therefore important to study virulence properties in different in vitro and in vivo models and the results obtained can provide complementary answers (Sachivkina et al. 2010; Last et al. 2021; Li et al. 2022).

One of the infection models that may help to unravel host–microbe interactions is in vitro models, which have been used since the 1960s (Morozov et al. 2011; Last et al. 2021). *In vitro* models are generally easier to handle, the majority of factors can be controlled, the evaluation of drug activity is more accurate, and, in some cases, they are cheaper than using animal models. These models can also be cataloged as *ex vivo* models (Sachivkina et al. 2010; Li et al. 2022), like cultured cells, removed organs, and skin equivalents or dermis equivalents (Morozov et al. 2011; Wagener et al. 2012; Deng et al. 2015). As good as the in vitro models are, they do not fully reproduce the host–microbe interactions that occur, for example, on the skin.

Contrary to in vitro models, the in vivo models mimic the complexity of the host response better (Spence et al. 2008; Deng et al. 2015). These are rather diverse and can vary from mammalian models to insect models. Mammalian models are phylogenetically the closest to human beings and, generally, are regarded as more accurately reproducing the host–microbe interaction, known as fidelity (Sachivkina 2010). Additionally, many of these models are well characterized, allowing for genetic modifications to reach a desirable condition. The drawbacks of these models are the high cost of feeding and maintenance, the limited number of individuals and the need for trained personnel to handle the animals (Wagener et al. 2012; Deng et al. 2015). All mammalian models are limited by ethical considerations—the use of mammalian infection models must be justified and subject to institutional and national regulation. This limits the use of mammalian models to address certain questions, such as large-scale studies of strain-specific differences in virulence or screening of antifungal compounds. These drawbacks can be solved by the implementation of alternative animal models, like invertebrates.

Invertebrate animal models have recently gained importance in fungal research since studies have shown that the microbial virulence factors involved in infections in mammals are the same as those involved in invertebrate infections (Last et al. 2019; Scheler et al. 2024). In fact, it seems that different aspects of the innate immune response in vertebrates and invertebrates are shared and represent a

conserved trait, which means that human pathogens, at least in part, interact similarly with both immune systems (Spence et al. 2008; Sparber et al. 2019). The innate immune responses in invertebrate models are comparable to, for example, the human immune response to fungi via Toll-like receptors, which were originally discovered in *Drosophila melanogaster* (Jacobsen 2019), a model system already used with *Malassezia* (Almaliki et al. 2020; Torres et al. 2020), and also present in *Caenorhabditis elegans* (Torres et al. 2020; Rios-Navarro et al. 2021). Besides, the well-developed phagocytic system in lepidopterous and coleopterous larvae parallels the process of phagocytosis in mammalian systems (Sparber et al. 2019; Almaliki et al. 2020).

Experimental models have been used to better understand the pathogenesis of M dermatitis. Cutaneous responses to the application of viable and killed "lipid-dependent" M in laboratory animals - guinea pigs, mice, rabbits; generally comprised focal areas of scaling that most often resolve without treatment upon discontinuation of inoculation (Zhang et al. 2019; Jacobsen 2019). Similarly, in laboratory beagle dogs, application of MP was associated histologically with epidermal hyperplasia, occasionally with parakeratosis, superficial perivascular dermal inflammation with primarily neutrophils and lymphocytes, and sometimes mast cells (but not eosinophils); features were more severe at sites that were occluded (Sell et al. 2022; Merkel et al. 2024). Histological changes markedly reduced within 7 days of withdrawal of yeast challenge. Inoculation of suspensions of MP into the middle ear and dermis of immunosuppressed mice led to transient infection that resolved within 21 days (Zhang et al. 2019; Rios-Navarro et al. 2021). Recently, Merkel et al. developed a minihost (invertebrate) experimental model wherein the pathogenicity of MP was evaluated in wild-type (WT) and Toll deficient *Drosophila melanogaster* (Merkel et al. 2024). WT flies were resistant to the infection, whereas Toll-deficient flies showed inoculum dependent mortality rates. Experimental models may prove valuable in the further elucidation of both yeast virulence and host immune factors that are important in disease processes in various species. Models of vaginal candidiasis in mice were developed at the Department of

Microbiology of the Peoples' Friendship University of Russia in 2009-2010 (Sachivkina et al. 2009; Sachivkina et al. 2010). Female mice in estrus were maintained by subcutaneous injections of the hormonal drug Mesalin (Intervet, USA). When creating dysbiosis in laboratory animals, the antibiotics doxycycline and benzylpenicillin were used.

Microbiological aspects antimycotic phytotherapy.

Since the 1950s, with the beginning of the widespread use of antibiotics, the use of medicinal plants as antimicrobial therapy, to a certain extent, has lost its relevance. At the same time, due to the frequent and uncontrolled use of antimicrobial drugs, such problems as the development of allergic reactions, toxic conditions, accompanied by a violation of colonization resistance of the intestine, began to arise (Ahmad et al. 2011; Bohmova et al. 2019; Sim et al. 2019). Along with this, the appearance of antibiotic-resistant microorganisms in the composition of microsymbiocenoses of the animal organism was noted, which was accompanied by a lack of effect in the treatment of diseases of fungal etiology, especially in immunocompromised animals.

The urgency of the problem of phytotherapy in microbiology, therapy and, in particular, in the treatment of diseases of fungal etiology, is due to a number of reasons.

First of all, this is the lack of efficacy and toxicity of traditional antimicrobial, including antimycotic drugs used to treat mycoses.

Secondly, it is a wide spectrum of action of medicinal plants that, along with antifungal activity, have antibacterial, antiviral and antiprotozoal activities.

Thirdly, it is a prolonged therapeutic effect due to the low rate of decomposition of plant components (Chen et al. 2010; Langford et al. 2010; Capoci et al. 2015).

CHAPTER 2. MAIN CONTENT OF THE WORK. MATERIALS AND RESEARCH METHODS

2.1. Research Plan

1. Choice of Malassezia strain from dogs (30 isolates) and cats (5 isolates).
2. Determination of the accompanying microflora in the ear exudate in case of MO.
3. Determination of antimycotic resistance of selected strains.
4. Determination of the ability of M to form biofilms.
5. Determination of the ability of M to adhere to buccal cells of dogs.
6. Determination of the ability of M to be phagocytosed with rat alveolar macrophages *in vitro*.
7. Based on points 3-6, we select one strain that has resistance to several modern antimycotics, which adheres better, but phagocytizes worse, and which produces the "strongest" biofilms.
8. Prove that Farnesol can destroy strongest MP biofilms *in vitro.*
9. Prove that Farnesol can change antimycotic resistance of selected strains *in vitro*.
10. Develop 2 models *in vivo* with M infection in mice and rabbits.
11. Infect 35 rabbits with this strain: 5 control, and 30 – experience.
12. Carry out treatment "Surolan", "Otifri", "Otoxolan" "Surolan" + Farnezol, "Otifri" + Farnezol, "Otoxolan" + Farnezol.
13. Carry out rabbit biochemical blood tests.
14. We look at how quickly the recovery comes; we fix the presence of M in the seeding of ear exudate.
15. Conclusions

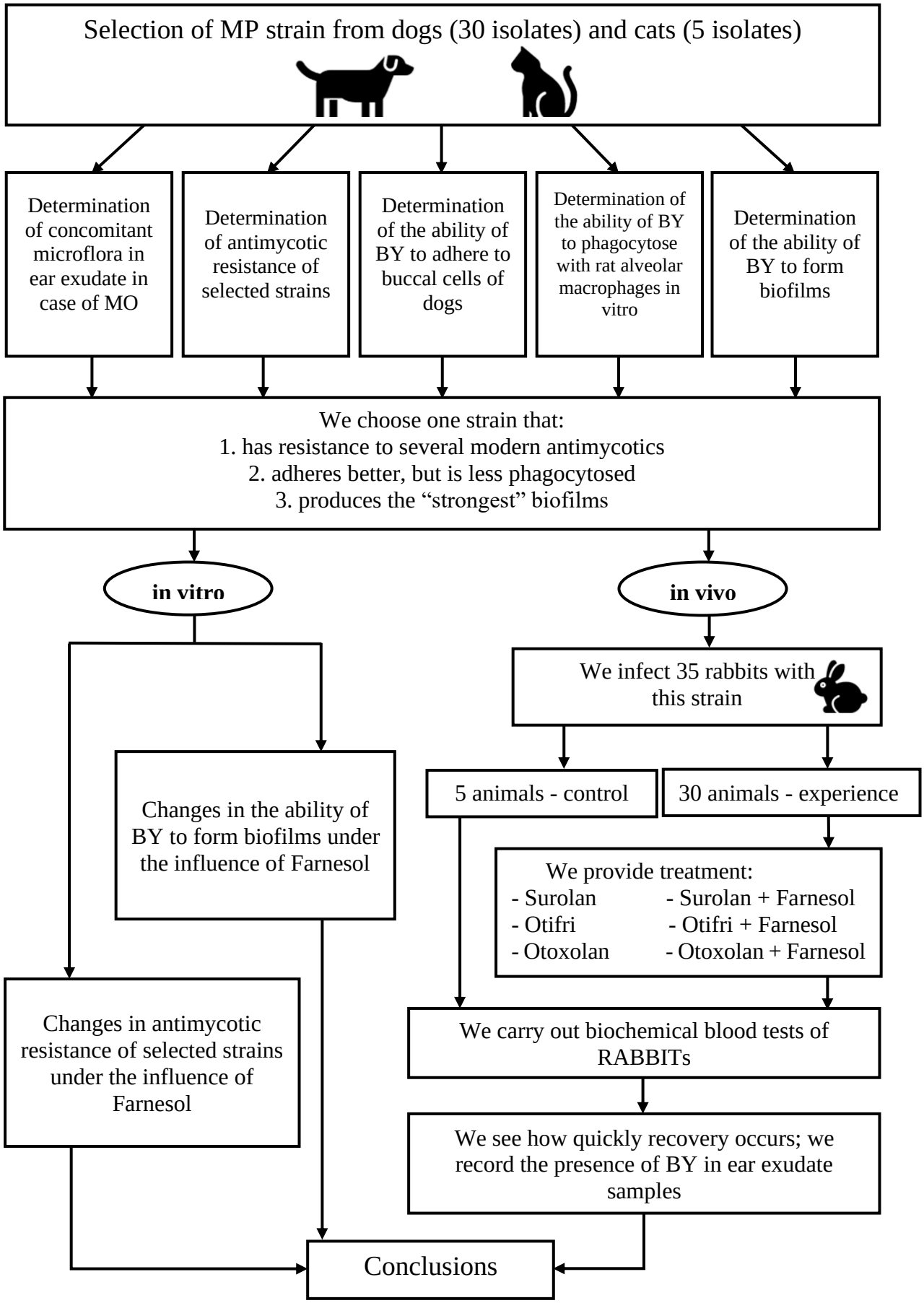


Figure 1. Research plan.

2.2. Obtaining clinical strains of *Malassezia pachydermatis*.

In the center of Veterinary Innovative Medicine of the RUDN (Moscow) for the period 2021-2022, 76 cases of dog otitis externa were investigated and 10 - cat otitis externa, of which 30 canine cases (Cd1-Cd30) (Fig.2) and 5 feline (Cc1-Cc5) (Fig.3) were confirmed otitis with MP etiology.

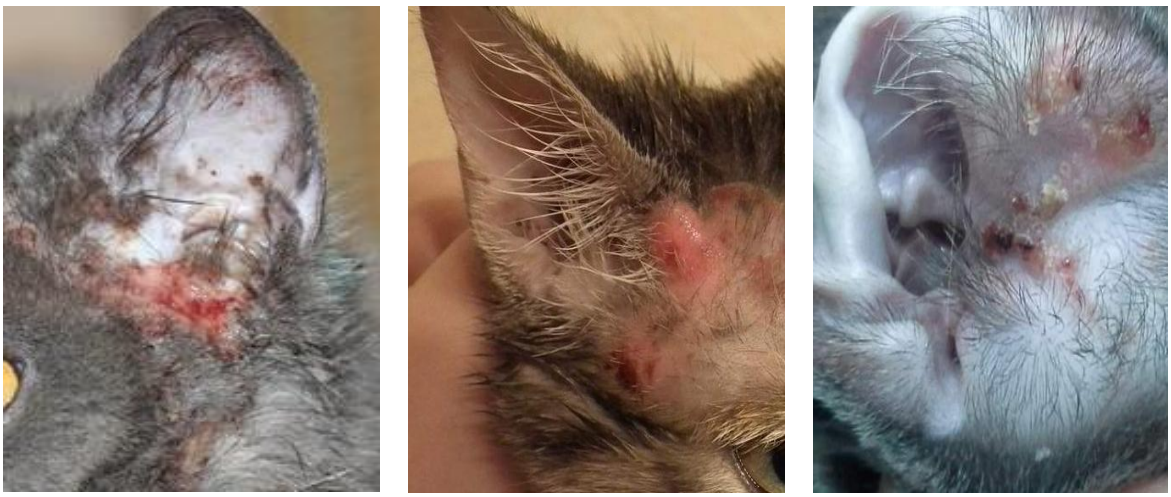
For our experiments, BY received clinically was used. Preliminary identification of strains to the genus M level was carried out by phenotypic signs using microscopy of an ear smear contents and staining with gentian violet. Ear exudate was applied to the surface of the nutrient medium Sabouraud dextrose agar (Difco, Bordeaux, France) and cultured at 37°C for 48-72 hours. The cultivation of microorganisms was carried out in a thermostat with a Heidolph Unimax 1010 shaker (Germany). Then identification of microorganisms was carried out using the matrix-activated laser desorption/ionization technology "Bruker Daltonik MALDI Biotyper" (Bruker Daltonik Inc., Germany). Identification with a Score of more than 2,000 was considered reliable. The obtained spectra were compared with the library of mass spectrum profiles of Biotiper3 MALDI (Kolecka et al. 2014; Honnavar et al. 2018). The strains were stored at − 80°C (Lenchenko et al. 2020).

If the culture was not identified as MP, then the data from these animals were not included. This work presents the results of an experiment with 100% identification of MP. There were precedents for the identification of another type of microorganism and, despite the fact that the animals were prescribed therapy and blood was taken for research, these results were not taken into account.

The study involved 30 dogs of different breeds, gender and age from one to 14 years. All the animals had apartment maintenance with walking. The diet consisted of dry food. Treatments for ectoparasites (external) and endoparasites (internal) were carried out in all participants of the experiment regularly and on time. From anamnesis: there was itching in the ears, an unpleasant smell for several weeks. At the receptions, it was noted: hyperemia of the auricles, stenosis of the auditory canal, in some cases alopecia, a large amount of yellow-brown discharge with a sharp sour smell (Fig.2 a-i).

Figure 2. Different clinical forms of dog's external otitis: A, B - erythematous otitis; C, D - erythematous-ceruminous; E, F - ceruminous.

Also, the study involved 5 cats of different breeds, gender and age from 7 month to 10 years. (Fig.3 a-c) The cats were kept at home, received various types of food - dry or liquid prepared food or fresh meat, according to the owners.



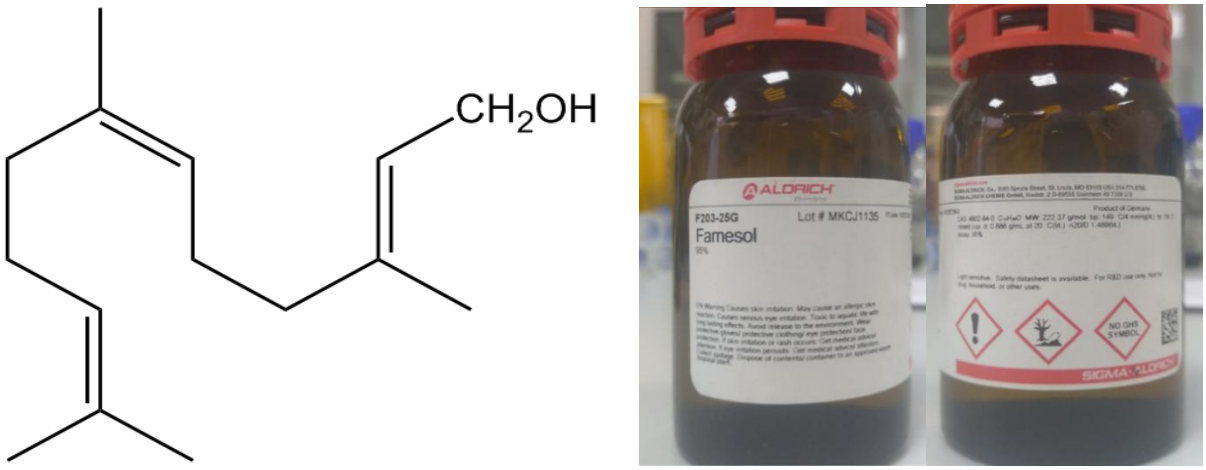
A B C

Figure 3. Different clinical forms of cat's external otitis.

In our work, several clinical forms of external otitis in dogs and cats were noted. Erythematous otitis is accompanied by erythema, edema, the degree of itching varied. Erythematous-ceruminous was manifested by erythema, itching, the release of abundant ear secretions (cerumen) of a yellow-brown color, often with an unpleasant odor. Ceruminous otitis was characterized by abundant discharge of earwax, but without signs of inflammation. The proliferative form was characterized by hyperplasia of the sebaceous glands, the formation of papules merging in the form of "cauliflower", which is typical in chronic cases. With purulent otitis, purulent discharge was observed, with palpation the ear was painful, sometimes crepitation was heard. In most cases, we observed MO with erythematous-ceruminous form.

2.3. Reagents.

1) Farnesol (farnesol) (trans, trans-farnesol; Sigma-Adrich, Darmstadt, Germany), molar mass = 222.37 gr/mol, mass of the substance = 0.886 g/mL, the amount of the substance in moles = 0.886:222.37 = 0.004 M/mL, or 4000 µM/mL (Fig.4).



A B

Figure 4. A- the chemical formula of Farnesol; B- the appearance of the drug Farnesol (Far) (Sigma-Aldrich, Germany).

2) Surolan (Elanco, Portugal) contains as active ingredients in 1 ml: miconazole nitrate 23 mg, polymyxin B sulfate 0.53 mg, prednisolone acetate 5 mg/ml, as well as auxiliary ingredients: a mixture of colloidal silicon dioxide and liquid paraffin (in a ratio of 1:2) - up to 1 ml. The drug is a homogeneous, opaque, oily suspension of white color, with a slight specific odor. Miconazole, which is part of the drug, is a synthetic derivative of imidazole, inhibits the synthesis of ergosterol in the membrane and plasma membranes of fungi, changes the permeability of the cell wall of some gram-positive bacteria and, thus, has a fungicidal and bactericidal effect. Miconazole is active against dermatophytes and fungi, incl. genus *Candida*, *Trichophyton, Malassezia, Microsporum*. Polymyxin B causes disruption of the integrity of the cytoplasmic membrane of the bacterial cell and has a bactericidal effect on gram-negative bacteria, including *Escherichia coli, Salmonella spp., Klebsiella spp., Pseudomonas spp., Enterobacter spp.* Prednisolone acetate is a synthetic glucocorticoid that, when applied topically, has an anti-inflammatory and anti-exudative effect, helps reduce itching and promote tissue healing. When Surolan is used externally, miconazole and polymyxin B are practically not absorbed and have a local antimicrobial and antifungal effect. Prednisolone acetate is

absorbed through the skin in small quantities, biotransformed in the liver and excreted from the body, mainly in the form of metabolites in urine and feces.

Indications: combined drug for the treatment of otitis media and skin diseases of bacterial and fungal etiology in dogs and cats. The drug has a wide spectrum of antimicrobial and antifungal activity, has anti-inflammatory and antiallergic effects.

Doses and method of administration: for otitis, Surolan is instilled into the sore ear, 3-5 drops. First, the auricles and ear canal are cleaned of earwax. In order to distribute the drug evenly, the base of the auricle is massaged with light movements. If after using the drug the animal shakes its head, the head should be fixed for several minutes to stop splashing the drug.

3) Otifri (Vetoquinol, Russia) is a hygienic lotion for topical use. Ingredients: water, propylene glycol, emulsifier (Cremofol EL), calendula, basil oil. In appearance, Otifri is a transparent solution that has a beneficial effect on the skin of the auricle and, with regular use, normalizes the microflora of the auricle and ear canal.

Indications: Otifri is used for hygienic treatment of the auricle and external auditory canal in dogs and cats.

Doses and method of administration: slightly lift the pet’s ear. Carefully place the bottle applicator so that it does not come into contact with the inner surface of the auricle and ear canal. Fill the ear canal with the required amount of lotion. Massage the base of your pet's ear in the cartilage area to distribute the lotion evenly. The animal can get rid of the remaining lotion on its own by simply shaking its head.

4) Otoxolan (KRKA, Slovenia) refers to combination drugs with antimicrobial, antifungal and anti-inflammatory effects. Thanks to the combination of active components with different mechanisms of action, the drug has a wide spectrum of

antibacterial and fungicidal activity, and has an anti-inflammatory effect in the affected area. Marbofloxacin, which is part of the drug, belongs to the fluoroquinolone group, is active against gram-negative and gram-positive microorganisms, including *Staphylococcus intermedius, Pseudomonas aeruginosa, Escherichia coli* and *Proteus mirabilis*, and has no effect on anaerobes. The mechanism of action of marbofloxacin is to inhibit the DNA synthesis of microorganisms. Clotrimazole - Fungicidal compound of the imidazole group, changing the permeability of the cell membrane, causes destruction of cellular elements and inhibits intracellular molecular synthesis. Active against dermatophytes and yeast fungi, in particular *Malassezia pachydermatis*. Dexamethasone acetate is a synthetic glucocorticosteroid, has an anti-inflammatory and anti-exudative effect, helps reduce itching and heal damaged tissues.

Indications: Otoxolan is prescribed to dogs for the treatment of otitis media of bacterial and fungal etiology caused by microorganisms and fungi sensitive to marbofloxacin and clotrimazole.

Doses and method of administration: before treatment, the dog's auricle and external auditory canal are hygienically treated. Shake the bottle with the drug thoroughly for 30 seconds, gently squeeze the bottle to fill the cannula, and then instill Otoxolan in each ear, 5-10 drops (depending on the size of the animal) and gently massage the base of the ear. The drug is used once a day until the animal recovers from 7 to 14 days.

Figure 5. Ear drops used in the experiment.

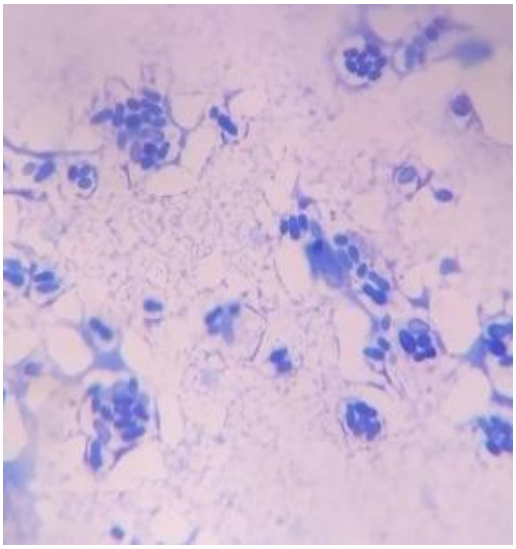
Ear drops are produced mainly packaged in 10 ml plastic bottles with a dropper and a screw cap with a control band, which are packaged individually in cardboard boxes complete with instructions for use. All 3 drugs in the experiment (Fig.5) in terms of the degree of impact on the body belong to low-hazard substances (hazard class 4 according to GOST 12.1.007-76), in recommended doses they do not have a locally irritating, resorptive-toxic or sensitizing effect.

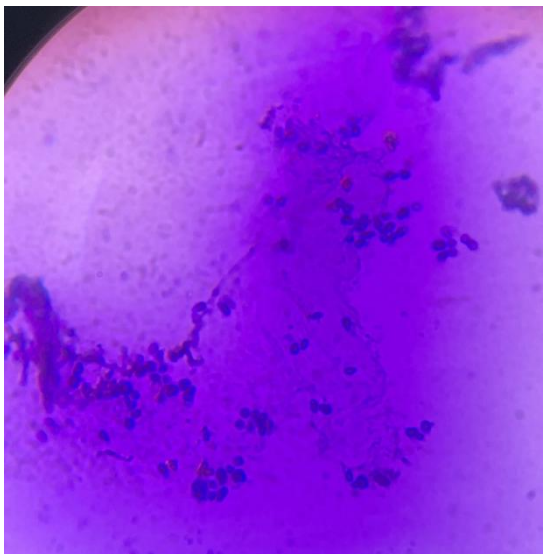
2.4. Labarotory work with fungal strains.

Issues of effective identification of species, as well as tracking their phylogenetic relationships, have been of interest throughout the development of biological science. The ability to distinguish representatives of species from each other using traditional methods can be complicated by high polymorphism within each of the species or, conversely, high interspecific morphological similarities. At the same time, the importance of the species identification procedure is beyond doubt, and more and more often, researchers resort to the use of modern methods based on the use of molecular genetics technologies.

The following nutrient media were used in the study: liquid and solid Sabouraud medium (BioMerieux, France) supplemented with glucose, penicillin, and streptomycin 100 IU/l; agar Sabouraud Chloramphenicol 2 (BioMerieux, France); "Heart-brain broth" (HiMedia, India); Agar Sabouraud with dextrose Sabouraud Dextrose Agar Eur. Pharm. (Conda, Spain); Sabouraud Dextrose Agar (HiMedia, India) (Rudenko et al. 2021).

During microbiological examination of the smears from the ears of sick dogs and cats same cultures were assigned to the genus M by phenotypic characteristics (Fig.6). The preparations were microscopized using an MBI-15-2 light microscope manufactured by LOMO, RF. When cultivating the material on the surface of the nutrient medium SDA or MPA after 48 hours of cultivation at 37°C, a typical growth of S forms of mucous, separately located colonies was observed, d=3-5 mm; or merged colonies of milky-white color (Fig.7).





