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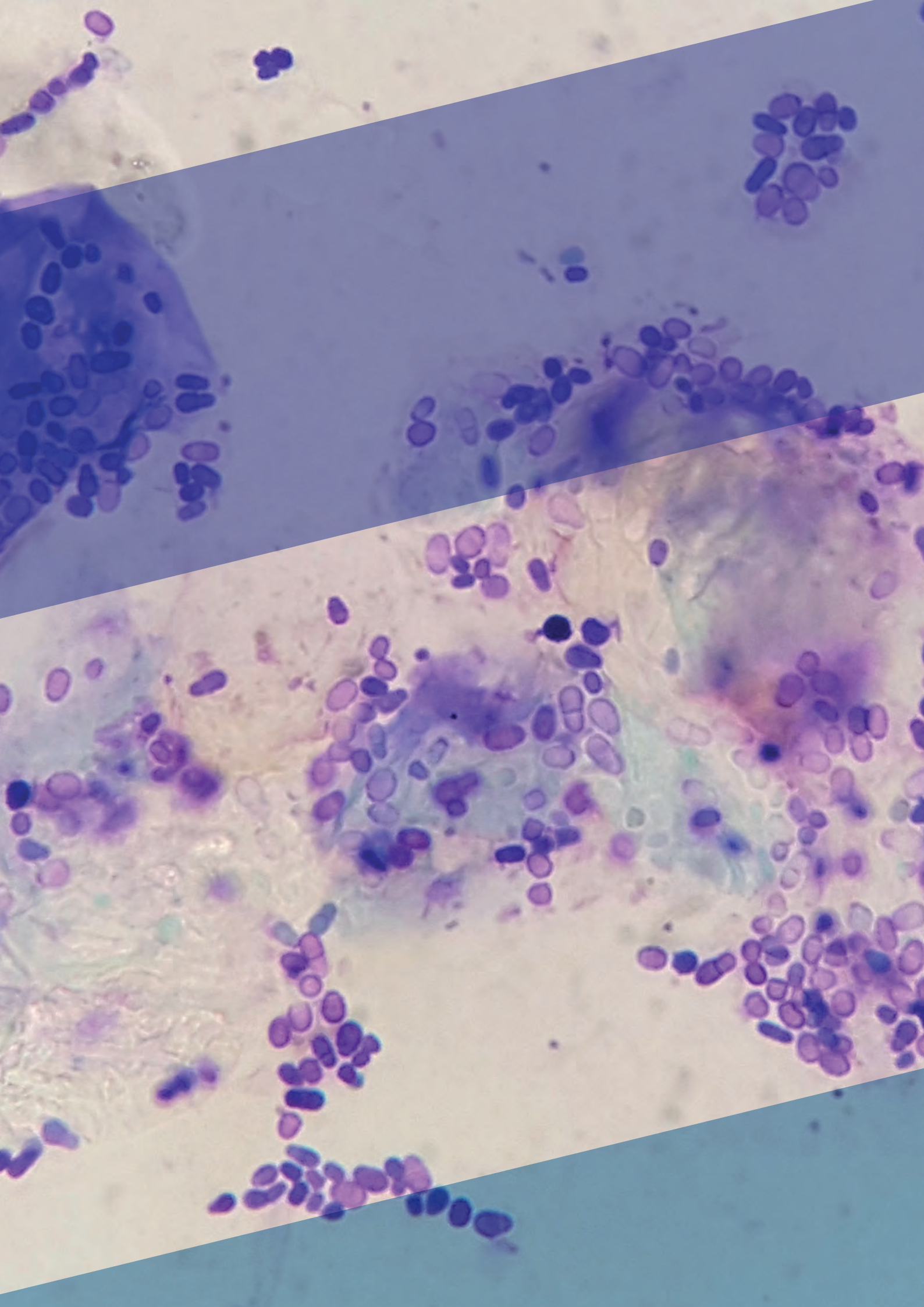

