Short communication

Kali carbonicum 6cH controls *Malassezia pachydermatis* growth *in vitro*

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Abstract

External otitis caused by *Malassezia pachydermatis* is a common disease in the veterinary practice routine, whose treatment has been challenging due to the risk of antimicrobial resistance. Thus, Homeopathy is seen as a possible alternative to control the infection. To screen potential medicines to be used in a non-individualized clinical homeopathy approach, a series of *in vitro* assays were performed, in which a suspension of *M. pachydermatis* colonies in 0.5 McFarland scale was diluted 1:1000 in sterile saline solution, and 10 µL of pre-selected homeopathic medicines was added. *Sulphur* 6cH, *Dolichos pruriens* 6cH, and *Kali carbonicum* 6cH were chosen from a pilot study, and the last potency was prepared in pure, sterile water. The controls were pure, sterile water and succussed pure, sterile water. An amount of 50 µL of each suspension has been sown in plates containing Sabouraud dextrose agar medium, with or without 1% Tween 80, for counting of colony-forming units (CFU) and cytomorphological analysis of *M. pachydermatis* samples taken from the same colonies. The analysis was made using Image J 1.53 by manually counting quiescent and germinative forms. *Kali carbonicum* 6cH treatment decreased the germinative/quiescent ratio in relation to the controls, independent of Tween 80 presence in the medium. The UFC counting, however, did not show statistically significant differences among groups, probably due to the lack of cytotoxicity against the yeasts. In conclusion, *Kali carbonicum* 6cH was considered a promising medicine to be tested in clinical conditions, probably due to its capacity to control yeast growth without creating a selective pressure able to induce resistance mechanisms.

Keywords: *Malassezia pachydermatis,* Homeopathy, external otitis, antimicrobial resistance.

Introduction

Canine external otitis is often diagnostic in the small animals' clinical routine, being the yeast *Malassezia pachydermatis* one of the leading agents involved due to the relation with its natural microenvironment and the immune status of the patient (Angileri *et al.*, 2019). In the auditive channel, there is a bacterial and fungal microbiome composed mainly of *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, *Pseudomonas* spp., *Malassezia pachydermatis,* and *Candida albicans* (Petrov *et al*., 2013).

Malassezia pachydermatis is commonly associated with dermatitis and otitis when external influences change the natural microbiome in which it is inserted (Peano *et al*., 2020). The indiscriminate use of anti-microbials to treat infections caused by *Malassezia pachydermatis* predisposes microbial resistance (Angileri *et al*., 2019; Park *et al*., 2020). Thus, the need to search for new possibilities and strategies for treatment leads to the study of non-conventional therapies, whose effectiveness still needs to be demonstrated. This is the case of homeopathy.

Homeopathic treatment has been associated with reliable effectiveness against otitis media in children (Sinha *et al*., 2012). Still, in dogs and cats, the effectiveness of homeopathy is better reported in external otitis (Mathie *et al*., 2010). Recently, the therapeutic effects of homeopathy have been seen as a specific example of hormesis, an intrinsic process present in all living beings that allows the

establishment of adaptative processes to stressors. It is considered one of the "hallmarks of health" (Calabrese, Giordano, 2021; Lopez-Otin, Kroemer, 2021; Ullman, 2021).

This study proposed screening of homeopathic medicines able to inhibit the growth of *Malassezia pachydermatis in vitro* to be further tested in clinical studies.

Materials e Methods

Pilot test: A preliminary examination was made using the disk diffusion test in the Sabouraud agar medium, inoculated with a suspension of *M. pachydermatis* CBS-1696 in the 0.5 scales McFarland, being a physiologic solution as the negative control. Plates were incubated for 72 hours at 36ºC. The medicines used in this step were chosen from the Materia medica according to the main symptoms of this kind of infection (intense pruritus, hyperkeratosis, hyperpigmentation, fissures of the epidermis, and restlessness). They were *Psorinum* 8cH, and *Sulphur*, *Calcarea carbonica*, *Echinacea angustifolia*, *Dolichos pruriens*, *Graphitis*, *Mercurius solubilis*, *Kali carbonicum*, and the isotherapic made from *M. pachydermatis* CBS-1696, all prepared as 6cH.