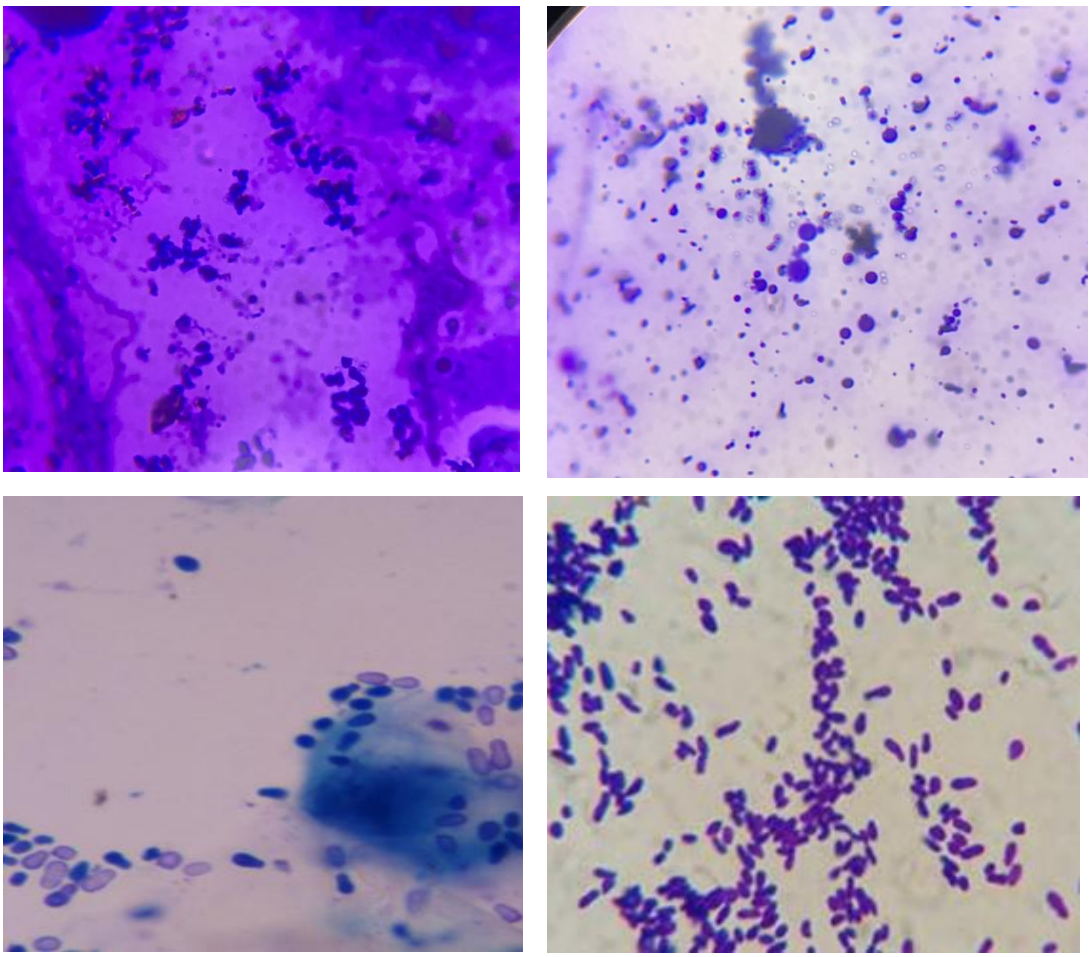
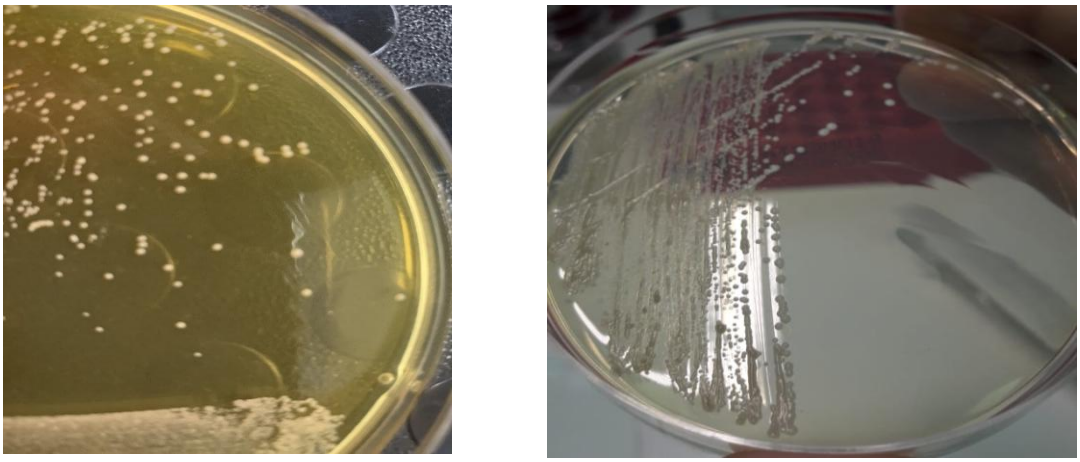


Figure 6. Morphology of *Malassezia pachydermatis* in the ear smear.



A B

Figure 7. Cultural properties of *Malassezia pachydermatis* on a solid media after 48 hours of cultivation: A – on SDA; B – on MPA.

But sometimes in a smear of ear exudate, we noticed other yeast-like fungi. They formed hyphae or filamentous tubes and could not be referred to MP. Our

assumptions were as follows: these are either representatives of the genus M, but lipophilic, which are capable of phylanentation, or *Candida spp*. (Fig.8).

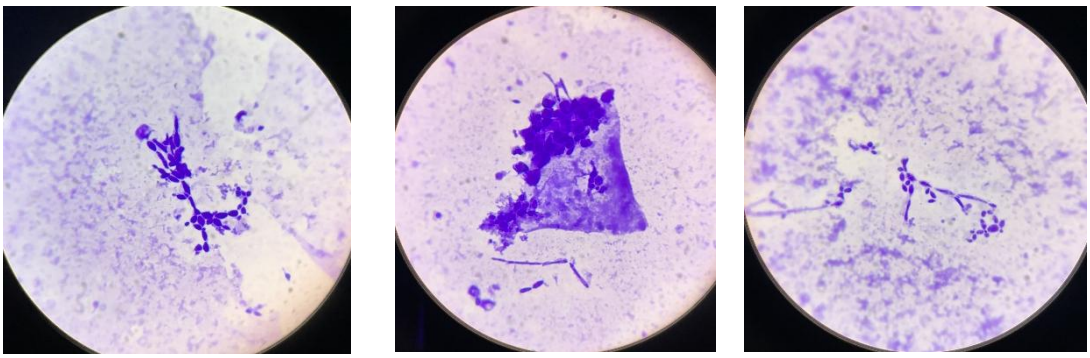


Figure 8. Other yeast-like fungi in a smesar under microscope.

In this case, for identification, we first used agar Sabouraud Chloramphenicol 2 (BioMerieux, France), and then chromogenic medium Hi Crome Candida Agar (HiMedia, India). Also, enzymatic features were recorded using the HiCandida Identification Kit (HiMedia, India) containing carbohydrates: urease, melibiose, lactose, maltose, sucrose, galactose, cellobiose, inositol, xylose, dulcitol, raffinose, trehalose (Urease, Melibiose, Lactose, Maltose, Sucrose, Galactose, Cellobiose, Inositol, Xylose, Dulcitol, Raffinose, Trehalose). Some properties of the isolate, such as saccharolytic activity and growth on a chromogenic medium, were compared with the properties of certified strains of *C. albicans* ATCC 2091 and *C. parapsilosis* ATCC 22019.

In any case, the identification of microorganisms was confirmed of using the Bruker Daltonik MALDI Biotyper (Bruker Daltonik Inc., Germany) - technology of matrix-activated laser desorption/ionization (Fig. 9).

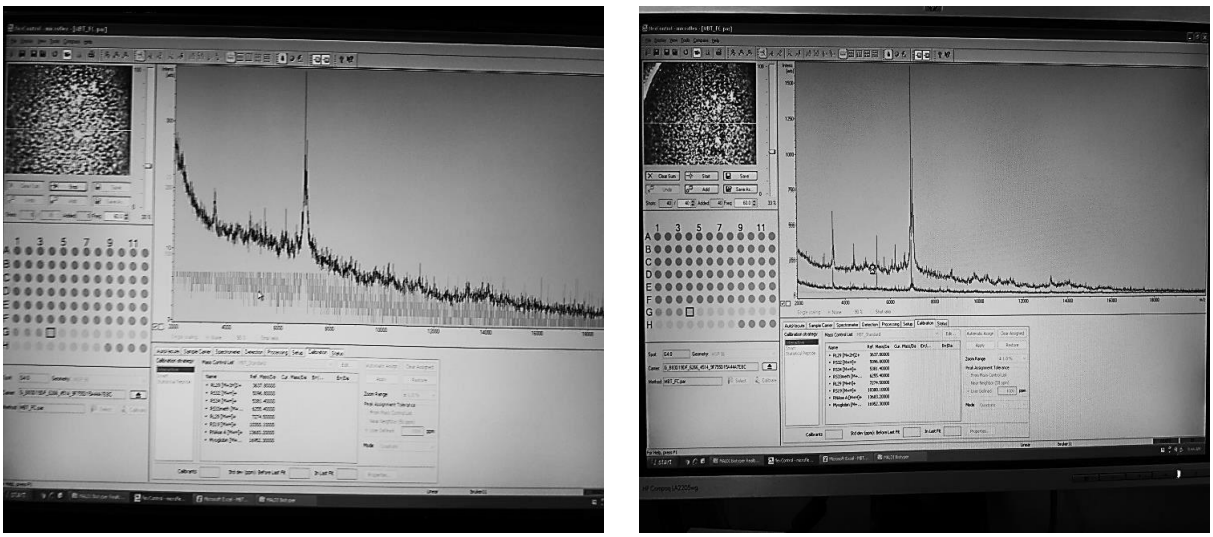


Figure 9. MALDI software picogram results.

2.5. Determination of the accompanying bacterial microflora in the ear exudate in case of *Malassezia* otitis.

Exudate from the ear of the animals was taken with a cotton swab. The material was placed in solution with sterile saline, then with the help of a bacterial loop mixture was inoculated into Petri dishes on the surface of solid nutrient media (Endo, MPA, MPB, Ressel, Blaurocca nutrient medium, MRS, bismuth sulfite agar, yolk-salt agar) (HiMedia, India and Obolensk, Russia) (Fig.10).

The morphology of bacteria was studied in smears stained according to Gram and Romanovsky-Giemsa. Further identification by biochemical properties was carried out in accordance with the "Bergey's Identifier for Bacteria". Gram-negative rods that gave a positive result in the test for the presence of catalase, negative in the test for cytochrome oxidase, oxidized and fermented glucose (in Hugh-Leifson's medium), reduced nitrates were assigned to the *Enterobacteriaceae* family. All isolated cultures were inoculated on semisolid Hiss media with different pH indicators and with glucose, maltose, lactose, mannose, sucrose, mannitol, and dulcite. Gram-positive rod-shaped bacteria were additionally subcultured onto Hiss medium with galactose, salicin, fructose, and arabinose. To determine the catalase activity of microorganisms, the bacterial mass removed with a loop from the agar surface was suspended in a drop of 3% hydrogen peroxide on a slide.

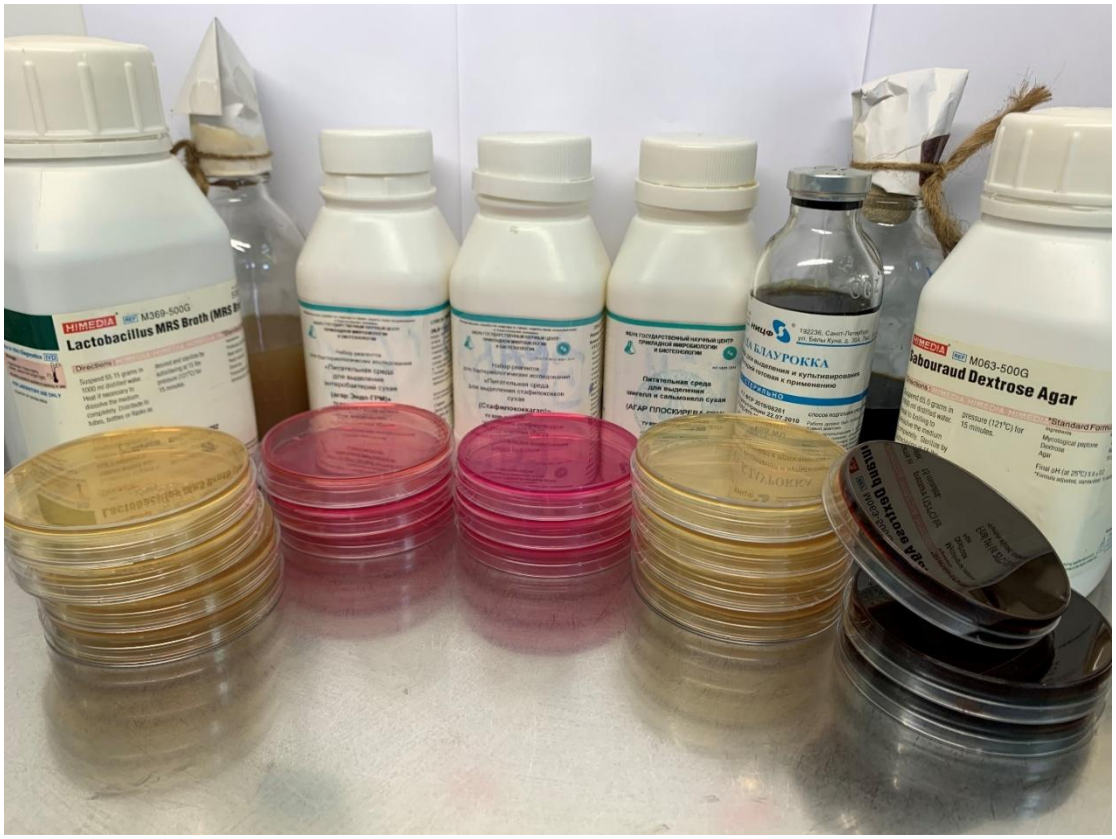


Figure 10. Culture media used in the study.

For further identification of the representatives of the *Enterobacteriaceae* family to the genus and species of culture, they were subcultured onto Olkenitsky's medium, a long-variegated row, which included media with mannitol, maltose, sucrose, xylose, rhamnose, dulcite, sorbitol, salicin, Rochelle salt (d-tartrate), milk with litmus and beef-extract broth for the study of indole, as well as tests for the utilization of citrate, acetate. In gram-negative rod-shaped bacteria, the fermentation of such carbohydrates as inositol and sorbitol was additionally determined using paper indicator systems (Nizhniy Novgorod, Russia).

To identify bacteria of the family *Pseudomonadaceae (Pseudomonas)*, the culture was grown on GRM medium (Makhachkala, Russia) in a thermostat at a temperature of 42° C for 24 hours. For the differentiation of bacteria of the genus *Staphylococcus* from the genus *Streptococcus* the presence of catalase was determined. For the differentiation of the genus *Staphylococcus* from the genus *Micrococcus* we used a glucose oxidation-fermentation test (Hugh-Leifson's medium). To identify the species of bacteria of the genus *Staphylococcus* tests were

carried out for the presence of coagulase, oxidation of mannitol, galactose, maltose, lactose, sucrose; the ability to grow in the presence of 10% NaCl.

In any controversial issues of identification, we resorted to confirming our results with the help of Bruker Daltonik MALDI Biotyper (Bruker Daltonik Inc., Germany) technology.

2.6. Densitometric indicators of microbial biofilms.

Colonies of four days cultures of MP from SDA were washed with physiological solution (PhS) (pH 7.0). The concentration of BY was 0.5, according to McFarland, which corresponded to 1.5×10^8 cells/mL. The tested samples were added to the wells of 96-well plate (Medpolymer Company, Russia), cultivated in the constant aerobic environment at 37 °C for 72 hours. The liquid was discarded and the wells were washed with 200 μl of phosphate-buffered solution (PBS) for three times (pH 7.3). The plates were shaken for 5 min at each stage of washing. The samples were fixed with 150 μl of 96% ethanol for 15 min and dried out at 37 °C for 20 min. The microbial biofilms were stained by adding 0.5% stain solution - crystal violet (Himedia, Mumbai, India) in each well and subsequent cultivation at 37 °C for 5 min. The optical density (OD) of the biofilm was measured by the degree of binding of crystal violet at a wavelength of 580 nm (OD_{580}) in an Immunochem-2100 microplate photometric analyzer (HTI, North Attleboro, USA) (Lenchenko et al. 2020; Sachivkina et al. 2020). That is, for weak biofilm producers, the optical density of the sample or the culture of microorganisms (density of the sample, ODs) is less than two times (ODs ≤ 0.194) greater than the optical density of the control, i.e., nutrient medium without in-oculum (density of the control, Dc); the optical density of the sample exceeds the optical density of the control by 2–4 times (ODs = 0.194–0.388) for moderate or average biofilm producers; the optical density of the sample exceeds the optical density of the control by more than four times (ODs ≥ 0.388) for strong biofilm producers. Density of control, Dc was 0.097±0.005. For statistical analysis (chi square), the Optical density (factorial sign) indicators were

converted into numbers - degree OD. Weak biofilm producer was given 1 point, average - 2 points, strong – 3 (Nardoni et al. 2014; Sachivkina et al. 2021).

2.7. Preparation *Malassezia pachydermatis* cultures and assessment of their susceptibility to antimycotics.

To determine the sensitivity of MP to antimycotic drugs was investigated using the Kirby–Bauer's disk diffusion method (Honnavar et al. 2016; Puigdemont et al. 2021; Sachivkina et al. 2022). The antifungals tested were nystatin (NS; 50 µg), amphotericin B (AP; 10 µg), ketoconazole (KT; 10 µg), clotrimazole (CC; 10 µg), voriconazole (VOR; 10 µg), fluconazole (FU; 25 µg), miconazole (MIC; 10 µg), and intraconazole (IT; 10 µg) (HiMedia™ Laboratories Pvt. Ltd., India). All strains recovered from animals with otitis externa were classified as susceptible (S) – 1 point, intermediate (I) – 2 points; or resistant (R) – 3 points according to the manufacturer's breakpoints for yeasts. These scores were entered into a table and calculated as Degree of resistance (Dr) to antifungal drugs (resulting sign) for the statistical analysis Chi-square test.

2.8. Determination of the ability of *Malassezia pachydermatis* to adhere to buccal cells of dogs.

To study the adhesive properties of BY on target cells, we used buccal epitheliocytes (BE) of a healthy 3-year-old male dog. BE were washed off with 5 ml of PBS from a cotton-gauze swab, then washed three times with PBS pH=7.2 by centrifugation at 1000 rpm for three minutes. The resulting cell suspension was tested in a Goryaev's chamber (Fig.11). The resulting suspension of BE was standardized by the number of cells in the Goryaev's chamber. A BY suspension was then prepared, 0.5 units turbidity standard, according to McFarland, which corresponded to 1.5×10^8 cells/mL. Cell suspension was combined in the ratio of 100 BY per one epitheliocyte.

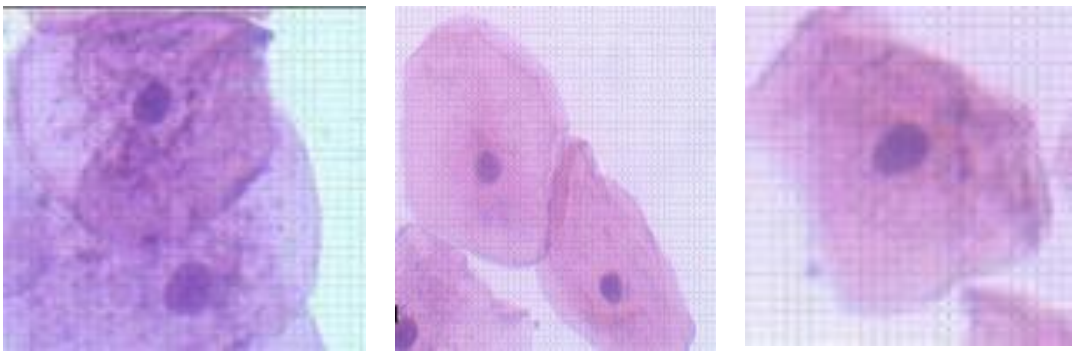


Figure 11. Buccal cells- epitheliocytes of dogs in a Goryaev's chamber.

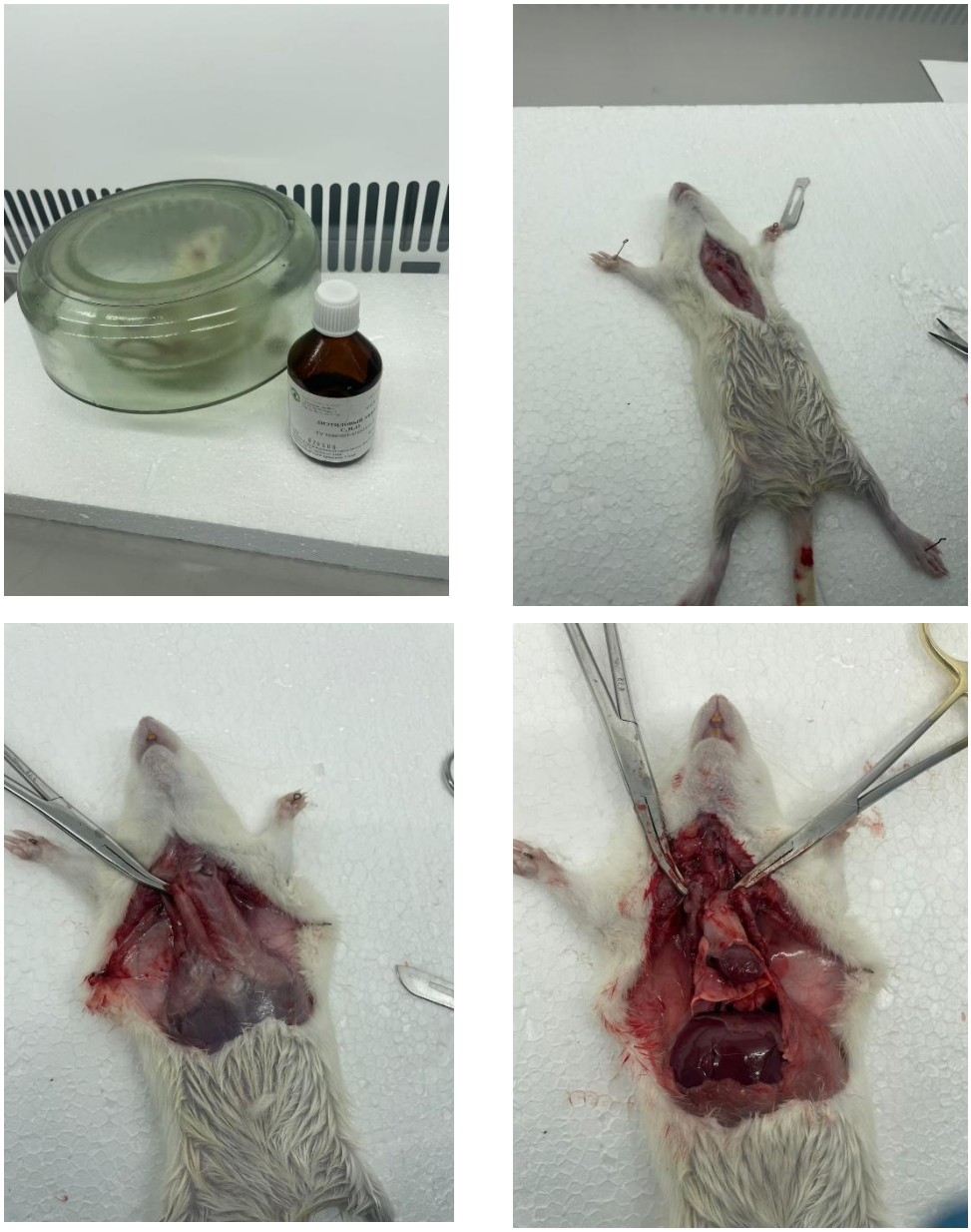
After 30 minutes of incubation, the cells were washed three times with SS and centrifuged for 2 minutes at 1000 rpm. Smears were prepared from the washed precipitate and stained by Gram. Epitheliocytes on which BY adhered were counted in 50 fields of view. The following indicators were determined: K - % of BE with adherent MP; and adhesion index (AI) – the amount of BY on one BE relative to the number of active epithelial cells (an indicator that characterizes MP adhesiveness). The experiment was repeated three times.

2.9. Setting the reaction of phagocytosis to check the ability of *Malassezia pachydermatis* to be phagocytosed with rat alveolar macrophages *in vitro*.

Alveolar macrophages of rats were used to determine the ability of BY to be phagocytized. For this experiment we used 20 Red - eyed albino rats of both sexes, weighing 120-160 gr.

To isolate alveolar macrophages in rats under ether anesthesia, the chest was opened, the lungs were removed along with the trachea and heart, after the trachea was tied with silk thread. The heart-lung complex was immersed 2-3 times in sterile physiological saline to remove blood. The solution for washing was injected into the trachea with a syringe, in an amount of 10 ml, until the lungs were completely filled. The lungs were lavaged with a liquid of the following composition: medium 199 (without antibiotics) containing heparin (5 IU/ml) and 1% DMEM/F12 nutrient medium. The medium pH was adjusted to 7.0-7.2 with NaOH using a pH meter. The lungs were gently massaged while the medium was aspirated with a syringe. The

wash liquid was then transferred to centrifuge beakers immersed in ice to prevent cells from sticking to the bottom of the beakers. The operation of washing the lungs was repeated 3 times (Fig.12).