Biofilm: microorganisms working together in cooperation

Dr. Chiara Noli, DVM, Dip ECVD

In the world of microorganisms, survival is often the result of cooperation and interaction. Biofilms, complex structures made up of colonies of microorganisms, are a perfect example of this kind of teamwork. A biofilm is a community of microorganisms, mainly bacteria, but also yeasts, that grow on a surface and surround themselves with an autonomously produced mucus-like material composed mainly of polysaccharides, proteins and DNA. This stratified structure allows microorganisms to adhere to a range of surfaces - both inert, such as glass or steel, and organic, such as the living cells of mucous membranes or the skin. The biofilm serves as a sort of shield protecting the microorganisms which hosts, offering effective defence against adverse environmental conditions and antimicrobial agents, including disinfectants and both systemic and topical antibiotics.
However, in many environments, biofilms play a crucial ecological role. For example, they are responsible for the biodegradation of many pollutants in aquatic ecosystems, such as in the treatment of wastewater.

How is a biofilm formed? (Figure 1)
Biofilms form when microorganisms in planktonic phase (single free-floating cells) perceive that they have reached a high level of density (*quorum sensing*). In these situations, intercellular chemical communication mechanisms are activated that lead to a change in behaviour. The first stage of biofilm formation is the attachment of the planktonic bacteria to a substrate, initially via weak and reversible bonds, and then via stronger adhesive structures, such as pili. These first cells provide anchoring sites for other microorganisms, including those of different species, and begin producing a water-rich mucinous matrix that may represent between 50 and 90% of mass. This protects the members of the colony from attacks from external agents (drying, bacteriophage viruses, natural or synthetic antimicrobial agents, antibodies or phagocytosis by cells from the immune system) and favours multiplication and intercellular communication. Some microorganisms may occasionally detach from the biofilm to be released into the environment and find other niches to colonise.

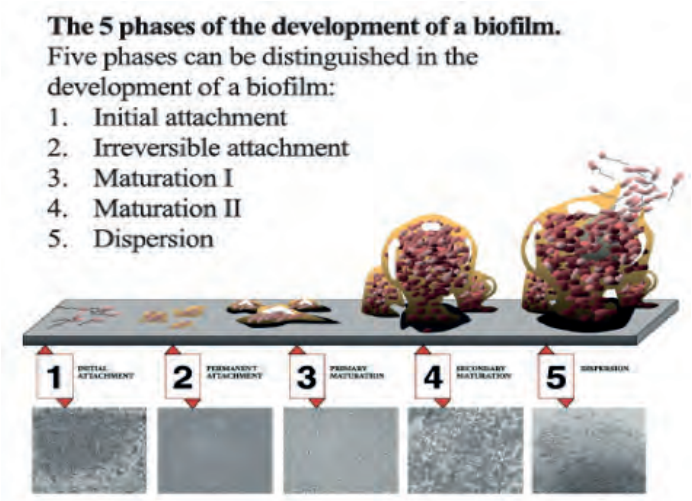

Figure 1. *The various stages of biofilm formation, from attachment of the first planktonic bacteria to dispersion from mature colonies.*

The role of biofilms in medicine
In medicine, biofilms are found in a range of pathological situations and can pose a significant challenge, as their unique structure presents considerable resistance to antibiotics. They are often responsible for chronic and persistent infections, such as sinusitis, tonsillitis, endocarditis, pneumonia, osteomyelitis, dental plaque, urinary infections, wounds and otitis.

Biofilm in cases of otitis
In the veterinary field, one of the most common problems found in dogs is otitis, a multifactorial inflammation of the ear, caused by bacterial or fungal infections, allergies, parasites or anatomical problems. It was recently revealed that biofilms play an important role in ear infections in dogs, and it was demonstrated than many common bacteria and yeasts responsible for otitis, such as *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus pseudintermedius, S. aureus*, as well as *Malassezia pachydermatis*, are capable of forming biofilms. In a study on strains of *Pseudomonas aeruginosa* isolated from cases of canine otitis, 40% were able to produce biofilms [10].

These complex aggregates of microorganisms can complicate management of otitis, representing a significant obstacle to treatment, as they render the infections more resistant to therapy and favour relapses. Antibiotics struggle to penetrate the biofilm matrix and to kill the bacteria inside, so much so that in biofilm conditions, bacteria require concentrations of antibiotics of between 2 and 4000 times higher than bacteria in planktonic form (not in biofilm) in order to be inhibited [5-7]. Many antibiotics mainly act on the replication phase of the microorganisms, but the majority of bacteria in biofilms are in a dormant state, and only a limited quantity of antimicrobial substances actually are able to penetrate, favouring the development of bacterial resistance and predisposing to a relapse of the infection with resistant strains.