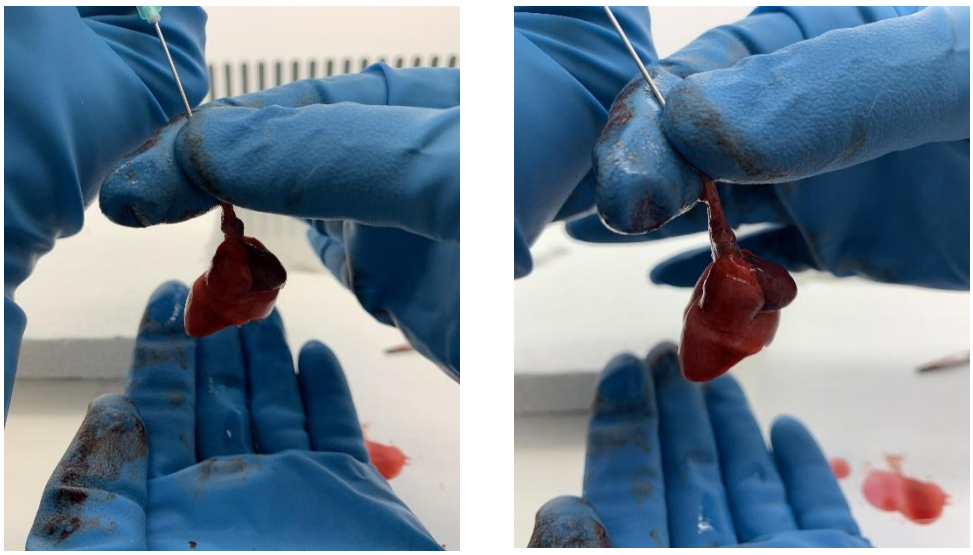


Figure 12. Obtaining alveolar macrophages from rat lungs.

To 1 ml of a suspension containing $0.5x10^6$ macrophages, 0.1 ml of a suspension containing $2.5x10^6$ MP cells was added (the optimal load is 50 BY per 1 macrophage).

A suspension of macrophages with M was placed in penicillin vials, 1 ml each, tightly closed with rubber stoppers, and placed in a thermostat at 37°C in an inclined position. The medium completely covered the coverslips.

To study the absorption activity of cells, the suspension was incubated in a thermostat for 60 minutes. To determine the digestive capacity - another 1 hour 30 minutes (total 150 minutes) in new bottles with a fresh portion of medium 199 containing 20% bovine serum.

After the end of the incubation, the coverslips with cells attached to them were taken out with tweezers, carefully washed in pure medium 199, and dried in air. Macrophages were fixed for 15 minutes in methyl alcohol. After fixation, the preparations were dried and stained according to Romanovsky-Giemsa for 30 minutes. Then the slides were thoroughly washed with distilled water, dried in air and dehydrated sequentially in 3 solutions, immersing the stained preparations in each of them for 1 minute.

first solution – acetone;

second solution - a mixture of equal parts of acetone and xylene;

third solution – xylene.

After that, the slides were glued with polystyrene to glass slides. Thus, ten preparations of the control and experimental groups were received. At least 100 cells were counted in one preparation, and the phagocytic number (PN) and phagocytic index (PI) were determined. The experience was repeated thrice.

The index of digestion ability (IDA) of macrophages was determined by the formula (Sachivkina 2010):

$$IDA = (PN_{60'} – PN_{150'}) / PN_{60'} \text{ x } 100\%, \quad (1)$$

where $PN_{60'}$ is the phagocytic number after 60-minute exposure,

$PN_{150'}$ – phagocytic number after 150-minute exposure.

2.10. Modeling of *Malassezia* infection on mice back.

At the beginning, we tried to simulate M infection in mice without reducing their immunity. A flat area was shaved on the back and 0.5 ml of suspension was applied. The mouse was fixed in the hands so that it did not lick the inoculum for a few minutes before drying. Then the mouse was released into the cage and observed for a week.

The model was unsuccessful. Malasseziosis did not develop, the hair on the back was overgrown and there were no signs of dermatitis. We repeated the infection on other mice. We took only 1.5 months mice into the experiment, caused them to go into heat with the help of the veterinary drug Mesalin and dysbiosis with the help of oral administration of antibiotics. Against this background, the MP was reinfected. After 3 days, the development of M dermatitis of the back was observed. The infection without treatment lasted from 7 to 20 days (Fig.13). Then spontaneous recovery occurred. The established facts allow us to consider the rational use of such a model. However, the use and testing of therapeutic agents is difficult, since mice easily reach this area and physically remove the therapeutic solutions. Therefore, it was decided to conduct the MO experiment on rabbits.

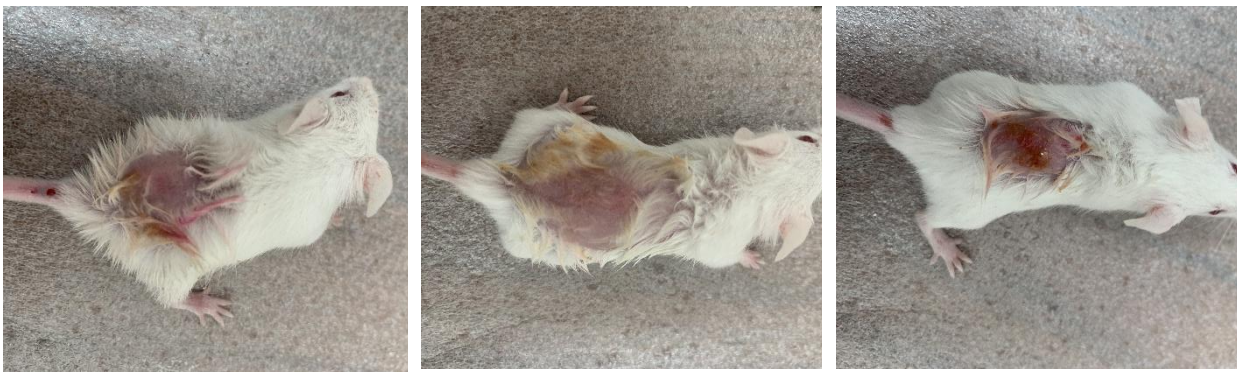


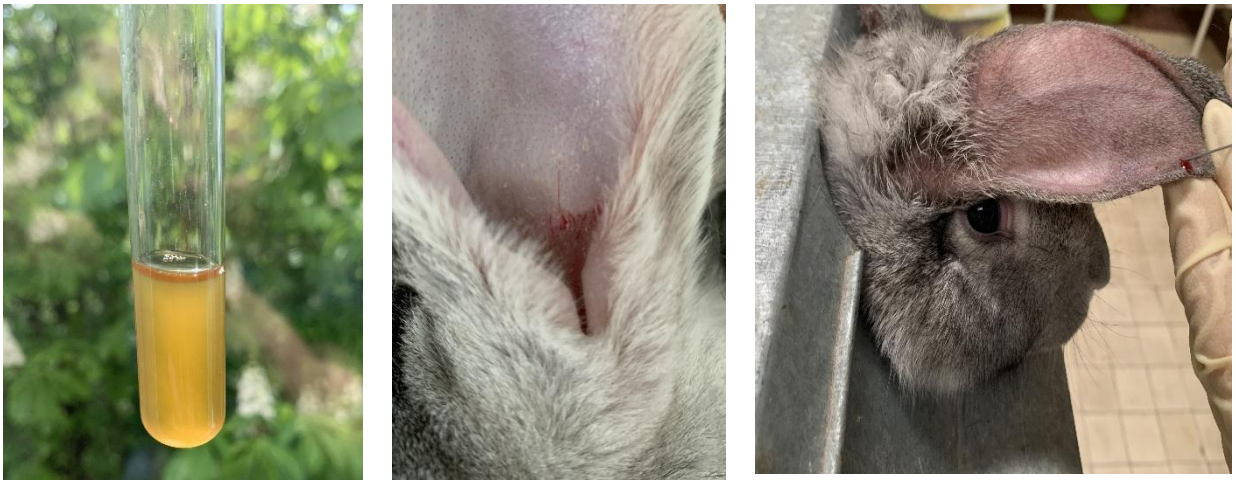
Figure 13. Modeling *Malassezia* infection on the back in mice.

2.11. Modeling of ear otitis in rabbits by a strain *Malassezia pachydermatis* from dogs.

Apparently, 20 healthy adult rabbits breed Gray Giant, 1 year old, weight 5.5 kg, male were used in experiment. Animals passed veterinary control and had all the documentation. Rabbits were under the supervision of a veterinarian during all days of the experiment from April to June 2022. Rabbits were kept in cages (length = 50.0 cm; width = 40.0 cm; height = 30.0 cm) (Fig.14). The ambient temperature was 20 ± 1 ° C, and the relative humidity was 45% ± 1%. Two groups of rabbits (control and experience) were fed the same food. Rabbits were fed with granular feed, which in-cluded meadow and mountain herbs, apple pomace, sunflower seed flour, flax seed, linseed oil, oligosaccharide fructans, dried cucumber, dried carrots, dried celery, dried zucchini, calendula flowers, red clover flowers, hop cones, yeast extract, and yucca extract. Throughout the test, water and food were provided freely.

Figure 14. Stages of preparation of the experiment on rabbits of the gray giant breed.

For infection MP suspension in SDB was prepared, 0.5 units turbidity standard, according to McFarland, which corresponded to 1.5×10^8 cells/mL. Notches were made on the inner surface of the ear with a sterile blade and 1 ml of a suspension of microorganisms was applied to these scratches. Of course, some of the solution got into the ear. The rabbits were well fixed and could not comb out this solution with their hind legs. After half an hour, they were released into the cage when the suspension had already dried in the ear (Fig.15).



A B C

Figure 15. Stages experiment on infection: A - preparing *Malassezia pachydermatis* suspension in SDB; B - scratches in the ear; C – rabbit fixation.

2.12. Clinical signs of MO model.

Clinical signs such as diarrhea, loss of body weight, tremors, skin coloration, erythema, itching, the release of abundant ear secretions (cerumen) of a yellow-brown color, often with an unpleasant odor were observed from mild to very severe.

2.13. Blood and serum analysis.

Three mL of blood was collected from the marginal ear vein for hematological and biochemical analysis. Hematological parameters such as red blood cell count, hemoglobin, hematocrit, and total and differential white blood cell count were

evaluated by using a hematological analyzer. The serum was used to estimate different liver function tests such as ALT and ALP which were determined according to earlier protocol plasma glucose concentrations, plasma urea concentrations, plasma creatinine, serum calcium, plasma albumin, total protein, cholesterol, and triglycerides, and uric acid was determined by using a Chem Analyzer model, BTS BioSystem, Spain, and diagnostic kit manufactured by BioMed Diagnostics, GmbH, Germany, following the user's manual.

2.14. The onset of complete recovery in experimental animals and tracking of relapses of *Malassezia* infection.

Ear exudate was applied to the surface of the nutrient medium Sabouraud dextrose agar (Difco, Bordeaux, France) and cultured at 37°C for 48-72 hours. The cultivation of microorganisms was carried out in a thermostat with a Heidolph Unimax 1010 shaker (Germany). After 48 hours in a thermostat, the results of the reaction were taken into account by the presence or absence of growth - the number of CFU was calculated.

2.15. *Malassezia pachydermatis* biofilms processing with Farnesol *in vitro*.

A method for studying Far different concentrations influence on YLF biofilms especially on *Candida spp.* we described in our previous studies (Sachivkina at al 2020). So, we did the same without any modification. Briefly, the automatic pipette was inserted into the wells of a 96-well plate (Medpolymer, St. Petersburg, Russia):

- 100 µL SDB in each of the 12 holes of the first row A.
- 100 µL of Far was added at an initial concentration of 400 µM to the second well of the first row. The first hole was left as a control. In the second hole, the volume was 200 µL, and the concentration of farnesol was 200 µM. By successive transfer of 100 µL of the solution from the second well to the third, from the third to the fourth…, etc., we reduced the concentration of Far by half each time.

• Then, in each well of the first row, starting from the first, we added 100 µL of MP culture in SDB at a concentration of 4 units (McFarland). An overview of the sequence of stages of the study into the effect of Far on the formation of MP biofilms is presented in Table 1.

The total volume of the wells was 200 µL. The experiment was repeated 3 times, 3 µL plates were used, and four rows were used per plate. The microliter plates were cultured with the lid closed at 37°C for 72 h.

The average decrease was measured and used to calculate the biofilm inhibition percentage by Far, where OD AS is optical density average of samples MP in experiment, OD AC is optical density average of isolates in control without Far:

$$Average\ decrease\ OD(\%) = \frac{OD\ AS\ \text{x}\ 100}{OD\ AC} - 100 \qquad (2)$$

Table 1: Stages of the study of the Farnesol effect on the MP biofilm formation.

	1	2	3	4	5	6	7	8	9	10	11	12
Action 1	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL	SDB 100 µL
Action 2		+Farnesol										
Action 3	Not titrated	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL	Transfer 100 µL
The concentration of farnesol	Control – no farnesol	200 µM	100 µM	50 µM	25 µM	12.5 µM	6.3 µM	3.1 µM	1.6 µM	0.8 µM	0.4 µM	0.2 µM
Action 4	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture	+100 µL of culture
Action 5	wait for 72 h											

2.16. Change in the sensitivity of the *Malassezia pachydermatis* strain to antimycotics under Farnesol.

Antibiotic resistance testing was carried out with selected strain using the standard disk diffusion method on SDA with or without Far five concentrations. A

daily culture of microorganisms was applied to Petri dishes with a lawn, then disks with an antibiotic were applied, plus 25 μl of SS or five different concentrations of Far (12.5 - 200 μM/ml). We decided to focus on these 5 concentrations because, according to our previous experience with *Candida* and *Malassezia* strains, higher dilutions of Far were not very effective (Sachivkina et al. 2022a; Sachivkina et al. 2022c). Experiments with disks were carried out in triplicate.

2.17. Clinical trials.

The study involved 30 dogs of different breeds, sexes and ages from 1 to 10 years. The animals were divided into 2 groups: experimental (n=15) and control (n=15). The experimental group was prescribed Surolan veterinary ear drops manufactured by Elanco Animal Health (Indiana, USA). Form - suspension, active ingredient - polymyxin B, prednisolone, miconazole, 2 times a day, for 14 days. Plus a solution of Farnesol (Far) (Sigma-Adrich, USA), which was prepared in advance at a farnesol concentration of 100 μM per ml, dilution was carried out in sterile physiological solution (SS) pH 7.0. After using Surolan drops, Farnesol was applied as a spray also twice a day. The control group was a group of animals that were prescribed only Surolan.

2.18. Statistical analysis.

All the results were expressed as mean ± standard error of mean (SEM) of at least three replicates. Criterion "chi-square" for the analysis of contingency tables. Such a table is a good means of representing the joint distribution of two variables, designed to explore the relationship between them. The cross table is the most versatile tool for studying statistical relationships, as it can represent variables with any level of measurement. The lines of the contingency table correspond to the values of one variable - optical density, the columns - to the values of another variable - the resistance of microorganisms.

The data for these two criteria were preliminarily grouped into intervals. The statistical significance was set at $p \leq 0,05$ and where applicable, the difference between samples was assessed using the statistical software XLSTAT 2020 (Addinsof Inc., New York, USA). The effect of different concentrations of Far was evaluated on the degree of biofilm formation was evaluated with a logarithmic-logistic distribution. This model was chosen because the Cox model's proportional hazards assumption did not fit all covariates. All the graphs were plotted using Microsoft Excel (Microsoft Excel for Office 365 MSO, Microsoft COP., Redmond, WA, USA).

CHAPTER 3. RESULTS OF OWN RESEARCH

3.1. Obtaining clinical strains *Malassezia pachydermatis*.

The study involved 30 dogs of different breeds, gender and age from one to 14 years (Table 2) and 5 cats 7 month to 10 years (Table 3).

Table 2: Characteristics of dogs from which strains of microorganisms were obtained.

Microorganism	Breed	Age, years	Clinical signs of disease
MP Cd1	Shar Pei	12	Purulent otitis media, itching
MP Cd2	English cocker spaniel	3	Erythema, scratches, abrasions, abrasions of the auricle, cerumen
MP Cd3	Irish Setter	7	Partial alopecia, unpleasant odor
MP Cd4	German Shepherd	2	Scabs, pustules of the ear
MP Cd5	Pekingese	1	Lichenification of the auricle
MP Cd6	Caucasian Shepherd	8	Change in coat color, itching
MP Cd7	Toy Terrier	10	Conjunctivitis, cerumen
MP Cd8	Dachshund	4	Chronical otitis media, erythema of the auricle
MP Cd9	Outbred	1	Itching, cerumen
MP Cd10	Husky	10	Loss of appetite or body weight, unpleasant odor
MP Cd11	Labrador	8	Erythema of the auricle, itching
MP Cd12	Outbred	3	Itching, erythema of the auricle
MP Cd13	Akita Inu	8	Cerumen, unpleasant odor
MP Cd14	American Bulldog	9	Partial alopecia, unpleasant odor
MP Cd15	Basset Hound	2	Scabs, pustules of the ear, cerumen
MP Cd16	Bloodhound	5	Lichenification of the auricle
MP Cd17	Rottweiler	3	Scabs, loss of appetite or body weight, unpleasant odor
MP Cd18	American Cocker Spaniel	10	Erythema of the auricle, cerumen
MP Cd19	Outbred	14	Partial alopecia, unpleasant odor
MP Cd20	Husky	1	Scabs, pustules of the ear
MP Cd21	Beagle	7	Lichenification of the auricle
MP Cd22	Chihuahua	4	Unpleasant odor, cerumen
MP Cd23	Labrador	11	Erythema of the auricle, cerumen, unpleasant odor
MP Cd24	Pomeranian Spitz	8	Chronical otitis media, erythema of the auricle
MP Cd25	American Bulldog	13	Erythema of the auricle
MP Cd26	American Cocker Spaniel	2	Chronical otitis media, erythema of the auricle
MP Cd27	Scottish Setter	4	Partial alopecia, unpleasant odor, cerumen
MP Cd28	Basset Hound	5	Scabs, pustules of the ear

MP Cd29	Chihuahua	9	Unpleasant odor, chronical otitis media, erythema of the auricle
MP Cd30	Outbred	14	Scabs, unpleasant odor, cerumen

Table 3: Characteristics of cats from which strains of microorganisms were obtained.

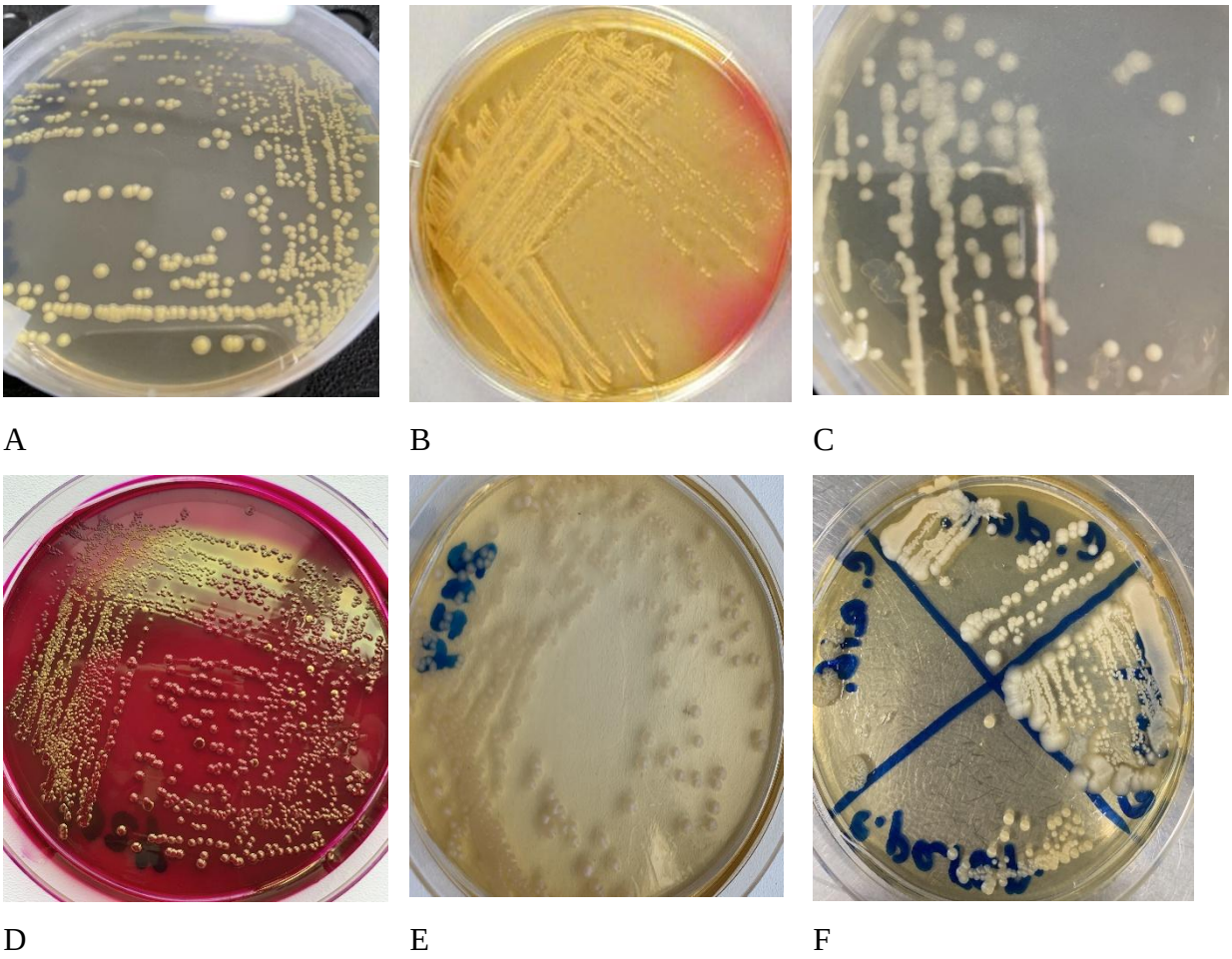
Microorganism	Breed	Age, years	Clinical signs of disease
MP Cc1	Abyssinian	10	Erythema of the auricle, cerumen
MP Cc2	American Bobtail	8	Partial alopecia, unpleasant odor, cerumen
MP Cc3	Outbred	7 months	Scabs, pustules of the ear, cerumen
MP Cc4	Outbred	5	Lichenification of the auricle
MP Cc5	Sphinx	4	Unpleasant odor, cerumen

As we can see, the MO disease does not depend on age or breed. The disease is most often characterized by: Erythema of the auricle, cerumen, partial alopecia, unpleasant odor, scabs, pustules of the ear, lichenification of the auricle ets.

3.2. Study of the species composition and biological properties of microorganisms isolated from external otitis in dogs and cats, as well as the characteristics of intermicrobial interactions *in vitro*.

The results of the microbiological studies are shown in tables 4 and 5 and in the following figures (Fig. 16 A-E). A comparative analysis of the species diversity of microorganisms isolated from the external auditory canal of dogs with different forms of otitis due to infection of the external auditory canal was performed. It was found that M were detected in monoculture in 13.3% of dogs and 20.0% of cats; association with 1 bacterial species was found in 10.1% of dogs and 20.0% of cats; association with 2 bacterial species was found in 6.6% and 20.0%, respectively; association with 3 bacterial species was found in 6.6% and 0%; association with 4 or more bacterial species was found in 16.7% and 0%; association with 1 fungal species was found in 6.6% and 20.0%; association with 1 fungal species and 1 bacterial species was found in 6.6% and 0%; association with 1 fungal species and 2 bacterial species was found in 10.1% and 20.0%. association with 1 type of fungi and 3 types of bacteria - 10.1% and 0%; association with 1 type of fungi and 4 or more types of bacteria - 13.3% and 0%, respectively. It should be noted that

associations of MP in dogs were most often with staphylococci and streptococci, and in cats MP was most often sown with enterobacteria and staphylococci.



A B C

D E F

Figure 16. A, B - growth of *staphylococci*, C-*streptococci*, D-*enterobacteria*, E-*lactobacteria*, F-yeast-like fungi.

Table 4: Results of quantitative comparison of microflora of ear exudate of dogs and cats (lg).

Genus of microorganism	Samples from dogs (n=30)	Samples from cats (n=5)
Staphylococcus sp. p.	10,15±0,52	9,72±0,64
Streptococcus sp. p.	8,58±0,43	7,60±0,37
Escherichia sp. p.	4,72±0,48	3,23±0,49
Pseudomonas sp. p.	3,86±0,55	2,16±0,41
Lactobacillus sp. p.	5,20±0,70	5,59±0,40
Bifidobacterium sp. p.	2,35±0,64	1,04±0,33
Klebsiella sp. p.	2,03±0,25	1,25±0,61
Bacillus sp. p.	1,78±0,52	1,45±0,24
Proteus sp. p.	1,74±0,46	1,35±0,36

Candida sp. p.	2,90±0,65	1,95±0,50
Trichophyton sp. p.	2,44±0,41	1,26±0,31
Microsporum sp. p.	1,64±0,42	-

A search was conducted for various forms of surgical infection in small domestic animals for the microbial landscape isolated from purulent exudate. However, Malassezia fungi were isolated only from dogs and cats with purulent otitis. Therefore, the further choice of the direction of the study fell on the study of the pathogenetic features of the course of Malassezia otitis.

Table 5: Species spectrum of microflora of ear exudate of dogs and cats.

Genus of microorganism	Samples from dogs (n=30)		Samples from cats (n=5)	
	Absolute number	%	Absolute number	%
S. saprophyticus	3	3,7	3	4,7
S. intermedius	5	6,1	2	3,1
S. epidermidis	1	1,2	1	1,6
S. aureus	5	6,1	2	3,1
S. hyicus	3	3,7	3	4,7
S. faecalis	6	7,3	4	6,3
S. uberis	1	1,2	1	1,6
E. coli	11	13,4	8	12,5
P. aeruginosa	3	3,7	2	3,1
P. vulgaris	2	2,4	3	4,7
K. oxytoca	3	3,7	2	3,1
B. subtilis	2	2,4	2	3,1
L. plantarum	5	6,1	4	6,3
L. rhamnosus	5	6,1	5	5,3
L. xylosus	3	3,7	1	1,2
L. acidophilus	4	4,9	4	6,3
B. adolescentis	2	2,4	3	4,7
B. animalis	4	4,1	5	7,8
B. bifidum	7	8,5	6	9,4
C. albicans	4	4,9	2	3,1

T. mentagrophytes	2	2,4	1	1,2
M. canis	1	1,2	-	-
TOTAL	82	100	64	100

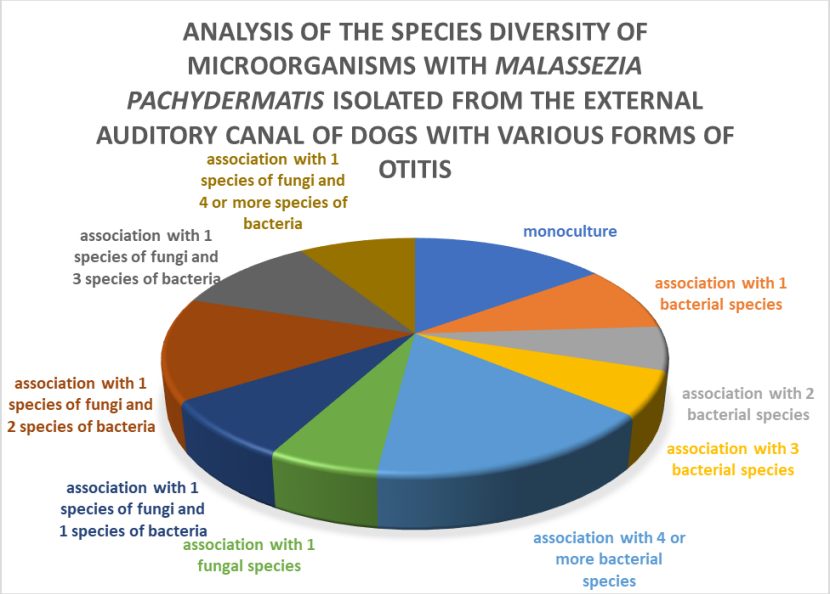


Figure 17. Structure of the microbiocenosis of the external auditory canal in dogs with otitis media.

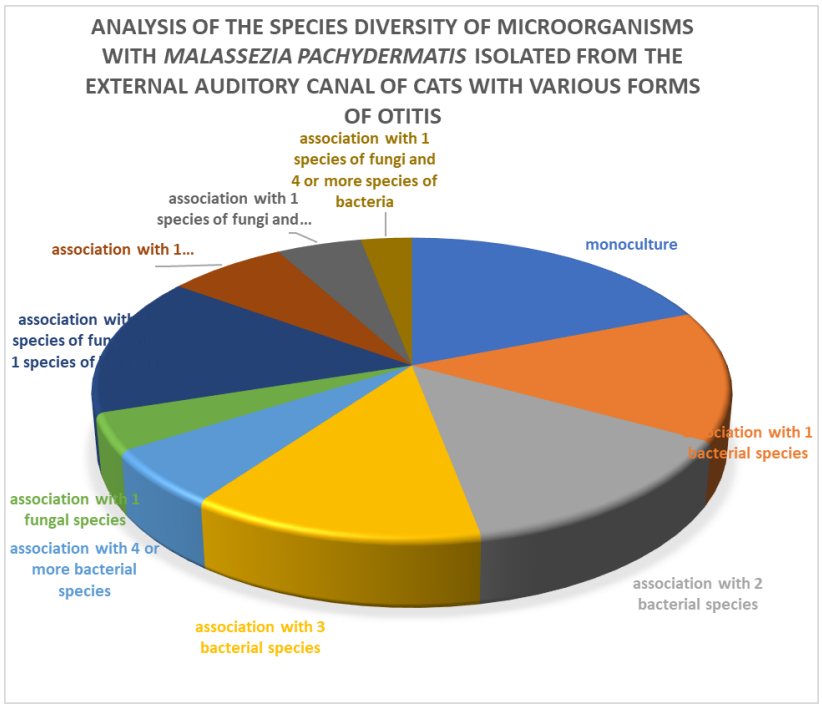


Figure 18. Structure of the microbiocenosis of the external auditory canal in cats with otitis media.

It should be noted that associations of MP in dogs were most often with *staphylococci* and *streptococci*; and in cats, MP were most often inoculated with *enterobacteria* and *staphylococci*.

3.3. Densitometric indicators of *Malassezia pachydermatis* biofilms and their susceptibility to antimycotics.

Densitometric indicators of the sample are in the Table 6 and 7. According to our gradation, 2 (6.7%) strains belong to weak producers of biofilms, 25 (83.3%) strains belong to moderate producers and 3 (10%) to strong ones. It is worth emphasizing the great importance of belonging to a high index of biofilm formation in our clinical strains MP. A moderate level of biofilm formation indicates good adhesion of these BY. Our team has experience in determining the degree of biofilm formation in clinical *Candida spp*. Compared to the genus M, the genus *Candida* can reach more higher level in biofilm production (Sachivkina et al. 2022b). Analyzing the densitometric indicators, it was found that studied MP Cd23, Cd27, and Cd3 is strongest producer of biofilms.

Table 6: Determination of the MP stains from dogs (Cd1 – Cd30) biofilm formation intensity by optic density and their susceptibility to antimycotics.

Micro-organism	Optic density	Degree OD	Antimycotic drugs								Degree of resistance
			NS	AP	KT	CC	VOR	FU	MIC	IT	
MP Cd1	0.203 ± 0.016	2	1	1	3	1	1	2	1	1	11
MP Cd2	0.351 ± 0.018	2	1	2	1	2	2	3	1	1	13
MP Cd3	0.400 ± 0.012	3	1	1	1	2	2	3	2	1	13
MP Cd4	0.287 ± 0.018	2	1	3	1	2	1	1	1	3	13
MP Cd5	0.261 ± 0.011	2	2	1	1	2	2	1	1	1	11
MP Cd6	0.312 ± 0.029	2	1	1	1	1	1	3	1	1	10
MP Cd7	0.255 ± 0.010	2	1	1	1	1	1	1	1	1	8
MP Cd8	0.243 ± 0.026	2	1	1	1	2	1	1	1	1	9
MP Cd9	0.190 ± 0.016	1	1	3	1	1	1	3	1	2	13
MP Cd10	0.237 ± 0.015	2	2	1	1	1	1	1	2	2	11
MP Cd11	0.345 ± 0.011	2	1	1	3	1	2	2	1	1	12
MP Cd12	0.323 ± 0.017	2	1	2	1	1	2	1	1	1	10
MP Cd13	0.192 ± 0.012	1	1	1	1	2	1	2	1	1	10
MP Cd14	0.258 ± 0.011	2	1	1	1	2	1	1	1	1	9
MP Cd15	0.261 ± 0.010	2	1	1	2	2	1	1	1	2	11
MP Cd16	0.313 ± 0.007	2	1	1	1	1	1	2	1	1	9

MP Cd17	0.294 ± 0.019	2	1	1	3	1	1	3	1	1	12
MP Cd18	0.286 ± 0.020	2	1	1	1	2	1	1	1	1	9
MP Cd19	0.362 ± 0.014	2	2	1	2	2	1	1	1	1	11
MP Cd20	0.366 ± 0.015	2	1	2	1	1	3	1	1	1	11
MP Cd21	0.280 ± 0.016	2	1	2	1	1	1	1	3	1	11
MP Cd22	0.344 ± 0.018	2	1	1	1	1	1	2	1	1	9
MP Cd23	0.441 ± 0.016	3	2	3	3	2	2	3	1	2	18
MP Cd24	0.370 ± 0.015	2	1	1	2	2	1	1	1	1	10
MP Cd25	0.323 ± 0.017	2	2	1	1	1	1	2	3	2	13
MP Cd26	0.288 ± 0.012	2	1	2	1	1	1	1	1	1	9
MP Cd27	0.403 ± 0.026	3	1	2	2	1	3	1	1	1	12
MP Cd28	0.368 ± 0.014	2	1	1	1	1	1	1	1	1	8
MP Cd29	0.297 ± 0.011	2	1	1	1	1	3	2	1	1	11
MP Cd30	0.353 ± 0.019	2	1	1	2	1	1	3	3	1	13

Note: ODc = 0.097 ± 0.005

Table 7: Determination of the MP stains from cats (Cc1 – Cc5) biofilm formation intensity by optic density and their susceptibility to antimycotic.

MP Cc1	0.278 ± 0.011	2	1	1	1	2	1	1	1	1	9
MP Cc2	0.211 ± 0.012	2	1	1	2	2	1	1	1	2	11
MP Cc3	0.219 ± 0.008	2	1	1	1	1	1	2	1	1	9
MP Cc4	0.280 ± 0.013	2	1	1	3	1	1	3	1	1	12
MP Cc5	0.226 ± 0.010	2	1	1	1	2	1	1	1	1	9

Note: ODc = 0.097 ± 0.005

The isolates were reported as sensitive, intermediate, and resistant in general, according to the manufacturer on CLSI guidelineser's breakpoints for yeasts (Gue´ho et al. 2011, Lenchenko et al. 2020), most strains were sensitive (S) to antimycotics drugs. However, a few exceptions were observed: three strains were classified as resistant (R) to AP, MIC and VOR; four R to KT; seven R to FU and only one R to IT. There are no R results for NS and CC antifungal discs. Intermediate results: 5 isolates to NS, KT and IT; 6-AP and VOR; 12 to CC; 7 to FU; 2 to MIC (Fig.19).

Strain MP Cd 23 was classified as R to AP, KT and FU, I to NS, CC, IT and VOR, S only to MIC. Strain MP Cd 27 was classified as R to VOR, I to AP, KT, and S to others. Strain MP Cd 3 was classified as R to FU, I to CC, VOR and MIC.

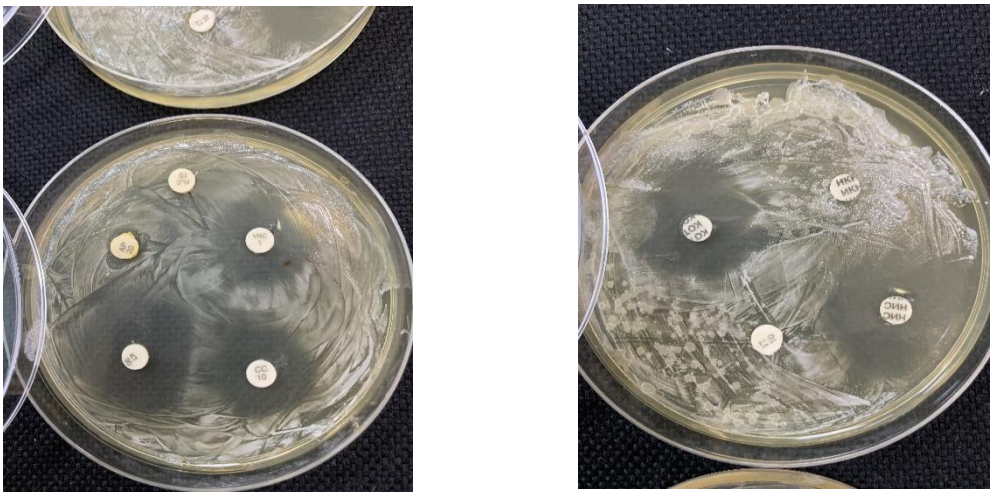


Figure 19. Different resistance *Malassezia pachydermatis* to the antifungals on the solid media.

It was very interesting to see if there is a relationship between the ability to form biofilms and resistance to the antifungals tested? One possibility to find out it was to calculate a resistance score, assigning a value of 1 if the isolate was sensitive, a value of 2 - intermediate, and a value of 3 - resistant. In this way, a numerical value was obtained for each isolate and we have adopted rules such that a yeast isolate is considered sensitive if the sum of the scores for all 8 antimycotic drugs is 8-10, intermediate - 11-13 and resistant - 14-18. Also, three categories have been defined for the ability to form biofilms (weak - 1, moderate - 2 and strong - 3). We carried out a table and perform statistical analysis «chi square» in Table 5.

Table 8: The relationship between the ability to form biofilms and resistance to the antifungals, «chi square» statistical analysis.

Biofilm optical density (factorial sign)	Degree of resistance to antifungal drugs (resulting sign)			
	sensitive 8-10	intermediate 11-13	resistant 14-18	total
weak - 1	1	1	0	2
moderate - 2	11	14	0	25
strong - 3	0	2	1	3
total	12	17	1	30

Calculation results: The value of the $\chi 2$ criterion is 10.421 (sum of all points chi squared). The critical value of $\chi 2$ at the significance level p=0.05 is 9.488. The relationship between the factorial and performance characteristics is statistically significant at a significance level of $p<0.05$ (the chi-squared value is greater than the critical value). Significance level p=0.034.

We combined all our observations from dogs (30 isolates) into 9 groups depending on the optical density and the degree of resistance to 8 antifungal drugs:

• 1st group: weak biofilm producer - sensitive to antifungal drugs: MP Cd 13

• 2nd group: weak biofilm producer - intermediate resistance to antifungal drugs: MP Cd 9

• 3rd group: weak biofilm producer - resistant to antifungal drugs: no isolates

• 4th group: moderate biofilm producer - sensitive to antifungal drugs: MP Cd 6,7,8,12,14,16,18,22,24,26,28

• 5th group: moderate biofilm producer - intermediate resistance to antifungal drugs: MP Cd 1,2,4,5,10,11,15,17,19,20,21,25,29,30

• 6th group: moderate biofilm producer - resistant to antifungal drugs: no isolates

• 7th group: strong biofilm producer - sensitive to antifungal drugs: no isolates

• 8th group: strong biofilm producer - intermediate resistance to antifungal drugs: MP Cd 3,27

• 9th group: strong biofilm producer - resistant to antifungal drugs: MP Cd 23

It has been statistically proven that there is a relationship between the optical density of MP biofilms and their sensitivity to drugs, and this relationship is statistically significant. Analyzing the densitometric indicators, it was found that studied Cd23, Cd27, and Cd3 is strongest producer of biofilms. Also, they have intermediate and resistant score to antifungal drugs. Therefore, it was decided to continue the experiment with these particular isolates of BY.

3.4. Determination of the ability of *Malassezia pachydermatis* to adhere to buccal epithelial cells.

Recent years have been marked by a noticeable increase in interest in the epithelium of the mucous membranes of animals and its protective functions. This is due to the recognition of the coordinating position of the mucosal epithelium in reactions that, through the interaction of immune mechanisms, ensure the onset and stabilization of inflammatory processes of the mucous membranes in response to stimulating influences of both endogenous (cytokines, etc.) and exogenous nature, in particular in interaction with microorganisms. Hence, the study of reactions between epithelial cells and pathogens such as M is of undoubted interest, since MO is the result of an imbalance between the clearance mechanisms in the mucosal epithelium system and the conditions that ensure active reproduction and persistence of fungi on the mucous membranes. The relevance of this chapter of the dissertation research is also associated with the fact that currently there is an increase in opportunistic infections in animals caused by MP. MP adhesion to epithelial cells is the first and obligatory stage of MO development. At the same time, the implementation of the adhesive potential in the “M - epithelial cells” system can be influenced by many factors, both from BY and from the host organism. In connection with the above, the model of artificial MP colonization on buccal epithelial cells is a simple and convenient way to assess the adhesive potential of BY and allows us to evaluate the impact of various factors of mucosal immunity on adhesive reactions in the “macroorganism-microorganism” system. Our first task was to investigate the question of choosing a strain for further research.

The conducted studies made it possible to establish the scale of MP strain differences in adhesive relationships with dog buccal epithelial cells. It was quite large for cultures from dogs (Average AI = 5.07 ± 0.72; K = 51.41 ± 5.39) and was less pronounced for strains isolated from cats (Average AI = 3.26 ± 0.51; K=48.76 ± 4.07). The MP Cd23 strain was chosen for further research, which was distinguished by a high adhesion index value of 8.28 ± 0.62 and an adhesion coefficient of 70.62 ± 4.91.

Table 9: Adhesion of 30 Malassezia pachydermatis strains from dogs to buccal epithelial cells.

Microorganism	AI - adhesion index, the amount of BY on one BE relative to the number of active epithelial cells	K - % of buccal epitheliocytes with adherent MP
MP Cd1	5,79±0,87	68,20±6,96*
MP Cd2	2,42±0,35**	45,60±5,87
MP Cd3	4,89±1,06	55,05±8,91
MP Cd4	2,80±0,57**	54,90±6,22
MP Cd5	6,33±1,41	46,53±3,36
MP Cd6	4,09±0,64	62,22±5,81
MP Cd7	5,18±1,01	61,46±5,37
MP Cd8	2,35±0,43**	24,13±2,89**
MP Cd9	3,12±0,40**	30,84±4,45**
MP Cd10	6,15±1,13	48,57±4,76
MP Cd11	8,29±1,67*	73,10±7,03*
MP Cd12	4,89±0,70	57,60±5,32
MP Cd13	3,37±0,39 **	47,61±6,09
MP Cd14	3,81±0,49**	44,80±5,61
MP Cd15	8,20±0,96*	75,06±8,91*
MP Cd16	2,90±0,72**	29,80±5,10**
MP Cd17	4,84±0,65	39,75±6,80
MP Cd18	5,50±0,89	22,78±2,03**
MP Cd19	7,19±0,85*	74,29±4,61*
MP Cd20	5,32±0,78	48,90±7,11
MP Cd21	3,56±0,32**	43,44±4,31
MP Cd22	6,61±0,68*	58,01±3,86
MP Cd23	8,28±0,62*	70,62±4,91*
MP Cd24	7,67±0,58*	65,31±5,84*
MP Cd25	5,31±0,49	44,08±3,05
MP Cd26	5,09 ± 0,67	43,19±4,46
MP Cd27	5,73 ± 0,56	61,75±5,00
MP Cd28	4,38 ± 0,43	39,80±5,10**
MP Cd29	3,17 ± 0,52**	41,75±6,80
MP Cd30	4,76 ± 0,75	62,78±5,03*
Average value	**5,07 ± 0,72**	**51,41±5,39**

Note: * – statistically higher indicators relative to the average value ($p<0.05$); ** – statistically lower indicators relative to the average value ($p<0.05$)

Table 10: Adhesion of 5 Malassezia pachydermatis strains from cats to buccal epithelial cells.

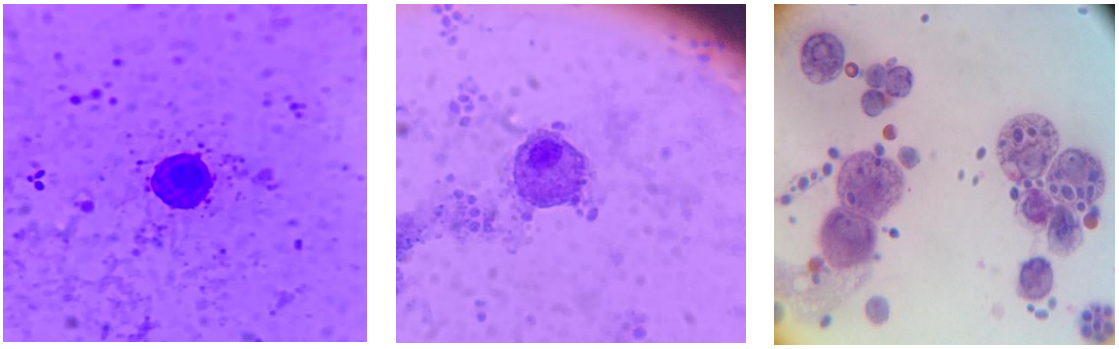
Microorganism	AI - adhesion index, the amount of BY on one BE relative to the number of active epithelial cells	K - % of buccal epitheliocytes with adherent MP
MP Cc1	3,29±0,41	40,62±3,91
MP Cc2	3,18±0,73	55,30±5,04
MP Cc3	4,12±0,50	64,08±4,05*
MP Cc4	2,09±0,38**	33,19±2,46**
MP Cc5	3,64±0,55	50,62±4,91
Average value	**3,26 ± 0,51**	**48,76 ± 4,07**

Note: * – statistically higher indicators relative to the average value ($p<0.05$); ** – statistically lower indicators relative to the average value ($p<0.05$)

For any microorganisms, adhesion is a starting mechanism in the formation of biofilms (a trigger mechanism for the infectious process), in which microbial cells are characterized by increased resistance to immune system effectors, antibiotics and disinfectants. In other words, molecular mechanisms of adhesion are universal for both pathogenic forms and representatives of normal flora, since they are based on ligand-receptor recognition. Also, the assessment of the ability of animal cells to phagocytosis is a research method that is intensively used in modern clinical and immunological diagnostics, since it is phagocytes, with their exceptional reactivity and high mobilization readiness, that constitute the first line of effector mechanisms of immunity. It follows from this that the ability of cells to full-fledged phagocytosis is the object of scientific research into the immune status and the basis for immunocorrective measures.

3.5. Phagocytosis of *Malassezia pachydermatis* by rat alveolar macrophages *in vitro*.

The most effective phagocytosis is carried out by macrophages, since they have a large number of effective antimycotic factors, in contrast to neutrophils, which are the first to rush to the site of infection. Macrophages are capable of direct adhesion of MP. Moreover, the most active and more differentiated are alveolar macrophages, which explains the very rare damage to the lungs when M. enters and colonizes them. To assess phagocytosis, a technique was used to obtain macrophages from rat bronchoalveolar fluid expressed in vitro, as described previously. Reaction of phagocytosis M by macrophages present on Figure 20.



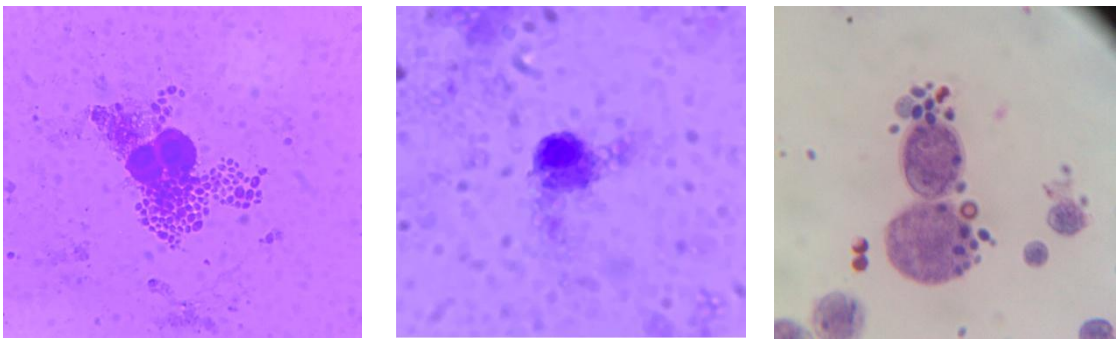


Figure 20. Microphotograph of the reaction of phagocytosis of M by rat alveolar macrophages in vitro. Increase 2000.

According to a study on biofilm formation, the most aggressive strains were MP Cd3, MP Cd23 and MP Cd27. The resistance indices of these three strains are also high: 12,13 and 18 (23 isolates). Accordingly, it was advisable to carry out a comparative analysis of M phagocytosis indicators on these three strains, since it is technically difficult to analyze phagocytic activity with 35 isolates due to the enormous amount of work. The data obtained were analyzed for these three strains and are presented in Table 11.

Table 11: Study of indicators of M phagocytosis on a model of rat alveolar macrophages.

Microorganism	Incubation time	PI, %	PN	IDA, %
MP Cd3	60 minutes	81,0±2,0	7,2±1,1	18,1
	150 minutes	46,0±1,9	5,9±1,2	
MP Cd23	60 minutes	88,7±2,8	8,6±1,0	- 9,3
	150 minutes	83,1±2,7	9,4±1,1	
MP Cd27	60 minutes	76,0±3,1	7,8±1,1	26,9
	150 minutes	51,0±2,4	5,7±1,2	

Note: PI - phagocytic index; PN - phagocytic number; IDA - index of digestion ability.

Based on the analysis of Table 11, it is important to note the following that after an hour of incubation, the PI and PN indicators of the 3 microorganisms did not differ significantly. However, during subsequent incubation (150 minutes), the PI and PN of MP Cd23 were significantly higher than those of the other two and amounted to 83.1 ± 2.7% and 9.4 ± 1.1, respectively. Moreover, when comparing the PI and PN of three strains at different times, we can come to the conclusion that

macrophages digested the main part of the microorganisms MP Cd3 and MP Cd27 at the end of the incubation period, while in MP Cd23 phagocytosis was still at the capture stage. It was found that the IDA values, which more clearly characterize the strain's resistance to phagocytosis, were reliably distinguishable among the selected isolates. The negative value of this indicator in MP Cd23 indicates that macrophages did not cope with their task, and BY could multiply and remain viable for a long time inside macrophages. Thus, MP Cd23 is a significantly more resistant microorganism to phagocytosis.

3.6. Modeling of ear otitis in rabbits by a strain *Malassezia pachydermatis* from dogs.

All strains of MP were stored at −80°C. Stain MP Cd23 was chosen for model development because, in our previous study, it was the strongest biofilm producer (optical density = 0.441 ± 0.016) and most resistant to antifungal drugs, and adhere & be phagocytosed better among other strains. Samples were cultured on SDA at 37°C for 72 h. After 3 days of incubation on agar, cultures were washed twice with sterile Brain Heart Infusion Broth (HiMedia™ Laboratories Pvt. Ltd.,), and suspended at McFarland 0.5 using a DEN1 McFarland Densitometer (Grant-bio, Grant Instruments Ltd., Cambridge, UK) for administration.

Infection of rabbits was carried out by adding 1ml BY suspension into both auricles with a syringe without needle into scratches made with a sterile blade beforehand (Figure 21). Animals were observed daily. Thus, the model of MO included a single infection of animals with MP at a dose of 10^8 CFU/ml. And we want to emphasize that this model is stable without any immunosuppression by additional preparations.