How to identify a biofilm in cases of otitis

The presence of biofilm is to be suspected in cases of otitis that resists standard therapy and/or is recurrent, above all if bacterial cultures and repeated antibiograms continuously show the same microorganism with the same sensitivity profile. A dark mucinous exudate is also an indication of the presence of biofilm (Figure 2).

Figure 2. *Dark mucinous exudate from the ear, an indication of the presence of biofilm (photo Dr. Giovanni Ghibaudo)*

A cytological exam of this exudate often confirms this theory. Samples are obtained with a cotton swab from the depths of the vertical ear canal. The cotton swab is then delicately rotated on the surface of the glass slide in order to deposit the material, avoiding smearing, as this may lead to signs of cellular breakage (striation of the nucleus).

Once coloured, biofilm appears as background strips of mucin with bacteria and/or yeasts, mostly in clumps. It is stressed that *Malassezia* is also often capable of producing a biofilm, and therefore the presence of this yeast in a cytological sample should not be taken as a reason to exclude the presence of a biofilm. (Figure 3)

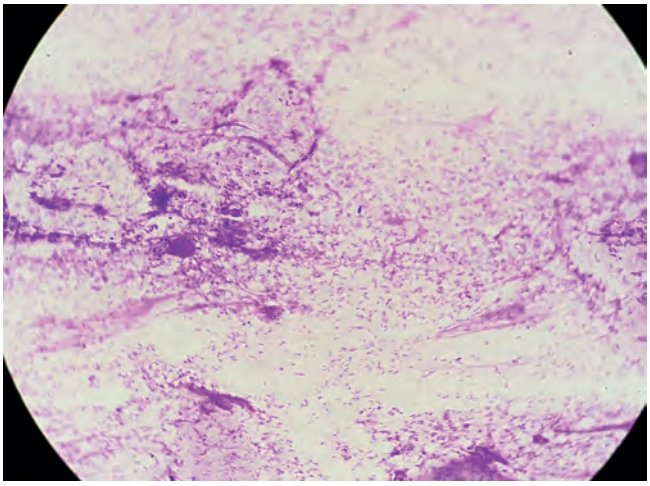

Figure 3. *Numerous bacteria and strips of mucin in a sample of ear exudate from a dog with otitis and presenting biofilm.*

Management and treatment of biofilm in the ear

It has recently been reported that the formation of biofilm in the ear is often underestimated and undetected [8], when instead it should be a suspect in all cases of otitis characterised by the presence of microorganisms.

A multimodal approach is required in the management of otitis with the presence of biofilm. First of all, it is fundamental that the mucinous patina is removed mechanically through what is known as "biofilm disruptors", before intervening with disinfectants and/or antibiotics. Disruptors of biofilm break down its structure, rendering the microorganisms more susceptible to antibiotics. The most well-known of these are N-acetylcysteine (NAC) and tris-EDTA, both of which are safe for intra-auricular therapy. Interestingly, both substances have been described as having intrinsic antimicrobial properties, combating both bacteria and *Malassezia* [1].

NAC is commonly used to dissolve mucus in cases of bronchitis, and a number of studies have also demonstrated its effectiveness in dissolving biofilms and favouring the penetration of antibiotics [2]. Furthermore, NAC has also been shown to work in synergy with some beta-lactam and quinolone antibiotics [3,11].

Tris-EDTA is capable of favouring the penetration of antibiotics into the walls of Gram-negative bacteria, such as *Pseudomonas spp.*[4,12], even when they are organised in biofilms [9], and strengthening the effect of the same.

In the most serious cases, Tris-EDTA and NAC need to be left *in situ* for at least 5 minutes, with repeated washing and aspiration cycles, preferably under general anaesthesia and with tracheal intubation, until the mass of biofilm and exudate has been completely removed from the ear canal (Figure 4).