Clinical signs such as loss of appetite or body weight, erythema, itching, the release of abundant ear secretions (cerumen) of a yellow-brown color, often with an unpleasant odor were observed from mild to very severe and entered in the table 12.

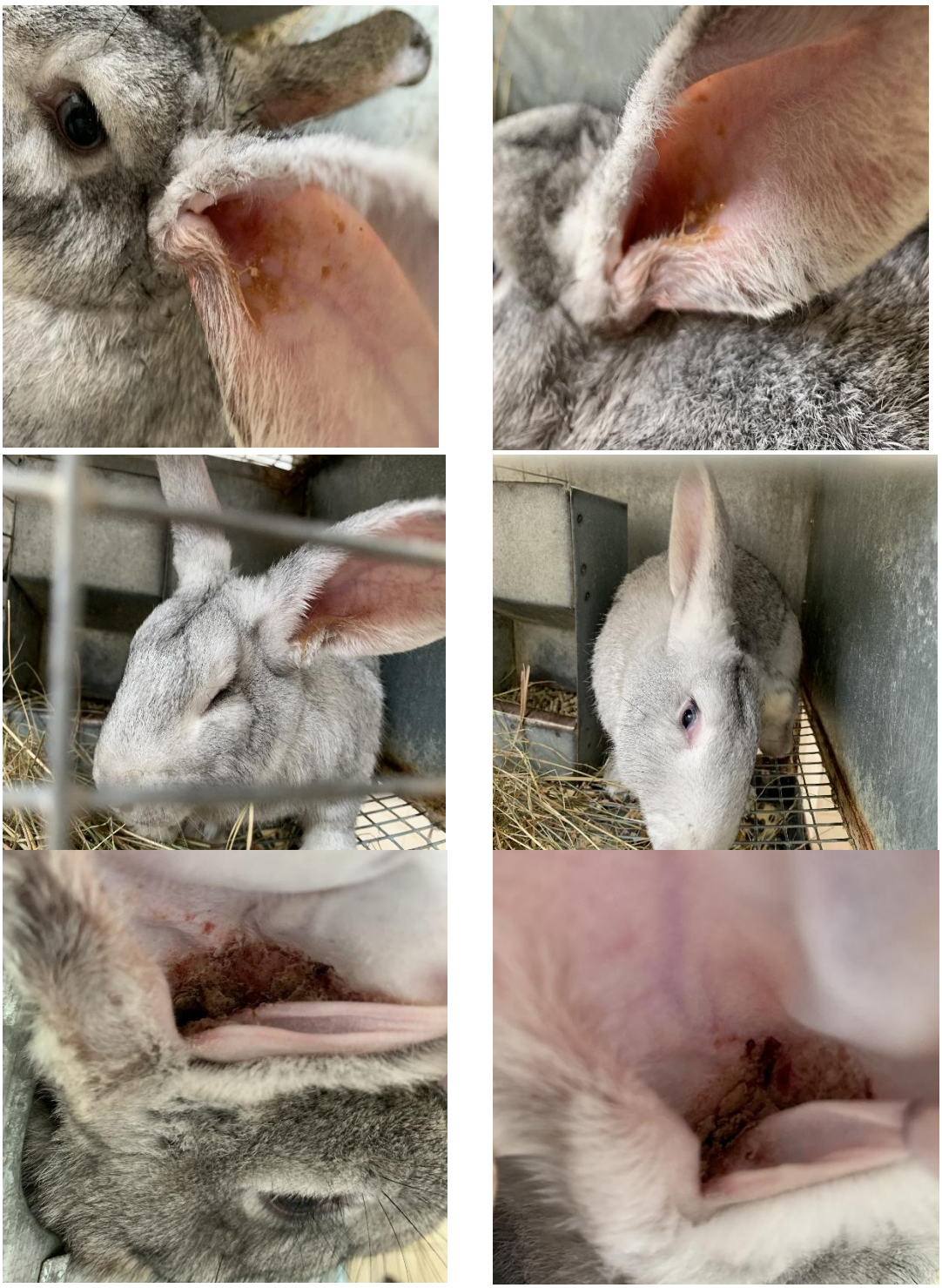


Figure 21. Clinical course of *Malassezia*-otitis media

Table 12: Severity of different clinical signs in rabbits' model of *Malassezia*-otitis media.

Parameters	Control	5 days experiment	10 days experiment	15 days experiment	20 days experiment	25 days experiment	30 days experiment
Loss of appetite or body weight	–	–	–	+	+	+	++

Erythema	–	+	++	++	+++	++++	++++
Itching	–	–	+	++	+++	++++	++++
Cerumen	–	–	+	+	++	++++	++++
Unpleasant odor	–	–	–	+	++	++++	++++
Note: Normal: –; mild: +; moderate: ++; severe: +++; very severe: ++++							

Ear smears were taken from rabbits every 5 days, followed by microscopy and seeding on nutrient media. MP was found in the smears under microscope and confirmed by growth on the SDA (table 13).

Table 13: Presence of Malassezia in ear secretion of experimental rabbits.

Parameters	**Control**	**5 days experi-ment**	**10 days experi-ment**	**15 days experi-ment**	**20 days experi-ment**	**25 days experi-ment**	**30 days experi-ment**
Presence BY in smears of contents from the ears	–	+	+	+	++	++	+++
growth *M. pachydermatis* on nutrient media	–	+	++	++	+++	++++	++++
Note: Absence: –; minor: +; moderate: ++; abundant: +++; massive: ++++							

The nature of growth and the degree of contamination was objectively assessed and the results can be seen in Table 13. However, in a follow-up study, in a repeat of experimental otitis in rabbits, we want to more accurately quantify BY and how the number and composition of other microorganisms in the ear canal changes.

In parallel with this study, blood was taken from the marginal ear vein in animals every 5 days to determine hematological and biochemical parameters according to conventional methods, as well as the activity of nonspecific immunity factors. The blood counts before infection served as controls.

Analysis of hematological parameters (Table 14) demonstrated progressive leukocytosis with each measurement, which indicates a growing inflammatory process in the body of infected animals. Thus, on the 5th day of the experiment, the number of leukocytes was 29.9% higher than in the control group, and on the 30th day - already 62.4%.

Table 14: Hematological parameters in rabbits' model of Malassezia-otitis media.

Parameters	**Physiological norm**	**Before the experiment - control**	**5 days experiment**	**10 days experiment**	**15 days experiment**	**20 days experiment**	**25 days experiment**	**30 days experiment**
Hb, g/l	100,00 – 160,00	140,80±6,42	129,10±6,38*	113,50±8,46*	90,80±6,06*	86,40±5,52*	84,90±3,87*	85,90±5,66*
ESR, mm/h	1 – 3	3,00±1,33	7,30±1,49*	14,50±2,32*	16,30±3,33*	20,30±3,80*	22,10±2,80*	20,90±2,07*
RBC, 10^{12}/l	4,50 – 7,50	5,62±0,65	5,25±0,61	4,70±0,44*	4,18±0,40*	3,74±0,37*	3,50±0,20*	3,44±0,21*
Leukocytes, 10^9/l	6,50 – 9,50	6,96±0,30	9,93±0,85*	12,23±0,72*	13,90±1,00*	15,96±1,44*	16,54±1,70*	18,51±1,13*
R, %	5 – 9	4,70±1,41	14,80±1,81*	15,00±1,69*	20,70±2,00*	25,90±2,68*	26,00±1,49*	30,30±2,41*
S, %	33 – 39	25,00±2,00	34,60±2,11*	37,00±1,76*	41,10±2,02*	40,90±1,96*	43,00±3,23*	44,00±2,53*
E, %	0 – 2	0,50±0,70	0,40±0,51	0,60±0,69	0,70±0,48	0,90±0,56	0,80±0,63	0,80±0,63
M, %	2 –10	4,80±1,22	4,90±1,19	5,00±1,15	5,10±1,19	5,30±1,41	5,20±0,78	4,70±0,94
B, %	0 – 4	2,20±0,91	2,30±0,82	2,40±0,84	2,80±0,78	3,20±1,03*	3,30±0,94*	2,90±1,10
Lymphocytes, %	43 – 62	62,80±3,64	44,80±6,35*	40,00±2,44*	29,60±3,50*	23,80±3,19*	21,50±3,53*	17,30±3,43*
Platelets, 10^9/l	125,0 – 250,0	223,30±16,39	204,80±17*,68	208,00±14,99*	203,10±10,94*	197,20±14,31*	180,60±11,89*	182,50±12,21*
Hematocrit, %	35 – 45	35,10±0,94	35,23±0,68	35,08±0,86	35,10±0,78	34,93±0,68	35,33±0,78	35,19±0,50
MCH, Pg	17,5 – 23,5	25,32±2,72	24,95±3,67	24,42±3,55	21,83±1,66*	23,28±2,53	24,34±1,99	25,07±2,53
MCHC, g/l	300,0 – 400,0	401,27±18,65	366,50±18*,60	323,53±23,32*	258,79±18,55*	247,31±14,88*	240,36±11,96*	244,09±15,64*
MCV, мкм³	57,0 – 70,0	63,12±	67,81±8,38	75,25±7,92*	84,76±9,30*	94,25±10,34*	101,50±6,34*	102,91±6,25*

*Note: here and below, data are presented as mean ± SD. * - statistically significant difference between the experiment and the control (P<0.05).*

The leukogram showed that with the clinical manifestation of experimental MO in the blood of rabbits, an increase in band neutrophils by 3.15 times and segmented neutrophils by 1.38 times is recorded already on the 5th day of the study. It should be noted that by the 30th day of the pathology, band and segmented neutrophils were increased by 6.45 and 1.76 times, respectively, when compared with the output data. In addition, progressive lymphopenia was recorded: on the 5th

day of the inflammatory process, the number of lymphocytes in the blood of rabbits was reduced by 28.7%, from 62.80±3.64% to 44.80±6.35%, and by the 30th day of the experiment - by 72.4%, from 62.80±3.64% to 17.30±3.43%. It was established that during the experimental course of MO, thrombocytopenia within the physiological norm is recorded in the blood of rabbits. Thus, on the 5th day of the study, the number of platelets decreased by 1.09 times, on the 15th day - by 1.10, and by the 30th day - by 1.22 times, when compared with the data before the start of the experiment. Also, in the dynamics of the inflammatory process in MO in rabbits, a decrease in the average concentration of hemoglobin in the erythrocyte and an increase in the average volume of erythrocytes were observed in the blood. Thus, on the 5th day of the study, the MCHC indicator decreased by 8.66%, and the MCV indicator increased by 6.91%, and by the 30th day of the study, the MCHC indicator had already decreased by 39.17%, and the MCV analyte increased by 38.66% when compared with the control data.

Analysis of biochemical parameters of rabbit blood serum showed that concentrations of high-density lipoproteins, cholesterol and triglycerides increased significantly after experimental infection by MP, while blood sugar and plasma fibrinogen levels decreased after infection compared with control ($P < 0.05$) (Table 15). Indicators such as LD lipoproteins, urea, uric acid, creatinine, calcium, serum globulin, albumin and total protein were without statistically significant changes.

Table 15: The biochemical parameters in rabbits' model of Malassezia-otitis media.

Parameters	**Control**	**5 days experi-ment**	**10 days experi-ment**	**15 days experi-ment**	**20 days experi-ment**	**25 days experi-ment**	**30 days experi-ment**
Blood sugar	120.77 ± 4.81	105.38 ± 4.02∗	103.00 ± 5.93∗	108.32 ± 4.74∗	106.88 ± 4.81∗	105.61 ± 4.60∗	104.02 ± 5.09∗
Cholesterol	118.36 ± 2.75	127.25 ± 3.81∗	128.82 ± 4.01∗	126.15 ± 4.78∗	128.25 ± 3.27∗	127.91 ± 5.03∗	127.90 ± 4.62∗
Triglyceride	138.62 ± 4.23	149.07 ± 3.90∗	150.84 ± 3.52∗	148.11 ± 3.46∗	147.90 ± 4.32∗	151.87 ± 4.73∗	150.28 ± 4.92∗
Uric acid	1.73 ± 0.39	1.96 ± 0.35	2.01 ± 0.40	1.98 ± 0.37	2.00 ± 0.42	1.87 ± 0.55	1.93 ± 0.50
Urea	30.90 ± 4.13	33.61 ± 5.17	32.74 ± 5.91	33.00 ± 4.47	34.58 ± 4.90	33.04 ± 5.88	34.23 ± 4.65
Creatinine	0.75 ± 0.14	0.80 ± 0.13	0.82 ± 0.15	0.81 ± 0.12	0.86 ± 0.14	1.00 ± 0.23	0.95 ± 0.20

Calcium	9.45 ± 1.80	10.63 ± 1.44	10.26 ± 1.82	11.03 ± 2.05	10.43 ± 1.91	10.52 ± 1.04	10.37 ± 1.68
Albumin	5.44 ± 0.83	4.36 ± 1.25	5.03 ± 1.74	4.93 ± 1.62	5.06 ± 1.24	4.99 ± 2.23	4.81 ± 1.76
Total protein	7.60 ± 1.33	8.42 ± 1.28	6.90 ± 2.04	8.36 ± 1.31	8.53 ± 1.83	7.52 ± 1.75	8.04 ± 1.80
HD lipoprotein (mg/dl)	40.26 ± 3.05	49.10 ± 2.97∗	45.83 ± 2.25	46.03 ± 2.40	49.13 ± 2.20∗	47.99 ± 1.94∗	50.08 ± 1.96∗
LD lipoprotein (mg/dl)	14.10 ± 1.12	15.17 ± 1.48	16.32 ± 2.01	15.66 ± 1.29	16.03 ± 1.41	16.82 ± 1.27	17.02 ± 2.04
Plasma fibrinogen	610.1 ± 33.7	564.7 ± 25.7	460.2 ± 23.7∗	464.8 ± 28.1∗	430.7 ± 19.7∗	434.1 ± 25.6∗	404.8 ± 21.5∗
Serum globulins	2.36 ± 0.43	2.80 ± 0.82	2.74 ± 0.51	2.68 ± 0.63	2.63 ± 0.39	2.70 ± 0.48	2.51 ± 0.36
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*							

Liver function tests showed that all parameters such as Alanine aminotransferase, Alkaline phosphatase, Bilirubin, Aspartate transaminase and Lactate dehydrogenase have grown in rabbits after experimental infection by MP (P < 0.05) (Table 16)

Table 16: Liver function enzymes in rabbits' model of *Malassezia*-otitis media.

Parameters	**Control**	**5 days experi-ment**	**10 days experi-ment**	**15 days experi-ment**	**20 days experi-ment**	**25 days experi-ment**	**30 days experi-ment**
Alanine aminotransferase (IU/L)	113.56 ± 5.80	123.07 ± 6.18	121.38 ± 5.82	129.37 ± 4.99∗	130.05 ± 6.16∗	127.83 ± 5.84∗	131.86 ± 6.01∗
Alkaline phosphatase (IU/L)	199.05 ± 12.86	230.46 ± 15.05∗	228.71 ± 9.42∗	231.88 ± 10.92∗	226.76 ± 11.51∗	235.04 ± 10.83∗	239.67 ± 9.59∗
Bilirubin (mg/dL)	0.50 ± 0.04	0.59 ± 0.03∗	0.60 ± 0.05∗	0.58 ± 0.03∗	0.58 ± 0.03∗	0.61 ± 0.04∗	0.59 ± 0.03∗
Aspartate transaminase (IU/L)	95.00 ± 10.60	127.94 ± 8.30∗	131.17 ± 14.65∗	129.36 ± 12.58∗	128.53 ± 9.58∗	130.04 ± 11.74∗	126.00 ± 10.31∗
Lactate dehydrogenase (IU/L)	420.20 ± 36.51	509.63 ± 48.50	495.10 ± 38.50	510.93 ± 41.32∗	519.40 ± 38.65∗	512.94 ± 39.66∗	515.70 ± 42.23∗
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*							

3.7. Studies of blood counts in rabbits with *Malassezia* otitis and how Far affects when added to the treatment regimen.

Clinical blood testing is one of the most important diagnostic methods that displays the reaction of hematopoietic organs to the influence of various physiological and pathological factors, it also allows you to monitor the effectiveness of therapy. 35 rabbits were divided into 7 groups of 5 animals. Each group received one of the following drugs:

1) Surolan, the active ingredients of which are: miconazole, polymyxin B, prednisolone;

2) Otifri lotion for cleaning ears with calendula which contains components such as: water, propylene glycol, emulsifier (Cremophor EL), calendula;

3) Otoxolan contains as active ingredients: marbofloxacin, clotrimazole, dexamethasone; and as auxiliary components propyl gallate, medium chain triglycerides, sorbitan oleate, anhydrous colloidal silicon oxide;

4) Surolan + Far 200 μM/mL in equal proportion;

5) Otifri + Far 200 μM/mL in equal proportion;

6) Otoxolan + Far 200 μM/mL in equal proportion;

7) no drugs at all – control.

All drugs were sprayed onto the entire affected surface of the ear (Fig. 22). The treatment was done once every day, rabbits were fixed, the duration of treatment was 30 days.

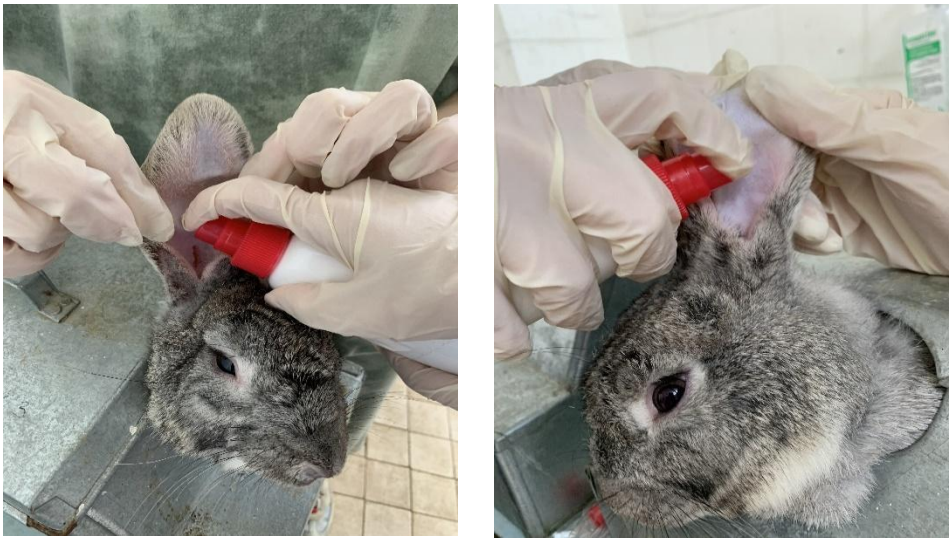


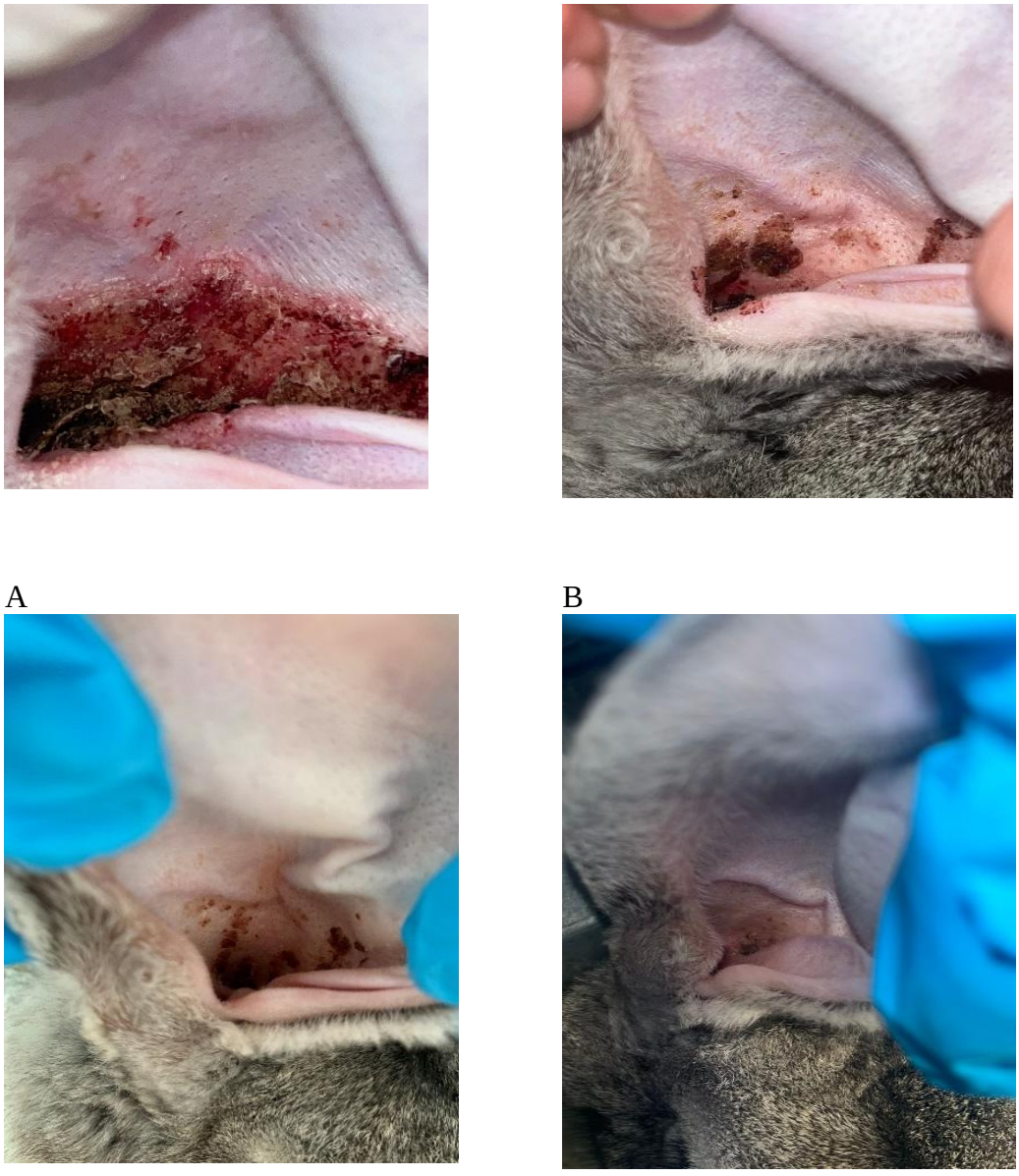
Figure 22. Spraying medications into the ear.

Every five days for a month, smears of ear contents were taken from animals in the control and 6 experimental groups and clinical signs of the disease were recorded (Table 17,18 and Fig.23).

Table 17: Clinical signs recovery when using different treatment regimensin on rabbits' model.

Parameters		**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Loss appetite/ body weight	**5 days treatment**	++	++	++	++	++	++	+++
Erythema		+++	++++	++++	+++	++	++	++++
Itching		++++	+++	+++	++	++	++	++++
Cerumen		+++	+++	+	++	+	+	++++
Unpleasant odor		+++	+++	+	++	+	+	++++
Loss appetite/ body weight	**10 days**	+	++	+	+	+	+	++++
Erythema		++	++++	+++	+	+	+	++++
Itching		++	+++	++++	+	++	−	++++
Cerumen		+	+++	++++	+	+	+	++++
Unpleasant odor		+	+++	++++	+	−	−	++++
Loss appetite/ body weight	**15 days**	+	+	−	−	−	−	++++
Erythema		++	++	+	+	−	−	++++
Itching		++	++	−	−	−	−	++++
Cerumen		+	++	−	−	−	−	++++
Unpleasant odor		+	+	−	−	−	−	++++
Loss appetite/ body weight	**20 days**	−	−	−	−	−	−	++++
Erythema		+	+	+	−	−	−	++++
Itching		+	++	−	−	−	−	++++
Cerumen		+	+	−	−	−	−	++++
Unpleasant odor		−	−	−	−	−	−	++++
Loss appetite/ body weight	**25 days**	−	−	−	−	−	−	++++
Erythema		+	+	+	−	−	−	++++
Itching		+	+	−	−	−	−	++++
Cerumen		−	+	−	−	−	−	++++
Unpleasant odor		−	−	−	−	−	−	++++
Loss appetite/ body weight	**30 days**	−	−	−	−	−	−	++++
Erythema		−	+	−	−	−	−	++++
Itching		−	−	−	−	−	−	++++
Cerumen		−	−	−	−	−	−	++++
Unpleasant odor		−	−	−	−	−	−	++++

Note: Normal: −; mild: +; moderate: ++; severe: +++; very severe: ++++



A B C D

Figure 23. Recovery clinical signs on rabbits' model: A - 5 days treatment with Surolan; B - 10 days; C - 15 days; D - 20 days.

The use of medicinal drugs + Farnesol in the animals of the experimental group reduced the signs of hyperemia, swelling, itching, the amount of exudation on the 5-7th days of treatment, and complete clinical recovery of the animals occurred on the 20th day. When using only drugs in animals (Surolan; Otifri; Otoxolan), on average, an improvement in the clinical condition occurred on 25 days, and final recovery followed after a full course of treatment - 30 days, and then when using Otifri once a day, redness of the ears persisted.

Animals in the control group maintained clear clinical signs of the disease throughout the experiment. Their condition worsened and did not recover on their own, which proves the excellent effectiveness of the MO model we developed in rabbits.

Analyzing the results obtained, we can say that both treatment regimens (with and without the addition of Far) turned out to be effective, but the regimen used in the experimental group Far + Surolan/Otifri/Otoxolan gave faster results due to the wide spectrum of action of the drug Farnesol in relation to microorganisms that most often cause otitis media and its anti-inflammatory effect.

Table 18: Confirmation of recovery using microbiological methods on rabbits' model.

Parameters		**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Presence MP in smears from ears	**5 days**	++	++	++	++	++	++	+++
Growth MP on nutrient media		+++	++++	++++	++	++	+++	++++
Presence MP in smears from ears	**10 days**	+	++	+	+	+	+	++++
Growth MP on nutrient media		++	+++	+++	+	+	+	++++
Presence MP in smears from ears	**15 days**	+	++	+	–	–	–	++++
Growth MP on nutrient media		++	++	++	+	+	–	++++
Presence MP in smears from ears	**20 days**	+	+	+	–	–	–	++++
Growth MP on nutrient media		+	+	+	–	–	–	++++
Presence MP in smears from ears	**25 days**	–	–	–	–	–	–	++++
Growth MP on nutrient media		–	+	–	–	–	–	++++
Presence MP in smears from ears	**30 days**	–	–	–	–	–	–	++++
Growth MP on nutrient media		–	–	–	–	–	–	++++
Note: Absence: –; minor: +; moderate: ++; abundant: +++; massive: ++++								

It is worth noting that in the experimental group there was not a single case of the presence of MP in the smear and on the nutrient medium after combination therapy with the drug + Farnesol after 20 days of therapy (Table 18). And in the control group of 5 animals, all of them had BY observed during microscopy of smears of ear exudate and were sown on the nutrient medium in high concentrations for all 30 days of the experiment.

It is important to note that when Farnesol was added to the drug, microbiological clearance from M occurred 5-10 days earlier. The best result in this series of experiments was with the combination of Otoxolan + Far, since clearing of the ears from BY was recorded on the 10th day of therapy.

Clinical blood testing is one of the most important diagnostic methods, reflecting the reaction of the hematopoietic organs to the influence of various physiological and pathological factors; it also allows you to monitor the effectiveness of the therapy. Clinical blood parameters of rabbits with MO before treatment were characterized by low erythrocyte count values - $5.20 \pm 0.34\ 10^6/\mu L$, which cannot be called anemia, but is a borderline value (Table 19-24).

Also, in sick animals, a decrease in hemoglobin was observed at the beginning - 9.18 ± 1.07 g/dL. After treatment, the amount of hemoglobin in the blood of experimental rabbits increased to values of 10 g/dL and higher, and a significant difference was present between the experimental groups, which indicates the positive effect of Farnesol in the treatment of Malassezia otitis.

We began to observe statistically significant differences between the experimental groups by the 15-20th day of treatment. For example, the number of platelets ($10^3/\mu L$) in the blood of sick animals in control was significantly higher, but after therapy the number of platelets became lower and this decrease occurred earlier in the groups with the combination of the drug and Far.

Table 19: Hematological parameters in rabbits' model on day 5 of treatment.

Parameters	**5 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/µL)		5.78 ± 0.52	5.64 ± 0.60	5.70 ± 0.61	5.66 ± 0.60	5.74 ± 0.65	5.60 ± 0.68	6.34 ± 0.41
Lymphocytes (10^3/µL)		2.70 ± 0.41	2.55 ± 0.40	2.62 ± 0.48	2.71 ± 0.40	2.70 ± 0.39	2.62 ± 0.41	2.71 ± 0.40
Monocytes (10^3/µL)		0.54 ± 0.12	0.58 ± 0.15	0.52 ± 0.19	0.57 ± 0.16	0.55 ± 0.14	0.55 ± 0.12	0.60 ± 0.13
Granulocytes (10^3/µL), of them		3.24 ± 0.30	3.19 ± 0.39	3.18 ± 0.37	3.27 ± 0.38	3.29 ± 0.43	3.06 ± 0.35	3.28 ± 0.45
Neutrophils (10^3/µL)		2.18 ± 0.31	2.24 ± 0.45	2.21 ± 0.40	2.10 ± 0.39	2.17 ± 0.32	2.13 ± 0.37	2.18 ± 0.32
Red blood cells (10^6/µL)		5.51 ± 0.50	5.68 ± 0.36	5.70 ± 0.40	5.62 ± 0.37	5.22 ± 0.35	5.38 ± 0.38	5.20 ± 0.34
Hemoglobin (g/dL)		8.93 ± 0.91	8.62 ± 0.95	8.73 ± 0.94	8.56 ± 1.02	8.78 ± 0.95	8.63 ± 0.90	9.18 ± 1.07
Hematocrit (%)		34.70 ± 3.46	32.71 ± 2.86	34.75 ± 3.27	36.01 ± 3.86	35.61 ± 3.06	33.70 ± 2.90	30.98 ± 2.88
Mean corpuscular volume (fL)		67.95 ± 2.83	66.39 ± 2.90	69.25 ± 3.13	68.24 ± 2.93	65.25 ± 2.68	68.30 ± 2.43	68.17 ± 2.86
Mean corpuscular hemoglobin (pg)		20.01 ± 0.95	19.71 ± 0.90	19.49 ± 0.96	19.81 ± 1.05	19.92 ± 0.94	19.84 ± 0.97	17.95 ± 1.07
Mean corpuscular hemoglobin concentration (g/dL)		31.891 ± 1.86	31.69 ± 1.56	32.83 ± 1.98	30.92 ± 1.48	32.84 ± 1.76	33.01 ± 2.16	30.50 ± 2.08
Platelets (10^3/µL)		218.20 ± 24.05	220.49 ± 23.91	221.83 ± 21.85	220.35 ± 25.48	221.75 ± 22.05	220.24 ± 23.07	232.94 ± 18.94
Note: there are no statistically significant differences in this table between experience and control ($P < 0.05$).								

On day 5 of our experiment, no statistically significant differences were observed between the 5 experimental groups and one control group. Conclusion: the duration of therapy is very short for visible results.

Table 20: Hematological parameters in rabbits' model on day 10 of treatment.

Parameters	**10 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/µL)		5.18 ± 0.53	5.41 ± 0.64	5.84 ± 0.58	5.17 ± 0.52	5.23 ± 0.54	5.15 ± 0.66	6.33 ± 0.48
Lymphocytes (10^3/µL)		2.53 ± 0.41	2.85 ± 0.40	2.62 ± 0.48	2.71 ± 0.40	2.70 ± 0.39	2.62 ± 0.41	2.61 ± 0.44
Monocytes (10^3/µL)		0.54 ± 0.12	0.58 ± 0.15	0.50 ± 0.19	0.57 ± 0.16	0.58 ± 0.14	0.55 ± 0.12	0.54 ± 0.11
Granulocytes (10^3/µL), of them		3.24 ± 0.30	3.19 ± 0.39	3.18 ± 0.37	3.27 ± 0.38	3.29 ± 0.43	3.06 ± 0.35	3.43 ± 0.55
Neutrophils (10^3/µL)		2.18 ± 0.31	2.24 ± 0.45	2.21 ± 0.40	2.10 ± 0.39	2.17 ± 0.36	2.13 ± 0.37	2.28 ± 0.35
Red blood cells (10^6/µL)		5.84 ± 0.43	5.61 ± 0.48	5.47 ± 0.43	5.64 ± 0.41	5.61 ± 0.43	5.61 ± 0.43	5.62 ± 0.38
Hemoglobin (g/dL)		9.05 ± 1.00	9.25 ± 1.30	9.24 ± 1.20	9.21 ± 1.34	9.26 ± 1.10	9.27 ± 1.28	9.05 ± 1.17
Hematocrit (%)		33.24 ± 3.75	35.36 ± 4.20	32.24 ± 4.02	35.08 ± 4.04	36.04 ± 4.08	35.14 ± 3.95	33.20 ± 4.73
Mean corpuscular volume (fL)		67.84 ± 3.12	67.80 ± 2.56	67.04 ± 3.02	66.36 ± 3.08	65.24 ± 3.00	67.09 ± 2.93	67.92 ± 2.86
Mean corpuscular hemoglobin (pg)		19.08 ± 0.92	18.86 ± 0.90	19.21 ± 0.74	19.28 ± 0.93	19.06 ± 0.72	19.15 ± 0.84	18.50 ± 1.05
Mean corpuscular hemoglobin concentration (g/dL)		30.60 ± 2.02	30.60 ± 2.02	31.83 ± 1.98	30.42 ± 1.48	32.84 ± 1.76	32.83 ± 1.98	30.40 ± 2.18
Platelets (10^3/µL)		216.90 ± 18.72	211.94 ± 20.79	215.23 ± 19.85	220.35 ± 25.48	226.75 ± 22.05	217.83 ± 21.85	235.74 ± 20.22
Note: there are no statistically significant differences in this table between experience and control ($P < 0.05$).								

On day 10 of our experiment, also no statistically significant differences were observed between the 5 experimental groups and one control group. Conclusion: the duration of therapy is very short for visible results according to Hematological parameters in rabbits' model.

Table 21: Hematological parameters in rabbits' model on day 15 of treatment.

Parameters	**15 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/μL)		5.01 ± 0.37	5.23 ± 0.58	5.83 ± 0.45	5.10 ± 0.65	5.41 ± 0.71	5.32 ± 0.47	6.33 ± 0.56
Lymphocytes (10^3/μL)		3.06 ± 0.38	2.96 ± 0.40	2.76 ± 0.45	2.58 ± 0.68	3.17 ± 1.28	2.66 ± 0.58	2.61 ± 0.44
Monocytes (10^3/μL)		0.29 ± 0.13	0.40 ± 0.17	0.41 ± 0.14	0.36 ± 0.18	0.42 ± 0.15	0.40 ± 0.17	0.50 ± 0.11
Granulocytes (10^3/μL), of them		3.08 ± 0.28	3.19 ± 0.39	3.16 ± 0.37	3.04 ± 0.31	2.88 ± 0.24	3.43 ± 0.58	3.43 ± 0.55
Neutrophils (10^3/μL)		2.00 ± 0.33	2.18 ± 0.41	1.99 ± 0.46	2.20 ± 0.58	2.14 ± 0.50	2.11 ± 0.42	2.28 ± 0.35
Red blood cells (10^6/μL)		5.29 ± 0.41	5.61 ± 0.48	5.47 ± 0.43	5.59 ± 0.51	5.59 ± 0.51	5.59 ± 0.51	5.62 ± 0.38
Hemoglobin (g/dL)		9.43 ± 1.21	9.25 ± 1.30	9.24 ± 1.20	9.40 ± 1.27	9.48 ± 1.01	9.53 ± 1.21	9.05 ± 1.17
Hematocrit (%)		35.90 ± 4.80	32.02 ± 3.17	33.94 ± 3.42	34.65 ± 3.81	34.90 ± 3.77	33.52 ± 3.83	33.20 ± 4.73
Mean corpuscular volume (fL)		64.01 ± 2.37	64.31 ± 2.65	68.21 ± 2.28	60.53 ± 2.67	69.01 ± 3.08	65.91 ± 2.64	67.92 ± 2.96
Mean corpuscular hemoglobin (pg)		19.00 ± 1.04	21.45 ± 0.86	19.74 ± 0.69	19.80 ± 1.02	20.32 ± 0.94	20.80 ± 0.73	18.60 ± 0.98
Mean corpuscular hemoglobin concentration (g/dL)		33.53 ± 1.20	32.61 ± 1.37	32.84 ± 1.42	32.41 ± 1.24	33.54 ± 1.26	32.51 ± 2.07	30.40 ± 2.18
Platelets (10^3/μL)		232.60 ± 20.34	225.60 ± 17.04	219.60 ± 21.05	218.60 ± 19.36	210.60 ± 16.04	200.32 ± 14.04*	240.34 ± 20.24

*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*

On the 15th day of our experiment, statistically significant differences between 5 experimental groups and one control group are visible in the level of platelets in rabbits that received daily therapy Otoxolan + Far.

Table 22: Hematological parameters in rabbits' model on day 20 of treatment.

Parameters	**20 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/μL)		5.08 ± 0.63	5.23 ± 0.51	5.11 ± 0.62	5.64 ± 0.55	5.09 ± 0.58	5.34 ± 0.43	6.13 ± 0.48
Lymphocytes (10^3/μL)		2.46 ± 0.38	2.41 ± 0.40	2.26 ± 0.45	2.38 ± 0.68	3.10 ± 1.23	2.26 ± 0.58	2.61 ± 0.44
Monocytes (10^3/μL)		0.29 ± 0.13	0.40 ± 0.17	0.41 ± 0.14	0.36 ± 0.18	0.42 ± 0.15	0.40 ± 0.17	0.50 ± 0.11
Granulocytes (10^3/μL), of them		2.87 ± 0.38	2.76 ± 0.39	3.10 ± 0.50	3.02 ± 0.41	2.85 ± 0.39	2.70 ± 0.34	3.43 ± 0.45
Neutrophils (10^3/μL)		2.00 ± 0.33	2.18 ± 0.41	1.99 ± 0.46	2.20 ± 0.58	2.14 ± 0.50	2.11 ± 0.42	2.28 ± 0.35
Red blood cells (10^6/μL)		5.28 ± 0.42	5.14 ± 0.40	4.97 ± 0.57	5.24 ± 0.45	5.35 ± 0.39	5.40 ± 0.42	5.62 ± 0.38
Hemoglobin (g/dL)		9.62 ± 0.81	9.22 ± 0.94	9.43 ± 1.05	8.82 ± 0.90	9.52 ± 0.81	9.41 ± 0.94	9.05 ± 1.17
Hematocrit (%)		35.38 ± 3.64	36.83 ± 4.08	36.19 ± 3.06	35.25 ± 4.03	32.83 ± 3.41	35.36 ± 4.15	32.20 ± 2.93
Mean corpuscular volume (fL)		64.57 ± 1.82	65.53 ± 1.94	65.69 ± 2.02	65.97 ± 2.13	66.05 ± 1.90	65.74 ± 1.95	67.92 ± 2.96
Mean corpuscular hemoglobin (pg)		19.90 ± 1.24	19.20 ± 1.13	18.56 ± 1.25	19.70 ± 1.20	18.84 ± 1.15	19.30 ± 1.02	17.94 ± 0.88
Mean corpuscular hemoglobin concentration (g/dL)		32.94 ± 1.54	35.90 ± 1.73	36.90 ± 2.02	37.65 ± 1.54*	36.82 ± 1.63*	37.24 ± 1.54*	30.40 ± 2.18
Platelets (10^3/μL)		225.78 ± 20.43	211.50 ± 21.43	209.26 ± 17.93	201.53 ± 20.42*	205.56 ± 18.79*	207.28 ± 17.43*	234.74 ± 20.22

*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*

On the 20th day of our experiment, statistically significant differences between 5 experimental groups and one control group are visible in the level of platelets and mean corpuscular hemoglobin concentration in rabbits that received daily therapy Otoxolan + Far; Surolan + Far; Otifri + Far.

Table 23: Hematological parameters in rabbits' model on day 25 of treatment.

Parameters	**25 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/μL)		4.96 ± 0.76	5.13 ± 0.52	5.24 ± 0.95	4.97 ± 0.90	4.82 ± 0.41∗	5.03 ± 0.51∗	6.23 ± 0.38
Lymphocytes (10^3/μL)		3.21 ± 0.44	3.14 ± 0.35	3.15 ± 0.38	3.06 ± 0.29	3.18 ± 0.30	3.01 ± 0.32	2.65 ± 0.44
Monocytes (10^3/μL)		0.29 ± 0.13	0.40 ± 0.17	0.41 ± 0.14	0.36 ± 0.18	0.42 ± 0.15	0.40 ± 0.17	0.50 ± 0.11
Granulocytes (10^3/μL), of them		2.87 ± 0.38	2.76 ± 0.39	2.80 ± 0.40	3.02 ± 0.51	2.85 ± 0.49	2.70 ± 0.34	3.43 ± 0.55
Neutrophils (10^3/μL)		2.08 ± 0.53	2.19 ± 0.61	2.26 ± 0.48	1.93 ± 0.66	2.24 ± 0.52	2.15 ± 0.60	2.18 ± 0.35
Red blood cells (10^6/μL)		5.28 ± 0.42	5.14 ± 0.40	4.97 ± 0.57	5.24 ± 0.45	5.25 ± 0.39	5.40 ± 0.42	5.62 ± 0.38
Hemoglobin (g/dL)		9.61 ± 1.03	9.73 ± 1.12	10.08 ± 0.85	10.22 ± 0.95	10.02 ± 1.10	9.43 ± 1.04	9.05 ± 1.17
Hematocrit (%)		34.39 ± 2.24	35.18 ± 2.57	33.75 ± 3.12	34.82 ± 3.00	35.19 ± 1.73	35.16 ± 3.10	32.10 ± 2.73
Mean corpuscular volume (fL)		64.92 ± 2.56	65.38 ± 2.48	65.36 ± 3.04	65.49 ± 2.93	65.12 ± 3.03	63.34 ± 2.50	67.82 ± 2.56
Mean corpuscular hemoglobin (pg)		19.90 ± 1.24	18.20 ± 1.13	18.56 ± 1.25	19.40 ± 1.20	18.84 ± 1.15	19.30 ± 1.02	18.60 ± 0.98
Mean corpuscular hemoglobin concentration (g/dL)		36.64 ± 2.26	35.60 ± 2.34	38.45 ± 1.79∗	39.61 ± 2.14∗	37.94 ± 2.83∗	38.62 ± 2.15∗	30.40 ± 2.18
Platelets (10^3/μL)		204.73 ± 16.19	201.26 ± 23.38	197.54 ± 20.70∗	201.50 ± 21.74∗	200.53 ± 15.67∗	199.50 ± 18.72∗	233.94 ± 22.48

*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*

On the 25th day of our experiment, statistically significant differences between 5 experimental groups and one control group are visible in the level of platelets & mean corpuscular hemoglobin concentration & total white blood cells in rabbits that received daily therapy Otoxolan + Far; Surolan + Far; Otifri + Far & just Otoxolan.

Table 24: Hematological parameters in rabbits' model on day 30 of treatment.

Parameters	**30 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Total white blood cells (10^3/μL)		4.90 ± 0.76	4.72 ± 0.87	4.97 ± 0.63	4.38 ± 0.74∗	4.21 ± 0.82∗	4.41 ± 0.55∗	6.93 ± 1.28
Lymphocytes (10^3/μL)		3.14 ± 1.20	3.75 ± 0.69	3.38 ± 0.70	3.24 ± 0.75	3.18 ± 0.84	3.22 ± 0.68	2.71 ± 0.48
Monocytes (10^3/μL)		0.29 ± 0.13	0.40 ± 0.17	0.41 ± 0.14	0.32 ± 0.18	0.42 ± 0.15	0.40 ± 0.17	0.50 ± 0.11
Granulocytes (10^3/μL), of them		2.87 ± 0.38	2.76 ± 0.39	2.83 ± 0.42	3.02 ± 0.51	2.84 ± 0.50	2.70 ± 0.34	3.43 ± 0.55
Neutrophils (10^3/μL)		2.18 ± 0.53	2.19 ± 0.61	2.16 ± 0.58	1.93 ± 0.60	2.24 ± 0.52	2.15 ± 0.60	2.28 ± 0.35
Red blood cells (10^6/μL)		5.52 ± 0.41	5.27 ± 0.40	5.20 ± 0.45	5.47 ± 0.50	5.28 ± 0.48	5.12 ± 0.36	5.60 ± 0.41
Hemoglobin (g/dL)		9.66 ± 0.47	9.84 ± 0.61	10.35 ± 0.53	10.06 ± 0.49	10.11 ± 0.79	10.60 ± 0.44	9.01 ± 1.10
Hematocrit (%)		41.03 ± 1.80∗	39.25 ± 1.96∗	40.01 ± 1.88∗	41.10 ± 1.90∗	40.56 ± 1.54∗	39.03 ± 1.90∗	32.20 ± 3.73
Mean corpuscular volume (fL)		62.92 ± 2.56	61.38 ± 2.48	65.36 ± 3.01	65.49 ± 2.73	64.12 ± 3.00	62.34 ± 2.50	67.92 ± 2.96
Mean corpuscular hemoglobin (pg)		21.90 ± 1.24	19.20 ± 1.13	20.56 ± 2.05	19.80 ± 1.64	19.87 ± 1.35	19.90 ± 1.52	18.60 ± 0.98
Mean corpuscular hemoglobin concentration (g/dL)		40.64 ± 2.26∗	41.60 ± 2.34∗	40.25 ± 1.79∗	41.61 ± 2.14∗	39.94 ± 2.03∗	40.62 ± 2.05∗	30.40 ± 2.18
Platelets (10^3/μL)		195.73 ± 11.88∗	198.06 ± 13.38∗	200.51 ± 15.70∗	196.40 ± 19.72∗	200.53 ± 15.76∗	197.55 ± 18.02∗	234.74 ± 23.82

*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*

On the 30th day of our experiment, statistically significant differences between 5 experimental groups and one control group are visible in the level of platelets & mean corpuscular hemoglobin concentration & total white blood cells & hematocrit in rabbits that received daily therapy in all 6 groups. In fact, the blood parameters of rabbits in the experimental groups returned by day 30 to the level of hematological

indicators before the experimental infection. This return of indicators was observed especially quickly in the group with the addition of Far.

Analysis of biochemical parameters of rabbit blood serum in an experiment using 6 different treatment regimens is presented in tables 25-30.

Table 25: The biochemical parameters in rabbits' model on day 5 of treatment.

Parameters	**5 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		103.84 ± 4.57	104.32 ± 3.90	102.85 ± 4.84	107.03 ± 3.28	105.63 ± 4.05	105.41 ± 3.60	104.00 ± 4.79
Cholesterol		127.92 ± 4.48	126.74 ± 3.57	127.90 ± 4.23	125.48 ± 5.01	126.94 ± 4.65	127.38 ± 4.03	128.80 ± 4.22
Triglyceride		150.84 ± 4.70	152.77 ± 4.61	150.32 ± 4.39	149.96 ± 4.75	151.72 ± 3.56	150.87 ± 4.23	152.18 ± 4.92
Uric acid		1.80 ± 0.59	1.93 ± 0.48	1.89 ± 0.36	2.05 ± 0.52	1.85 ± 0.45	1.87 ± 0.55	1.98 ± 0.51
Urea		33.64 ± 4.27	34.92 ± 4.00	33.35 ± 4.10	34.61 ± 3.91	34.02 ± 3.47	33.74 ± 4.88	35.20 ± 4.05
Creatinine		0.85 ± 0.18	0.79 ± 0.22	0.84 ± 0.20	0.86 ± 0.17	1.06 ± 0.19	0.89 ± 0.18	0.90 ± 0.21
Calcium		10.53 ± 0.84	10.80 ± 1.20	9.92 ± 1.06	10.39 ± 1.14	10.25 ± 0.98	10.42 ± 1.04	10.77 ± 1.28
Albumin		4.69 ± 1.67	4.72 ± 2.04	4.86 ± 1.71	4.90 ± 2.02	4.37 ± 2.05	5.02 ± 1.23	4.89 ± 1.66
Total protein		6.97 ± 1.92	7.45 ± 1.39	7.86 ± 1.55	6.85 ± 2.01	7.81 ± 1.74	7.92 ± 1.65	8.44 ± 1.70
HD lipoprotein (mg/dl)		47.66 ± 1.85	48.38 ± 1.80	50.05 ± 2.11	49.51 ± 1.72	47.82 ± 1.90	48.54 ± 2.04	50.48 ± 1.96
LD lipoprotein (mg/dl)		17.33 ± 1.94	16.95 ± 1.77	17.73 ± 1.28	16.75 ± 1.94	16.51 ± 1.46	16.84 ± 1.57	18.01 ± 2.03
Plasma fibrinogen		406.7 ± 19.8	427.3 ± 20.2	414.5 ± 16.4	416.3 ± 21.0	419.2 ± 17.4	424.6 ± 19.6	400.8 ± 20.5
Serum globulins		2.81 ± 0.53	2.77 ± 0.49	2.90 ± 0.50	2.73 ± 0.61	2.80 ± 0.54	3.05 ± 0.58	2.91 ± 0.46
Note: there are no statistically significant differences in this table between experience and control (P < 0.05).								

Table 26: The biochemical parameters in rabbits' model on day 10 of treatment.

Parameters	**10 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		104.88 ± 2.91	106.35 ± 4.20	105.30 ± 4.65	106.88 ± 3.95	106.93 ± 3.81	108.76 ± 4.46	102.53 ± 4.09
Cholesterol		126.92 ± 4.48	125.74 ± 3.57	126.90 ± 4.23	125.48 ± 5.01	126.94 ± 4.65	126.38 ± 4.03	130.40 ± 4.65

Triglyceride		150.84 ± 4.70	152.77 ± 3.91	150.32 ± 4.39	149.96 ± 3.75	148.72 ± 3.56	150.87 ± 4.23	150.28 ± 4.04
Uric acid		2.11 ± 0.48	1.92 ± 0.55	2.04 ± 0.49	1.74 ± 0.63	1.86 ± 0.52	2.00 ± 0.48	2.13 ± 0.50
Urea		33.64 ± 4.27	34.92 ± 4.00	33.35 ± 4.10	34.61 ± 3.91	34.02 ± 3.47	33.74 ± 4.88	34.23 ± 4.65
Creatinine		0.87 ± 0.18	0.79 ± 0.22	0.84 ± 0.20	0.83 ± 0.17	1.06 ± 0.19	0.89 ± 0.18	0.80 ± 0.13
Calcium		10.03 ± 0.84	10.80 ± 1.20	10.20 ± 1.06	10.01 ± 1.14	10.25 ± 0.98	10.46 ± 1.04	10.37 ± 1.68
Albumin		4.40 ± 1.67	4.72 ± 2.04	4.66 ± 1.71	4.20 ± 2.02	4.37 ± 2.05	5.02 ± 1.23	4.81 ± 1.76
Total protein		6.97 ± 1.92	7.45 ± 1.39	7.86 ± 1.55	6.85 ± 2.01	7.81 ± 1.74	7.92 ± 1.65	8.04 ± 1.80
HD lipoprotein (mg/dl)		47.66 ± 1.85	47.38 ± 1.80	50.05 ± 2.11	49.51 ± 1.72	47.82 ± 1.90	48.54 ± 2.04	51.08 ± 1.96
LD lipoprotein (mg/dl)		17.33 ± 1.94	16.95 ± 1.77	17.73 ± 1.28	16.75 ± 1.94	16.51 ± 1.46	16.84 ± 1.57	17.02 ± 2.04
Plasma fibrinogen		406.7 ± 19.8	427.3 ± 20.2	414.5 ± 16.4	416.3 ± 21.0	419.2 ± 17.4	424.6 ± 19.6	403.0 ± 20.5
Serum globulins		2.81 ± 0.53	2.77 ± 0.49	2.90 ± 0.50	2.73 ± 0.61	2.80 ± 0.54	3.05 ± 0.58	2.91 ± 0.56
Note: there are no statistically significant differences in this table between experience and control (P < 0.05).								