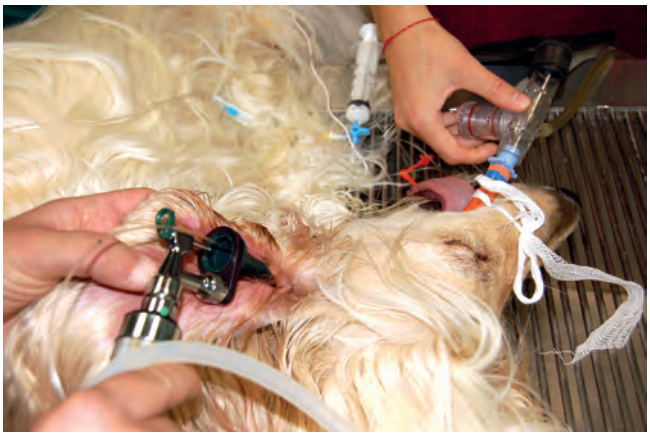

Figure 4. *Washing liquid is instilled and re-aspirated from the ear canal multiple times until the canal appears fully clean. At this point, antibiotic/antifungal drops with steroids can be instilled*

Afterwards, washing with disinfectants and drying products, usually based on chlorexidine, can be carried out before instilling antibiotic and/or antifungal drops, always accompanied by a topical steroid. This procedure is then to be repeated at home by the owner on a daily basis until no more biofilm is observed.
In less serious cases, following appropriate cleaning, Tris-EDTA and NAC can be applied into the ear canal with the animal awake, massaging the base of the ear, with a contact time of 5 minutes, after which time excess liquid is removed with a sterile gauze. After a wait of at least 30 minutes, antibiotic and/or antifungal drops are applied, as always accompanied by a topical steroid. In-vitro study has demonstrated that the presence of Tris-EDTA and NAC compromises neither the effectiveness nor the stability of topical steroids [13].

Cutaneous biofilm

It may be a less well-known fact that biofilms can also form in other areas of the body as well as the ears, for example in folds of skin. These include interdigital spaces, snout folds of brachycephalic dogs, the vulvar fold, the labial fold, the underside of the neck of dogs with abundant skin folds and salivation (Cockers, Bordeaux mastiffs), and the umbilical, subcaudal or pericaudal folds on dogs with screw tails.

The colonisation of these areas by bacteria and/or yeasts is pathological, and only occurs in the presence of predisposing factors: for example, allergic conditions in interdigital spaces with consequential lapping and an increase in humidity; in snout folds in case of excessive lacrimation; in the vulvar fold in case of urinary incontinence, and so on. There is no doubt that anatomical "folds" in these areas of the body do not allow for the proper aeration of the skin, favouring an increase in humidity, which may be worsened through licking or the pooling of urine, saliva or faeces.
Clinical appearance in these cases is represented by the presence of a creamy matter in the fold in question (Figures 5 a, b, c), which under cytological examination demonstrates an abundant and uniform population of microorganisms, but in the absence of cellular inflammation (a demonstration that the matter is therefore not pus) (Figure 6).

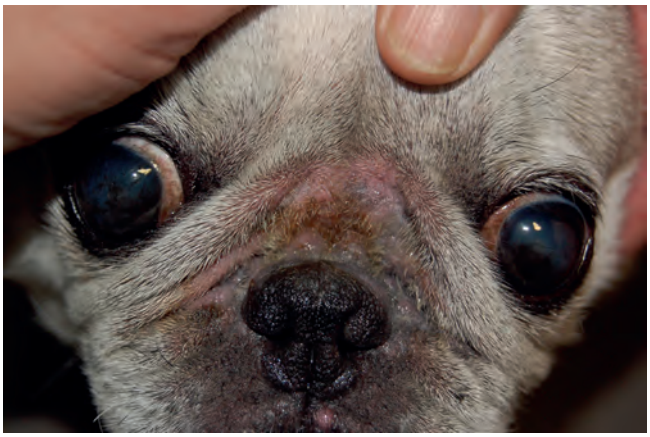

Figure 5a. *Presence of wet, mucinous and foul-smelling matter in the nasal fold of a brachycephalic dog*