As we can see, there is no statistical difference in the first 10 days of the experiment in biochemical analyses. Indicators: concentrations of high-density lipoproteins, cholesterol and triglycerides, while blood sugar and plasma fibrinogen levels, LD lipoproteins, urea, uric acid, creatinine, calcium, serum globulin, albumin and total protein statistically indistinguishable in the group of rabbits with and without treatment. This means that biochemical balance, even with adequate treatment, does not arise immediately in the macroorganism.

Table 27: The biochemical parameters in rabbits' model on day 15 of treatment.

Parameters	**15 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		109.28 ± 3.93	108.32 ± 4.50	109.64 ± 3.75	110.30 ± 4.62	107.51 ± 4.23	108.32 ± 4.75	104.02 ± 5.01
Cholesterol		125.15 ± 3.76	120.08 ± 4.15	122.43 ± 4.73	119.15 ± 3.88	121.15 ± 4.02	124.42 ± 3.70	127.94 ± 4.60
Triglyceride		145.11 ± 3.43	149.35 ± 3.68	150.31 ± 3.97	146.48 ± 3.64	147.85 ± 4.14	145.27 ± 4.22	150.28 ± 4.73
Uric acid		1.88 ± 0.30	1.90 ± 0.35	1.98 ± 0.37	1.98 ± 0.37	1.99 ± 0.39	1.83 ± 0.32	1.94 ± 0.50

Urea		32.64 ± 4.26	33.10 ± 3.83	32.95 ± 4.01	35.00 ± 4.27	33.83 ± 3.56	34.68 ± 4.31	34.23 ± 4.65
Creatinine		0.85 ± 0.28	0.79 ± 0.22	0.82 ± 0.20	0.83 ± 0.17	1.06 ± 0.19	0.89 ± 0.18	0.80 ± 0.13
Calcium		10.03 ± 0.84	9.80 ± 1.20	10.20 ± 1.06	10.01 ± 1.14	9.45 ± 0.98	9.86 ± 1.04	10.50 ± 1.60
Albumin		4.40 ± 1.67	4.72 ± 2.04	4.66 ± 1.71	4.20 ± 2.02	4.37 ± 2.05	5.02 ± 1.23	4.81 ± 1.76
Total protein		6.67 ± 1.42	7.52 ± 1.30	7.56 ± 1.50	6.85 ± 2.01	7.81 ± 1.74	7.92 ± 1.65	8.24 ± 1.84
HD lipoprotein (mg/dl)		48.34 ± 2.96	46.82 ± 2.47	47.57 ± 3.06	46.25 ± 2.68	42.57 ± 2.83∗	46.13 ± 2.40	51.28 ± 1.90
LD lipoprotein (mg/dl)		16.31 ± 1.94	15.95 ± 1.70	15.73 ± 1.78	16.75 ± 1.94	16.01 ± 1.46	16.24 ± 1.59	17.02 ± 2.04
Plasma fibrinogen		474.3 ± 24.9∗	434.2 ± 18.6	454.7 ± 24.7	475.2 ± 19.8∗	483.1 ± 22.6∗	464.7 ± 20.7∗	394.9 ± 23.5
Serum globulins		2.81 ± 0.53	2.77 ± 0.49	2.90 ± 0.50	2.73 ± 0.61	2.80 ± 0.54	2.68 ± 0.63	2.51 ± 0.36
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*								

On the 15th day of the experiment using different drugs, we see the first statistically significant differences in the blood biochemical indicators: plasma fibrinogen and HD lipoproteins.

Table 28: The biochemical parameters in rabbits' model on day 20 of treatment.

Parameters	**20 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		110.28 ± 3.93	108.32 ± 4.50	109.64 ± 3.75	110.30 ± 4.62	107.51 ± 4.23	108.32 ± 4.75	105.37 ± 5.09
Cholesterol		120.15 ± 3.76	122.09 ± 4.15	122.03 ± 4.73	119.15 ± 3.90	121.15 ± 4.02	124.42 ± 3.70	127.90 ± 4.62
Triglyceride		145.11 ± 3.43	149.35 ± 3.68	150.31 ± 3.97	146.48 ± 3.64	147.85 ± 4.14	145.27 ± 4.22	150.28 ± 4.92
Uric acid		1.88 ± 0.30	1.90 ± 0.35	1.98 ± 0.37	1.98 ± 0.37	1.99 ± 0.39	1.83 ± 0.32	1.93 ± 0.50
Urea		32.64 ± 4.27	34.92 ± 4.03	35.35 ± 4.10	34.61 ± 3.51	32.02 ± 3.47	33.74 ± 4.88	34.23 ± 4.65
Creatinine		0.85 ± 0.28	0.79 ± 0.22	0.82 ± 0.20	0.83 ± 0.17	1.06 ± 0.19	0.89 ± 0.18	0.80 ± 0.13
Calcium		10.03 ± 0.84	9.80 ± 1.20	10.20 ± 1.06	10.01 ± 1.14	9.45 ± 0.98	9.86 ± 1.04	10.37 ± 1.68
Albumin		4.80 ± 1.67	4.72 ± 2.04	4.66 ± 1.71	4.30 ± 2.02	4.37 ± 2.05	5.02 ± 1.23	4.81 ± 1.76
Total protein		6.67 ± 1.42	7.52 ± 1.30	7.56 ± 1.50	6.85 ± 2.01	6.81 ± 1.74	6.92 ± 1.65	8.04 ± 1.80
HD lipoprotein (mg/dl)		48.34 ± 2.96	46.82 ± 2.47	47.57 ± 3.06	44.25 ± 2.68∗	42.57 ± 2.83∗	46.13 ± 2.40	52.08 ± 2.04

LD lipoprotein (mg/dl)		16.31 ± 1.94	15.95 ± 1.70	15.73 ± 1.78	16.75 ± 1.94	16.01 ± 1.46	16.24 ± 1.59	17.02 ± 2.04
Plasma fibrinogen		474.3 ± 24.9∗	467.2 ± 20.6∗	470.1 ± 23.2∗	475.2 ± 19.8∗	483.1 ± 22.6∗	464.7 ± 20.7∗	404.8 ± 21.5
Serum globulins		2.64 ± 0.58	2.77 ± 0.49	2.80 ± 0.50	2.73 ± 0.65	2.80 ± 0.54	2.68 ± 0.63	2.51 ± 0.36
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*								

On the 20-25th day of the experiment, statistical differences with the control were noticed in the same two indicators.

Table 29: The biochemical parameters in rabbits' model on day 25 of treatment.

Parameters	**25 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		108.25 ± 5.17	105.31 ± 4.63	109.24 ± 3.62	110.08 ± 4.36	104.69 ± 3.74	107.38 ± 4.21	101.82 ± 4.19
Cholesterol		128.05 ± 3.78	127.00 ± 3.51	123.54 ± 4.26	125.25 ± 2.99	124.72 ± 4.21	125.85 ± 3.93	127.90 ± 4.62
Triglyceride		149.26 ± 3.88	150.69 ± 4.64	149.07 ± 3.69	146.82 ± 3.90	152.43 ± 4.10	149.07 ± 3.90	154.95 ± 4.60
Uric acid		1.78 ± 0.30	1.92 ± 0.35	1.98 ± 0.37	1.98 ± 0.37	1.96 ± 0.39	1.83 ± 0.32	1.93 ± 0.50
Urea		32.64 ± 2.97	33.92 ± 3.45	34.35 ± 4.10	33.61 ± 2.91	32.02 ± 3.47	32.74 ± 3.88	34.23 ± 4.65
Creatinine		0.83 ± 0.18	0.79 ± 0.22	0.81 ± 0.20	0.84 ± 0.17	0.76 ± 0.19	0.89 ± 0.18	0.85 ± 0.13
Calcium		9.03 ± 0.84	9.80 ± 1.20	10.02 ± 1.06	9.01 ± 1.14	9.25 ± 0.98	9.46 ± 1.04	10.37 ± 1.68
Albumin		4.50 ± 1.67	4.72 ± 2.04	4.36 ± 1.71	4.30 ± 2.02	4.37 ± 2.05	4.22 ± 1.23	4.81 ± 1.76
Total protein		8.42 ± 1.28	8.42 ± 1.28	8.42 ± 1.28	8.42 ± 1.28	8.42 ± 1.28	8.42 ± 1.28	8.04 ± 1.80
HD lipoprotein (mg/dl)		46.34 ± 2.96	43.82 ± 2.47∗	42.57 ± 3.06∗	45.25 ± 2.68∗	43.57 ± 2.83∗	45.13 ± 2.40∗	51.48 ± 1.96
LD lipoprotein (mg/dl)		16.31 ± 1.94	15.95 ± 1.70	15.73 ± 1.78	16.75 ± 1.94	16.01 ± 1.46	16.24 ± 1.59	17.02 ± 2.04
Plasma fibrinogen		494.3 ± 24.9∗	469.2 ± 22.6∗	500.1 ± 23.2∗	485.2 ± 19.8∗	493.1 ± 24.6∗	468.7 ± 20.9∗	404.8 ± 21.5
Serum globulins		2.64 ± 0.58	2.77 ± 0.49	2.80 ± 0.50	2.73 ± 0.65	2.80 ± 0.54	2.68 ± 0.63	2.51 ± 0.36
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*								

Table 30: The biochemical parameters in rabbits' model on day 30 of treatment.

Parameters	**30 days**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Blood sugar		120.34 ± 4.45∗	110.75 ± 4.01	119.86 ± 5.77∗	118.77 ± 3.90∗	116.43 ± 4.11∗	120.48 ± 4.39∗	103.02 ± 5.09
Cholesterol		117.65 ± 3.94	120.36 ± 3.56	120.96 ± 2.52	116.36 ± 3.75	118.36 ± 2.75	119.30 ± 2.85	126.84 ± 4.31
Triglyceride		140.62 ± 3.98	133.62 ± 5.76∗	138.62 ± 5.23	135.81 ± 4.80∗	136.91 ± 5.16	134.62 ± 4.13∗	150.38 ± 4.67
Uric acid		1.88 ± 0.30	1.92 ± 0.35	1.98 ± 0.37	1.98 ± 0.37	1.96 ± 0.39	1.83 ± 0.32	2.08 ± 0.50
Urea		31.64 ± 2.97	33.92 ± 3.45	33.35 ± 4.10	35.61 ± 3.51	32.02 ± 3.47	32.74 ± 3.88	34.23 ± 4.65
Creatinine		0.83 ± 0.18	0.79 ± 0.22	0.81 ± 0.20	0.84 ± 0.17	0.86 ± 0.19	0.89 ± 0.18	0.80 ± 0.13
Calcium		9.03 ± 0.84	9.80 ± 1.20	10.02 ± 1.06	9.01 ± 1.14	9.25 ± 0.98	9.46 ± 1.04	10.37 ± 1.68
Albumin		4.50 ± 1.67	4.72 ± 2.04	4.36 ± 1.71	4.30 ± 2.02	4.37 ± 2.05	4.22 ± 1.23	4.81 ± 1.76
Total protein		7.41 ± 2.24	6.82 ± 1.96	6.87 ± 1.49	7.58 ± 1.33	6.90 ± 2.73	7.80 ± 1.64	7.84 ± 2.03
HD lipoprotein (mg/dl)		44.34 ± 2.93∗	43.82 ± 2.47∗	42.57 ± 3.06∗	45.25 ± 2.68∗	43.57 ± 2.83∗	45.13 ± 2.40∗	52.08 ± 2.07
LD lipoprotein (mg/dl)		16.31 ± 1.94	15.95 ± 1.70	15.73 ± 1.78	16.75 ± 1.94	16.01 ± 1.46	16.24 ± 1.59	17.02 ± 2.04
Plasma fibrinogen		517.3 ± 24.9∗	492.2 ± 22.6∗	500.1 ± 23.2∗	485.2 ± 19.8∗	493.1 ± 25.6∗	500.7 ± 24.9∗	431.7 ± 21.9
Serum globulins		2.44 ± 0.58	2.47 ± 0.49	2.50 ± 0.50	2.73 ± 0.65	2.80 ± 0.54	2.38 ± 0.63	2.61 ± 0.46
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*								

By the 30th day of the experiment (Table 30), biochemical parameters: HD lipoprotein & plasma fibrinogen & blood sugar & triglyceride in experimental groups, especially with Far, returned to normal levels before experimental infection. There are no statistically significant differences in other indicators.

Table 31: Liver function enzymes in rabbits' model of *Malassezia*-otitis media.

Parameters	**5 days treatment**	**Surolan**	**Otifri**	**Otoxolan**	**Surolan + Far**	**Otifri + Far**	**Otoxolan + Far**	**control**
Alanine aminotransferase (IU/L)		128.00 ± 7.93	129.12 ± 6.59	127.98 ± 6.47	131.25 ± 7.31	128.37 ± 8.42	130.05 ± 6.08	131.86 ± 6.01
Alkaline phosphatase (IU/L)		226.61 ± 10.12	229.38 ± 9.50	232.54 ± 10.49	226.56 ± 10.61	234.78 ± 13.46	227.16 ± 9.32	239.67 ± 9.59

Bilirubin (mg/dL)		0.59 ± 0.05	0.60 ± 0.04	0.61 ± 0.04	0.62 ± 0.05	0.58 ± 0.04	0.60 ± 0.04	0.59 ± 0.04
Aspartate transaminase (IU/L)		132.00 ± 14.60	131.17 ± 13.73	127.20 ± 8.79	131.49 ± 15.92	130.45 ± 17.60	127.17 ± 14.43	126.00 ± 10.30
Lactate dehydrogenase (IU/L)		511.05 ± 28.29	517.46 ± 37.66	512.92 ± 31.30	520.73 ± 37.64	512.03 ± 29.72	510.47 ± 30.20	515.70 ± 40.52
Alanine aminotransferase (IU/L)	**10 days**	128.47 ± 7.93	130.12 ± 6.59	127.98 ± 6.47	131.25 ± 7.31	128.37 ± 8.42	130.05 ± 6.08	132.86 ± 6.85
Alkaline phosphatase (IU/L)		223.71 ± 11.12	229.38 ± 9.50	230.54 ± 12.49	226.56 ± 10.61	233.78 ± 13.46	226.16 ± 11.32	237.63 ± 9.46
Bilirubin (mg/dL)		0.59 ± 0.05	0.60 ± 0.04	0.61 ± 0.04	0.62 ± 0.05	0.58 ± 0.04	0.60 ± 0.04	0.68 ± 0.04
Aspartate transaminase (IU/L)		132.00 ± 14.60	131.17 ± 13.73	127.20 ± 8.79	131.49 ± 15.92	130.45 ± 17.60	127.17 ± 14.43	132.00 ± 9.87
Lactate dehydrogenase (IU/L)		520.05 ± 28.29	517.46 ± 37.66	519.92 ± 31.30	520.73 ± 37.64	512.03 ± 29.72	518.47 ± 40.20	515.70 ± 42.20
Alanine aminotransferase (IU/L)	**15 days**	124.50 ± 6.40	125.29 ± 6.45	122.46 ± 8.02	121.30 ± 7.46	125.51 ± 5.86	129.37 ± 5.99	131.86 ± 6.05
Alkaline phosphatase (IU/L)		231.21 ± 10.68	227.47 ± 14.21	234.52 ± 13.39	226.27 ± 12.58	235.58 ± 8.95	231.80 ± 10.90	239.64 ± 12.06
Bilirubin (mg/dL)		0.60 ± 0.05	0.62 ± 0.05	0.61 ± 0.04	0.62 ± 0.05	0.58 ± 0.04	0.60 ± 0.04	0.65 ± 0.04
Aspartate transaminase (IU/L)		132.17 ± 14.60	130.17 ± 13.73	126.28 ± 8.79	131.49 ± 15.92	130.45 ± 17.60	127.17 ± 14.43	131.50 ± 9.87
Lactate dehydrogenase (IU/L)		509.05 ± 28.29	517.46 ± 37.66	510.92 ± 31.30	520.73 ± 37.64	512.03 ± 29.72	510.47 ± 40.20	515.70 ± 35.48
Alanine aminotransferase (IU/L)	**20 days**	122.50 ± 6.40	125.29 ± 6.45	121.46 ± 8.02	121.30 ± 7.46	120.51 ± 5.99	126.38 ± 5.92	131.86 ± 6.46
Alkaline phosphatase (IU/L)		220.74 ± 10.68	225.43 ± 9.47	228.70 ± 9.68	226.68 ± 11.52	231.45 ± 8.46	228.71 ± 9.40	239.67 ± 10.00
Bilirubin (mg/dL)		0.60 ± 0.05	0.62 ± 0.05	0.61 ± 0.04	0.62 ± 0.05	0.58 ± 0.04	0.60 ± 0.04	0.68 ± 0.04
Aspartate transaminase (IU/L)		132.17 ± 14.60	130.17 ± 13.73	126.28 ± 8.79	131.49 ± 15.92	130.45 ± 17.60	127.17 ± 14.43	132.00 ± 9.87
Lactate dehydrogenase (IU/L)		490.10 ± 29.58	498.62 ± 38.84	495.10 ± 39.30	501.10 ± 30.72	492.19 ± 33.54	495.20 ± 37.50	515.70 ± 40.20

Alanine aminotransferase (IU/L)	**25 days**	122.56 ± 6.08	125.45 ± 6.23	120.88 ± 7.57∗	125.07 ± 6.24∗	127.43 ± 5.99	121.07 ± 5.18∗	136.80 ± 6.05
Alkaline phosphatase (IU/L)		230.76 ± 18.87	237.39 ± 17.55	228.21 ± 14.46	230.40 ± 19.63	232.32 ± 20.34	231.46 ± 15.01	240.67 ± 9.50
Bilirubin (mg/dL)		0.60 ± 0.05	0.62 ± 0.05	0.61 ± 0.04	0.62 ± 0.05	0.57 ± 0.04∗	0.59 ± 0.03∗	0.69 ± 0.04
Aspartate transaminase (IU/L)		125.99 ± 9.73	124.04 ± 8.50	131.80 ± 12.84	120.92 ± 8.48	115.36 ± 7.66	127.94 ± 9.30	130.00 ± 10.31
Lactate dehydrogenase (IU/L)		510.27 ± 40.20	501.05 ± 41.89	521.62 ± 32.80	508.49 ± 35.75	515.61 ± 40.34	509.63 ± 38.50	520.70 ± 40.23
Alanine aminotransferase (IU/L)	**30 days**	115.56 ± 6.45∗	113.56 ± 5.80∗	110.28 ± 7.46∗	112.42 ± 5.16∗	109.94 ± 6.41∗	113.36 ± 5.80∗	135.65 ± 6.43
Alkaline phosphatase (IU/L)		196.52 ± 14.63∗	195.35 ± 16.29∗	205.91 ± 21.36	202.05 ± 17.87∗	184.33 ± 15.24∗	199.64 ± 12.86∗	239.67 ± 10.59
Bilirubin (mg/dL)		0.58 ± 0.05	0.56 ± 0.04	0.52 ± 0.09	0.49 ± 0.04∗	0.55 ± 0.05	0.54 ± 0.07	0.68 ± 0.07
Aspartate transaminase (IU/L)		98.04 ± 19.84	110.30 ± 12.66	92.89 ± 16.45∗	104.75 ± 15.64	95.21 ± 14.53∗	85.60 ± 11.60∗	136.00 ± 16.31
Lactate dehydrogenase (IU/L)		419.80 ± 36.11∗	420.20 ± 38.21∗	409.64 ± 41.64∗	411.62 ± 36.53∗	427.70 ± 38.75∗	424.20 ± 32.54∗	525.80 ± 42.23
*Note: The data are represented as mean ± SD. * - statistically significant difference between experience and control (P < 0.05).*								

By day 25-30 of the experiment, analysis of liver samples showed that parameters such as alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase and lactate dehydrogenase in rabbits receiving topical treatment differed from those in the control group without treatment ($P < 0.05$) (Table 31). Bilirubin throughout the experimental infection with MP was approximately the same in all periods and did not statistically differ in different groups. Normalization of 4 out of 5 indicators was again observed more quickly in the groups where farnesol was added.

3.8. *Malassezia pachydermatis* biofilm inhibition by Farnesol.

The average decrease was measured and used to calculate the biofilm inhibition percentage by Far, where OD AS is optical density average of thee isolates (MP C23,27 and 3) in experiment, OD AC is optical density average of thee isolates (MP C23,27 and 3) in control without Far (Tabl. 32).

Table 32: The effect of different concentrations of Farnesol on *Malassezia pachydermatis* biofilms and optical density average decrease, % densitometric studies.

	1	2	3	4	5	6	7	8	9	10	11	12
Far concentration / Micro-organism	Control no farnesol	200 μM	100 μM	50 μM	25 μM	12.5 μM	6.3 μM	3.1 μM	1.6 μM	0.8 μM	0.4 μM	0.2 μM
MP Cd23	0.441 ± 0.016	0.120 ±0.008	0.135 ±0.011	0.234 ±0.011	0.226 ±0.019	0.233 ±0.010	0.249 ±0.014	0.302 ±0.015	0.368 ±0.017	0.407 ±0.016	0.439 ±0.011	0.453 ±0.012
MP Cd27	0.403 ± 0.026	0.117 ±0.016	0.121 ±0.009	0.144 ±0.013	0.186 ±0.018	0.272 ±0.016	0.284 ±0.011	0.307 ±0.008	0.320 ±0.014	0.379 ±0.012	0.393 ±0.010	0.399 ±0.010
MP Cd3	0.400 ± 0.012	0.123 ±0.018	0.142 ±0.014	0.160 ±0.021	0.154 ±0.017	0.185 ±0.009	0.234 ±0.014	0.252 ±0.012	0.262 ±0.009	0.279 ±0.010	0.383 ±0.014	0.398 ±0.008
Average OD of 3 isolates	0.415	0.120	0.133	0.179	0.189	0.230	0.256	0.287	0.317	0.390	0.405	0.417
Average decrease OD, %	0	71	68	57	54	55	38	31	24	6	2,4	-0,5

The Far demonstrated good antibiofilm effects at a concentration of more 1.6 μM/mL (24% OD decrease of biofilm) (Fig. 24), and its highest antibofilm effects (71-55% - more than a half) were observed at a concentration of 200-12.5 μM/mL. The non-linear log-logistic regression model was used to construct a dose-response curve for Far antifungal effect.

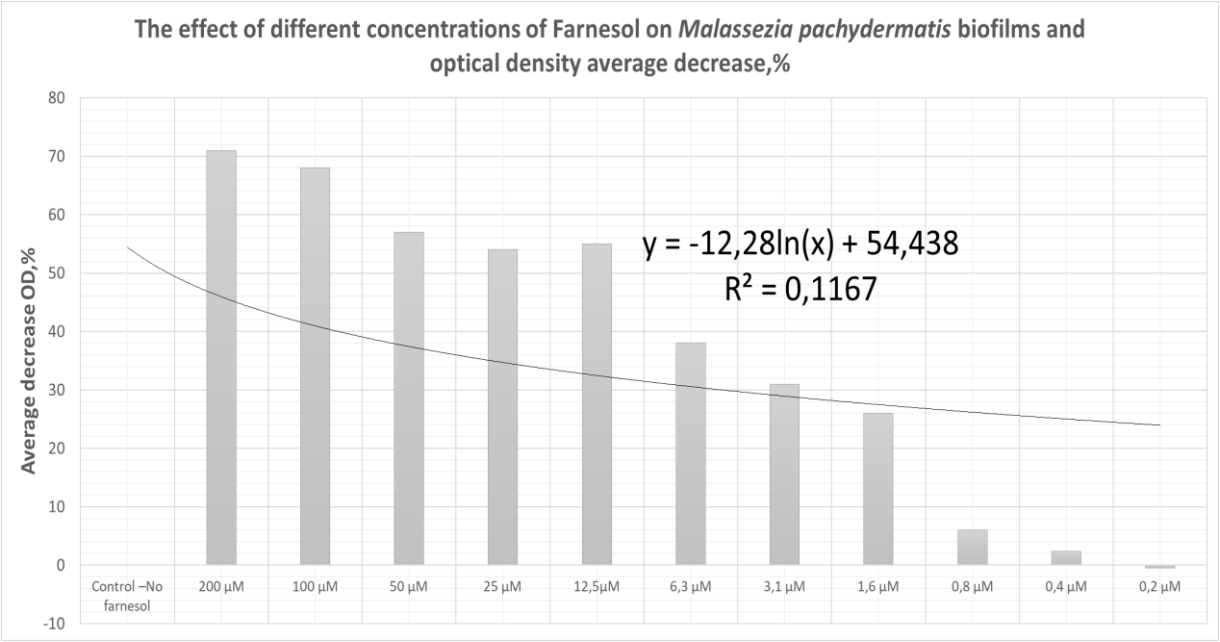


Figure 24. In vitro activity of Farnesol against *M. pachydermatis* (n = 3) biofilms and dose-response curve.

This logarithmic relationship is plotted with the most aggressive clinical strain. According to our practice on models of other fungi (like *Candida spp.*), the Far effect will be even stronger on microorganisms with weak pathogenic and adhesive properties.

3.9. Change in the sensitivity of the *Malassezia pachydermatis* strain to antimycotics and the influence of five concentrations of farnesol on these results.

Antibiotic resistance testing was carried out with the MP C23 strain using the standard disk diffusion method on SDA. Recall that this strain was resistant to AP, KT and FU. Moderately resistant to NS, CC, IT and VOR. And sensitive only to MIC. Experiments with disks were carried out in triplicate. The data in the table on sensitivity to antimicrobial drugs are given without +/- for a better understanding of the table. +/- did not exceed 0.4. The research results were processed by generally accepted statistical methods and were considered reliable at $p \leq 0.05$.

The results of the experiment on changing the sensitivity of MP to antimicrobial drugs when Far is added to the latter are presented in Table 33.

Table 33. Sensitivity of the *Malassezia pachydermatis* strain to antimycotics and the effect of five concentrations of farnesol on these results. The percentages by which sensitivity to antimycotics increases are indicated in parentheses.

Drug	hours	Antimycotic	Antimycotic + PhS (control)	Antimycotic + 12.5 Far	Antimycotic + 25 Far	Antimycotic + 50 Far	Antimycotic + 100 Far	Antimycotic + 200 Far
NS	24 h	10	10	17 (+ 70.0 %)	20 (+ 100.0 %)	21 (+ 110.0 %)	21 (+ 110.0 %)	21 (+ 110.0 %)
	48 h	10	10	16 (+ 60.0 %)	16 (+ 60.0 %)	18 (+ 80.0 %)	18 (+ 80.0 %)	18 (+ 80.0 %)
AP	24 h	0	0	8	10	11	11	11
	48 h	0	0	6	10	11	11	11
KT	24 h	11	11	18 (+ 63.7 %)	19 (+ 72.7 %)	19 (+ 72.7 %)	20 (+ 81.8 %)	22 (+ 100.0 %)
	48 h	11	11	18 (+ 63.7 %)	18 (+ 63.7 %)	19 (+ 72.7 %)	20 (+ 81.8 %)	22 (+ 100.0 %)
CC	24 h	14	14	14	14	14	14	15 (+ 7.1 %)
	48 h	13	13	13	13	13	13	15 (+ 15.4 %)
VOR	24 h	11	11	18 (+ 63.7 %)	19 (+ 72.7 %)	19 (+ 72.7 %)	20 (+ 81.8 %)	22 (+ 100.0 %)
	48 h	11	11	18 (+ 63.7 %)	18 (+ 63.7 %)	19 (+ 72.7 %)	20 (+ 81.8 %)	22 (+ 100.0 %)
FU	24 h	8	8	8	10 (+ 25.0 %)	11 (+ 37.5 %)	11 (+ 37.5 %)	11 (+ 37.5 %)
	48 h	6	6	6	10 (+ 66.7 %)	11 (+ 83.3 %)	11 (+ 83.3 %)	11 (+ 83.3 %)
MIC	24 h	17	17	18 (+ 5.9 %)	19 (+ 11.8 %)	19 (+ 11.8 %)	20 (+ 17.6 %)	20 (+ 17.6 %)
	48 h	16	17	18 (+ 5.9 %)	18 (+ 5.9 %)	19 (+ 11.8 %)	20 (+ 17.6 %)	20 (+ 17.6 %)
IT	24 h	13	13	13	14 (+ 7.7 %)	14 (+ 7.7 %)	14 (+ 7.7 %)	15 (+ 15.4 %)
	48 h	13	13	13	13	14 (+ 7.7 %)	14 (+ 7.7 %)	15 (+ 15.4 %)

- Farnesol increases the inhibition zone
- Farnesol does not affect the inhibition zone

It follows from the table that the clinical MP 23 strain was completely resistant to Amphotericin B (AP). And with Farnesol, even in small concentrations, this drug began to work.

However, we should also pay attention to the presence of results with some drugs where Farnesol did not affect sensitivity in any way: this is MP with Clotrimazole. The last drug still "gave up" when Farnesol was added at the highest concentration of 200 μM/ml. There are also unique indicators: the sensitivity of MP to Nystatin, Voriconazole and Ketoconazole doubled with the addition of Farnesol at a concentration of 25-200 μM/ml. And in fungi, it was noted that the zone of growth inhibition with increasing incubation time from 24 to 48 hours slightly decreases or remains the same.

Thus, in most cases, with the addition of Farnesol, an increase in the sensitivity of microorganism to therapeutic antifungal drugs is recorded in the experiment compared to the control.

3.10. Efficiency of Farnesol for the treatment of canine otitis complicated by *Malassezia pachydermatis.*

Clinical blood test is one of the most important diagnostic methods, reflecting the reaction of the hematopoietic organs to the influence of various physiological and pathological factors, it also allows monitoring the effectiveness of the therapy.

Clinical blood parameters of dogs with Malassezia otitis before treatment were characterized by low values of the number of erythrocytes - $5.94 \pm 0.72\ 10^{12}$ /l, which cannot be called anemia, but is a borderline value.

Also, in sick animals, a decrease in hemoglobin to 127.38 ± 9.34 g/l was observed (Table 34). The number of leukocytes in the blood of sick animals is within the normal range of $13.14 \pm 3.85\ 10^{9}$/l, but after therapy, the number of leukocytes became slightly lower. After treatment, the amount of hemoglobin in the blood of dogs increased to 168.54±8.34 g/l in the experiment and 137.60±9.34 g/l in the control. There is a reliable difference of 1.2 times between these indicators, which indicates a positive effect of Farnesol in the treatment of Malassezia otitis.

Table 34. Clinical indicators of dog blood in the experiment

Indicators	Reference values	Before treatment (n=30)	After treatment	
			Experience (n=15)	Control (n=15)
Red blood cells, 10^{12}/l	5,5 - 8,5	5,94±0,72	7,91±0,87	7,12±1,04
Hematocrit, %	37 - 55	38,12±3,98	51,54±3,86	46,02±4,37
Hemoglobin, g/l	120 -180	127,38±9,34	168,54±8,14*	137,60±9,34*
Leukocytes, 10^{9}/l	6 -17	13,14±3,85	8,54±1,98	11,14±2,61
Rod - shaped neutrophils	0 - 3	9,63±2,03	1,59±0,32*	2,25±0,34*
Segmented neutrophils	60 - 70	56,28±5,26	61,20±5,45	58,82±5,25
Eosinophils	2-12	10,41±3,16	5,86±1,72*	8,32±1,62*
Monocytes	3 -10	4,27±0,84	2,69±0,35	3,13±0,64
Basophils	0 - 1	0,06±0,02	0	0
Lymphocytes	12 - 30	22,41±3,34	28,66±2,24	27,48±2,57

Note: * - statistically significant difference between experience and control

A small eosinophilia (but within the normal range), which can be observed before treatment of malacesiosis otitis, is typical for many infectious and invasive diseases, as well as intoxications. Also, in dogs before treatment, an increase in band neutrophils was observed 9.63±2.03, which is higher than the physiological norm and also indicates an inflammatory process with small values of segmented neutrophils 56.28±5.26. After two weeks of treatment, we can observe an improvement in the clinical parameters of the dogs' blood and even see a statistically significant difference between the experiment and the control in terms of band neutrophils (1.59±0.32 in the experiment and 2.25±0.34 in the control), eosinophils (5.86±1.72 and 8.32±1.62) and hemoglobin levels (168.54±8.14 and 137.60±9.34). We characterize this process as a pronounced inflammatory reaction, manifested by vivid clinical signs, as well as cellular dynamics, which is an important criterion for indicating the disease. Thus, when adding Farnesol to the treatment regimen for fungal otitis in dogs, small changes in the clinical composition of the blood are

recorded, characterized by an increase in the number of erythrocytes and hemoglobin, a decrease in the number of leukocytes, while the indicators of eosinophils and band neutrophils in the experiment decrease compared to the control.

THE DISCUSSION OF THE RESULTS.

The transition from commensal to pathogen is common in dogs in particular, and to a lesser extent in cats, so that cases of Malassezia otitis externa and Malassezia dermatitis are common in small animal veterinarians' practices. For example, the prevalence of otitis externa among dogs presenting to primary care is approximately 10% (Goyal et al. 2016; King et al. 2016), and up to 70% of such cases may be associated with *M. pachydermatis* (Zhang et al. 2019; Rios-Navarro et al. 2021). Treatment of chronic and recurrent MO can be a significant challenge. Long courses of local etiotropic therapy lasting several months may be required. It should be borne in mind that during treatment the pathogen may develop resistance to the antimycotic used. With deep changes in ear tissue, systemic use of antifungals and/or antibiotics may be required, depending on the results of a comprehensive microbiological study.

Drugs intended for the treatment of MO should be used in accordance with the following general rules (Bismarck et al. 2020; Rios-Navarro et al. 2021): before using the drugs, it is necessary to thoroughly clean the ear canal of ear secretions, which may reduce the effectiveness of the action. For ceruminous forms of otitis, it is recommended to use oil-based drugs, and for exudative forms - water-based. All drugs should be injected into the ear canal warm. After administering the drug, it is recommended to massage the base of the ear for 1-2 minutes. Despite adherence to all recommendations, current treatment of otitis externa, including antifungal, antibiotic and glucocorticoid drugs, faces problems due to drug resistance and non-compliance by pet owners with long-term therapy. Complete recovery after initial therapy is quite rare (25% of cases). Clinical improvement is observed in 30%, persistence of infection in 22%, relapse in 23% (Wagener et al. 2012; Zhang et al. 2019). This research study examines alternative treatments beyond traditional antifungal drugs and provides recommendations for their practical use (Fig. 24). These methods include the inclusion of herbal medicine in the treatment protocol. Implementation of these methods will optimize traditional treatment protocols, minimize drug resistance, and improve control of canine otitis externa caused by M.

pachydermatis, consistent with the One Health concept promoting judicious use of antimicrobials.

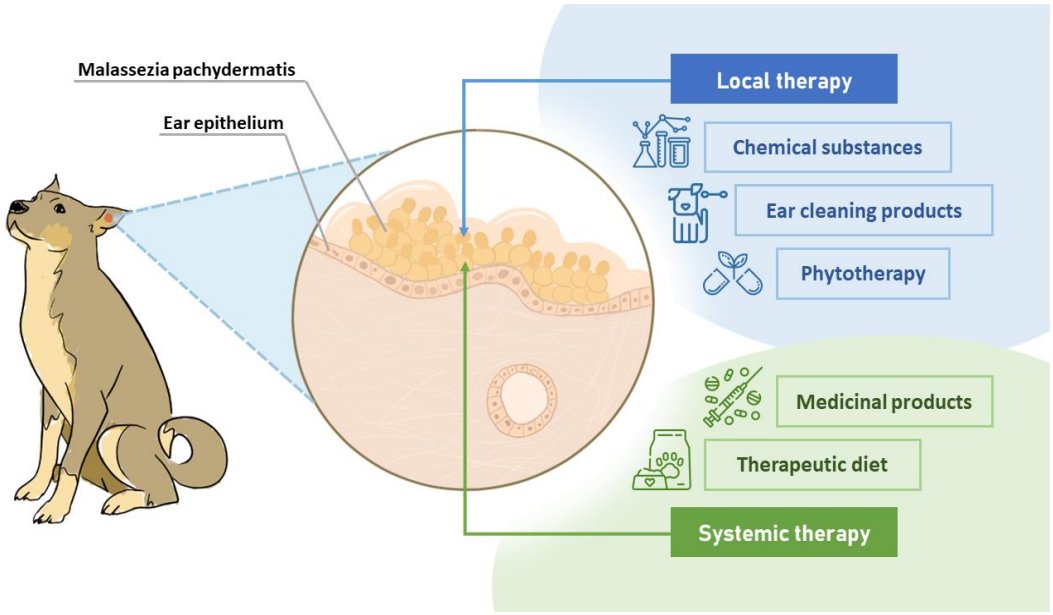


Figure 24. Methods used for the treatment of *Malassezia* otitis in modern veterinary medicine.

Essential oils and their phenolic components have been studied in several studies with promising results. Most studies have shown their antifungal activity (Bonin et al. 2020; Dutra et al. 2019; Matos et al. 2021). These compounds have antibacterial and anti-inflammatory properties that may help reduce clinical signs and can be used in mixed infections (Moulari et al. 2007; Nardoni et al. 2017; Sim et al. 2019a; Sim et al. 2019b; Puigdemont et al. 2021; Vercelli et al. 2021).

An in vitro study assessed the antifungal activity of 22 essential oils. The results showed that oregano, lemongrass, palmarosa and cinnamon oils were effective against MP (Bismarck et al. 2020). Another study found that Harungana madagascariensis leaf extract was active against MP, *Staphylococcus intermedius* and *Pseudomonas* species (Moulari et al. 2007).

The pomegranate extract and oral prednisone were as effective as conventional treatment and significantly reduced clinical signs *in vivo* (Puigdemont et al. 2021). Phenolic components such as carvacrol and thymol derived from oregano and thyme

oil have shown fungicidal activity and superior antimicrobial properties compared to their essential oils (Sim et al. 2019a).

Similarly, cinnamon essential oil and its phenolic component cinnamaldehyde have also shown promising antimicrobial potential against MP (Sim et al. 2019b). The essential oil combination was effective against clinical isolates of MP. Two of the five mixtures tested—one containing *Citrus paradisi, Salvia sclarea, Ocimun basilicum,* and *Rosmarinus officinalis,* and the other consisting of *Citrus limon, S. sclarea, R. officinalis,* and *Anthemis nobilis*—caused a reduction in clinical signs in animals (Henselm et al. 2009).

A commercial product containing essential oils from several plants was effective in vitro, improved clinical signs, and restored fungal homeostasis in the ears of the majority of dogs observed (Vercelli et al. 2021). Finally, three other commercial products based on oil of oregano, savory and thyme were studied separately and in mixtures. Savory oil alone performed better, presenting lower minimum inhibitory concentrations compared to the other two oils and the mixture of three. The combination of oils did not demonstrate superior antimicrobial activity compared to the other two isolated natural compounds. However, all compounds tested, including those that were combined, showed antifungal activity against isolates from dogs with otitis externa (Ebani et al. 2020).

To confirm the effectiveness of these alternative treatments and to incorporate them into clinical practice, we conducted microbiological studies, clinical trials, and comparative treatment studies for herbal medicine Farnesol. To substantiate the relevance of the chosen topic, we, first of all, monitored the spread of the incidence of MO among dogs and cats, and also selected microorganism strains for further research.

It has been established that MO disease does not depend on the age or breed of animals. The disease is most often characterized by: erythema of the auricle, cerumen, partial alopecia, unpleasant odor, scabs, pustules of the ear, lichenification of the auricle ets.

When analyzing the obtained microbiological data about species diversity, it was most often recorded that M in monoculture was detected in dogs in 15%, in cats - in 19%, association with 1 type of bacteria - 9% in dogs and 14% in cats; association with 2 types of bacteria -6 and 14%, respectively; association with 3 types of bacteria – 6 and 13%; association with 4 or more types of bacteria - 16 and 6%; association with 1 species of fungi – 6 and 4%; association with 1 species of fungi and 1 species of bacteria – 8 and 15%; association with 1 species of fungi and 2 species of bacteria - 14 and 7%; association with 1 species of fungi and 3 species of bacteria - 11 and 5%; association with 1 species of fungi and 4 or more species of bacteria – 9 and 3%. Also, we should note that associations of MP in dogs were most often with *staphylococci* and *streptococci*; and in cats, MP were most often inoculated with *enterobacteria* and *staphylococci*.

Analyzing the densitometric indicators, it was found that studied MP Cd23, Cd27, and Cd3 is strongest producer of biofilms. The resistance profile of three microorganisms, the most powerful biofilm producers, looked like this: strain MP Cd 23 was classified as R to AP, KT and FU, I to NS, CC, IT and VOR, S only to MIC. Strain MP Cd 27 was classified as R to VOR, I to AP, KT, and S to others. Strain MP Cd 3 was classified as R to FU, I to CC, VOR and MIC. Also, it was statistically proven that there is a relationship between biofilm optical density and sensitivity to drugs.

Interesting, in our opinion, is the analysis of the ability of MP strains to adhere on dog buccal epithelial cells. It was quite large for cultures from dogs (Average AI = 5.07 ± 0.72; K = 51.41 ± 5.39) and was less pronounced for strains isolated from cats (Average AI = 3.26 ± 0.51; K=48.76 ± 4.07). The MP Cd23 strain showed high adhesion index 8.28 ± 0.62 and an adhesion coefficient - 70.62 ± 4.91.

When conducting a study on phagocytosis, it was noted that after an hour of incubation, the PI and PN indicators of the 3 microorganisms did not differ significantly. However, during subsequent incubation (150 minutes), the PI and PN of MP Cd23 were significantly higher than those of the other two and amounted to 83.1 ± 2.7% and 9.4 ± 1.1, respectively. Moreover, when comparing the PI and PN

of three strains at different times, we can come to the conclusion that macrophages digested the main part of the microorganisms MP Cd3 and MP Cd27 at the end of the incubation period, while in MP Cd23 phagocytosis was still at the capture stage. It was found that the IDA values, which more clearly characterize the strain's resistance to phagocytosis, were reliably distinguishable among the selected isolates. The negative value of this indicator in MP Cd23 indicates that macrophages did not cope with their task, and BY could multiply and remain viable for a long time inside macrophages. Thus, MP Cd23 is a significantly more resistant microorganism to phagocytosis.

The rabbit model of otitis ear with MP Cd23 was successful and clinical signs of the disease were observed: loss of appetite or body weight, erythema, itching, the release of abundant ear secretions (cerumen) of a yellow-brown color, often with an unpleasant odor. Analysis of hematological parameters on the model of otitis media in rabbits showed that the total number of leukocytes (total WBC), granulocytes, neutrophils and platelets at first slightly in the first days of infection, and then significantly increased. Other indicators such as erythrocytes, hemoglobin, hematocrit, average volume of erythrocytes (MCH), average concentration of corpuscular hemoglobin (MCHC), lymphocytes and monocytes decreased during the month of infection to control values before inoculation ($P < 0.05$). Analysis of biochemical parameters of rabbit blood serum showed that concentrations of high-density lipoproteins, cholesterol and triglycerides increased significantly after experimental infection by MP, while blood sugar and plasma fibrinogen levels decreased after infection compared with control ($P < 0.05$). Indicators such as LD lipoproteins, urea, uric acid, creatine, calcium, serum globulin, albumin and total protein were without statistically significant changes. Liver function tests showed that all parameters such as Alanine aminotransferase, Alkaline phosphatase, Bilirubin, Aspartate transaminase and Lactate dehydrogenase have grown in rabbits after experimental infection by MP ($P < 0.05$).

The use of medicinal drugs (Surolan; Otifri; Otoxolan) + Farnesol in the animals of the experimental group reduced the signs of hyperemia, swelling, itching, the

amount of exudation on the 5-7th days of treatment, and complete clinical recovery of the animals occurred on the 20th day. When using only drugs in animals, on average, an improvement in the clinical condition occurred on 25 days, and final recovery followed after a full course of treatment - 30 days, and then when using Otifri once a day, redness of the ears persisted. Animals in the control group maintained clear clinical signs of the disease throughout the experiment. Their condition worsened and did not recover on their own, which proves the excellent effectiveness of the MO model we developed in rabbits.

Analyzing the microbiologocal indicators, we can say that both treatment regimens (with and without the addition of Far) turned out to be effective, but the regimen used in the experimental group Far + Surolan/Otifri/Otoxolan gave faster results. Normalization of hematological and biochemical parameters of rabbit blood was observed more quickly in the groups where Far was added.

Also, Far demonstrated *in vitro* good antibiofilm effects at a concentration of more 1.6 μM/mL (24% OD decrease of biofilm), and its highest antibofilm effects (71-55% - more than a half) were observed at a concentration of 200-12.5 μM/mL. The results of the experiment on changing the sensitivity of MP to antimicrobial drugs showed that Far increase such sensitivity. For example, Amphotericin B began to work with Far, even in small concentrations. Clotrimazole began to work with Far 200 μM/ml. There are also unique indicators: the sensitivity of MP to Nystatin, Voriconazole and Ketoconazole doubled with the addition of Far at a concentration of 25-200 μM/ml.

Summarizing the results of the research, it should be noted that we have achieved a solution to the current scientific problem of MO in veterinary medicine, and have improved methods for diagnosing and treating infectious otitis of a fungal nature. The introduction of Farnesol to therapeutic treatment regimens turned out to be pathogenetically justified, which is confirmed by the studies conducted.

CONCLUSIONS

The One Health approach recommends the judicious use of antimicrobials, given global concerns about antimicrobial resistance. This study included the herbal drug Farnesol for therapeutic use against otitis externa caused by M. pachydermatis. We have extensively searched for the most resistant and pathogenic strain among dogs and cats. Further research and testing of a laboratory rabbit model proved the effectiveness of integrating Farnesol into the clinical practice of a veterinarian for otitis media. For the first time, the correction of malassezia otitis media in a laboratory animal was theoretically and experimentally substantiated by administering farnesol once a day for 30 days against the background of antifungal agents. These alternative approaches have shown promise in reducing clinical symptoms and addressing the growing problem of drug resistance. In this search for alternative treatments, the goal is to improve the well-being of canine and feline patients and to refine and optimize traditional treatment protocols.

Results of the study

1. It was found that malossezia otitis does not depend on the age and breed of animals. When analyzing the obtained data on species diversity, M. pachydermatis was most often detected as a monoculture in dogs in 15%, in cats - in 19% of cases, in association with 1 bacterial species - in 9% of dogs and 14% of cats; in association with 2 bacterial species - in 6 and 14%, respectively; in association with 3 bacterial species - in 6 and 13%; in association with 4 or more bacterial species - in 16 and 6%; in association with 1 fungal species - in 6 and 4%; in association with 1 fungal species and 1 bacterial species - in 8 and 15%; in association with 1 fungal species and 2 bacterial species - in 14 and 7%; in association with 1 fungal species and 3 bacterial species - in 11 and 5%; in association with 1 type of fungi and 4 or more types of bacteria - in 9 and 3%. Associations of MP in dogs were most often with staphylococci and streptococci, and in cats - with enterobacteria and staphylococci.

2. Analyzing the densitometric indices of all isolated samples (n = 35), it was found that the MP isolates Cd23, Cd27 and Cd3 from dogs are the strongest producers of biofilms. The resistance profile of these three microorganisms was as follows: the MP strain Cd23 was resistant to 87.5% of antimycotics; Cd27 to 37.5%; Cd3 to 50%. The relationship between the ability to form biofilms and sensitivity to drugs was also statistically proven.

3. MP strains are able to adhere to the buccal epithelial cells of dogs. Adhesion was more productive for cultures from dogs and less pronounced for strains isolated from cats. The isolate MP Cd23 had the highest adhesion index of 8.28 ± 0.62 and the adhesion coefficient of 70.62 ± 4.91.

4. When conducting a study on phagocytosis, it was noted that the phagocytic number and the MP index of Cd23 were significantly higher than those of other strains and amounted to 83.1 ± 2.7% and 9.4 ± 1.1, respectively.

5. Malassezia otitis is effectively reproduced in vivo in rabbits with bright clinical signs of the disease. Biochemical studies of the blood serum of experimental animals showed an increase in liver enzymes already on the 5th day of the study: ALT increased by 8.37%, AST - by 34.67%. When assessing the results of the hematological study, it is possible to note the presence of an inflammatory process in the body of infected rabbits. Thus, on the 5th day of the experiment, the number of leukocytes was 4.67% higher than in the control group, and on the 30th day - already 32.27%. 6. The use of drugs (Surolan; Otifri; Otoxolan) + Farnesol in animals of the experimental group reduced the signs of hyperemia, edema, itching, the amount of exudate on the 5-7th day of treatment, and complete clinical recovery of animals occurred on the 20th day. When using only drugs in animals, an average improvement in the clinical condition occurred on the 25th day, and final recovery occurred after a full course of treatment - 30 days, and then when using Otifri once a day, redness of the ears persisted. In animals of the control group, clear clinical signs of the disease persisted throughout the experiment. Their condition worsened and did not recover on its own, which proves the excellent efficiency of the developed model of MO in rabbits. The analysis showed that the microbiological

parameters of both treatment regimens (with and without the addition of Farnesol) were effective, but the regimen used in the experimental group Far + Surolan/Otifri/Otoxolan gave faster results. When carrying out therapeutic measures in the first 10 days of the experiment, there were no statistical differences in the biochemical analyzes of the rabbits' blood, and by the 30th day of the experiment, some biochemical parameters returned to normal values \u200b\u200bbefore the experimental infection, especially in the groups of animals with the addition of Farnesol. Thus, high-density lipoproteins on the 30th day of treatment in the groups Surolan + Far, Otifri + Far and Otoxolan + Far were 13.11%, 16.34% and 13.34% lower, respectively, than in the control group. Similar changes are observed in the results of the hematological analysis. 7. Farnesol demonstrated good antibiofilm effects in vitro at concentrations above 1.6 μM/ml (24% reduction in biofilm OD), and its highest antibiofilm effects (71-55% - more than twice) were observed at concentrations of 200-12.5 μM/ml.

8. The results of the experiment on changing the sensitivity of MR to antimicrobial drugs showed that Farnesol increases such susceptibility. For example, Amphotericin B began to work with Farnesol even in low concentrations. The culture again became sensitive to Clotrimazole with Far 200 μM/ml. There are also unique indicators: the sensitivity of MR to Nystatin, Voriconazole and Ketoconazole doubled with the addition of Farnesol at a concentration of 25-200 μM/ml.

PRACTICAL PROPOSALS AND PROSPECTS FOR FURTHER DEVELOPMENT OF THE TOPIC:

1. Conduct diagnostics of isolated microorganisms from the ears of animals with malasseziosis for sensitivity to antibacterial drugs, since this study tested sensitivity only to antifungal agents in the main pathogen. Study of the accompanying flora will help to select the optimal therapeutic dose of Farnesol.

2. Therapy for Malassezia otitis should include local Farnesol at a concentration of ≥ 200 μM/ml once a day for 10 days. It is necessary to develop a convenient aerosol form of the drug that combines Farnesol + antibiotic + antimycotic.

3. Conduct clinical trials of MO therapy with Farnesol on dogs and cats. This study also creates prerequisites for studying Farnesol as the main drug for other mycoses in various animals.

4. The results can be used in scientific research, in the educational process when preparing students in the specialty "Veterinary Science", improving qualifications in diagnostics and therapy of animals with skin pathologies, otitis, as well as in the preparation of educational and reference manuals on veterinary medicine.

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