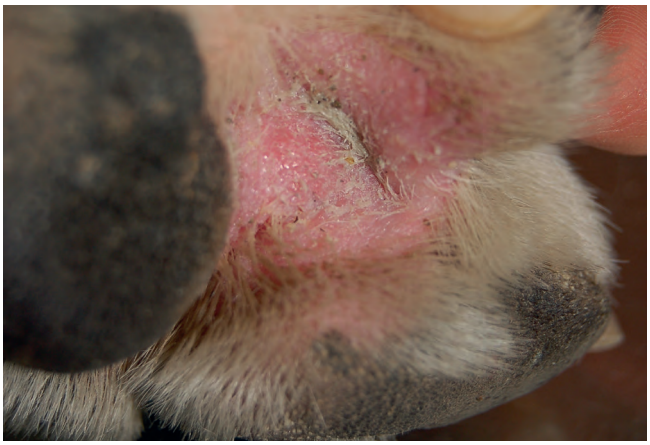

Figure 5b. *Presence of creamy matter in an interdigital space of a dog with atopic dermatitis.*

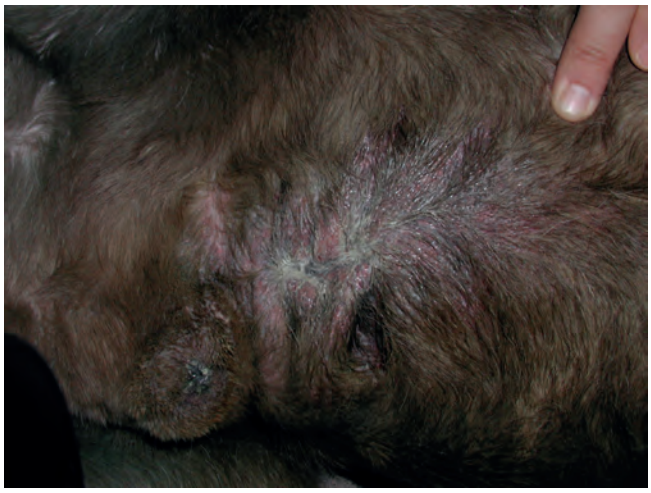

Figure 5c. *Foul-smelling matter in the umbilical fold of a molosser.*

These may be bacteria, but it should be remembered that *Malassezia* is also capable of forming a biofilm (Figure 6), which is often difficult to eradicate with common treatments.

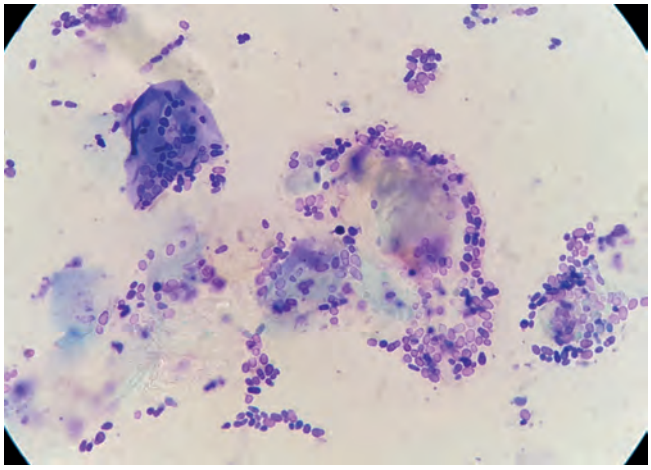

Figure 6. *Cytological sample showing the exclusive presence of a large quantity of Malassezia yeasts attached to the corneocytes on the surface of the skin.*

Often, topical treatment with antibiotic creams, or disinfectant gels or sprays, may not achieve the desired results, due to the presence of a biofilm. As with the ears, in this case treatment is based on the disaggregation of the biofilm with Tris-EDTA and NAC solutions, followed by the disinfecting of the area and the application of an antimicrobial cream or gel to inhibit the formation of new biofilm. It is, however, also essential to identify and resolve the predisposing factors (anatomical conformation with particularly accentuated folds, frequent bathing, etc.) or primary factors (underlying allergies, urinary incontinence, diarrhoea, etc.) in order to be able to definitively resolve the problem.

Conclusion

Otitis and dermatitis in folds are common conditions in dogs, and the presence of biofilm can render these problems difficult to manage. Recognising the significance of biofilms in ear infections and in intertrigo in dogs is a fundamental step in developing more effective treatment strategies, such as the use of disaggregating agents containing Tris-EDTA and NAC.

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