Medicines preparation: All medicines were prepared one day before, using sterile purified Type 1 water (18.2 MW.cm at 25ºC) obtained from a Direct-Q3 purification system (SmartPark Direct Q3) with Biopak filters (Millipore, Darmstadt, Germany) and manipulated in a laminar flow cabinet. The stock potencies were prepared in a pharmacy registered at the Brazilian Sanitary Surveillance Agency – ANVISA (BRASIL, 2011) in two previous potencies (4cH or 6cH, depending on the medicine), using 10% ethanol as a vehicle, in such a way that the working potency would have 0.001% ethanol to avoid the direct chemical effect of the ethanol on yeasts. The last working potency was prepared in the research laboratory and succussed 100 times vertically at the robotic arm Denise™ (AUTIC, São Paulo, Brazil).

Before being used, the working potencies were filtered in a 0.22 micrometers mesh filter (Millipore, Darmstadt, Germany), and the flasks were coded by a person not involved in any part of the experiment, allowing a blind procedure. Codes were broken only after the statistical analysis.

Main test: Yeasts were suspended in sterile physiologic solution at 0.5 McFarland scale and diluted 1:1000, adding the selected medicines from the pilot study (*Dolichos pruriens* 6cH, *Kali carbonicum* 6cH, and *Sulphur* 6cH) or the controls (*Ethylicum* 2cH, pure water 1cH, and non-succussed pure water) at 1%. Then, 50µL of each treated colony was poured on the Sabouraud agar medium in a Petri dish and spread using a Drigalski strap. The plates were prepared in triplicate in two series, one with medium containing 1% Tween 80 and the second with medium without Tween 80. The presence of Tween 80 is an essential variable in improving the growth of yeasts, making the effects more evident. The plates were incubated for 72 hours at 36ºC. Then, the number of colony-forming units (CFUs) was counted.

Additionally, each sample was harvested from the cultures with a strap and used to prepare a smear on 200µL of physiological solution poured on a glass slide. The smear was dried at room temperature and fixed with methanol P.A. Each smear was stained with Gram´s method and mounted between the slide and a coverslip, using a conventional resin for histological procedures.

The yeast morphology was evaluated considering two patterns: quiescent and germinative ones. A manual counting process was made over the total smear area using the software Image J 1.53.

Statistical analysis: The statistical analysis was made using the software Prism 8.0. The Shapiro-Wilk test tested the normality, and one-way ANOVA, followed by Tukey, evaluated the significance.

To evaluate quiescent and germinative forms of *Malassezia pachydermatis*, the X^2 test was used with Yates correction. In all cases, the significance level was determined as $p \leq 0.05$.

Ethics: It is an *in vitro* study; no Ethical committee approval was required.

Results

The analysis of CFUs showed high variance among groups, independent of Tween 80 insertion in the medium. So, no statistically significant difference was observed (Figure 1).

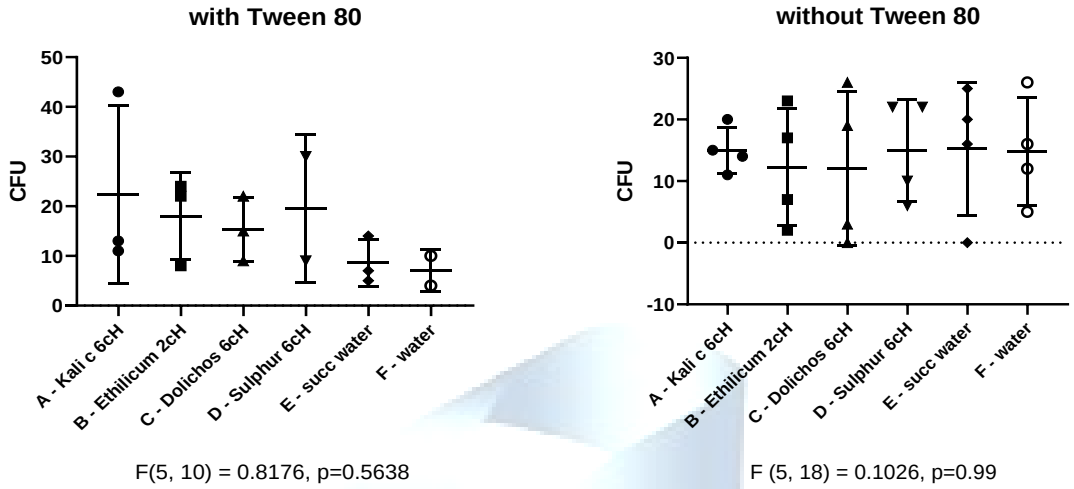
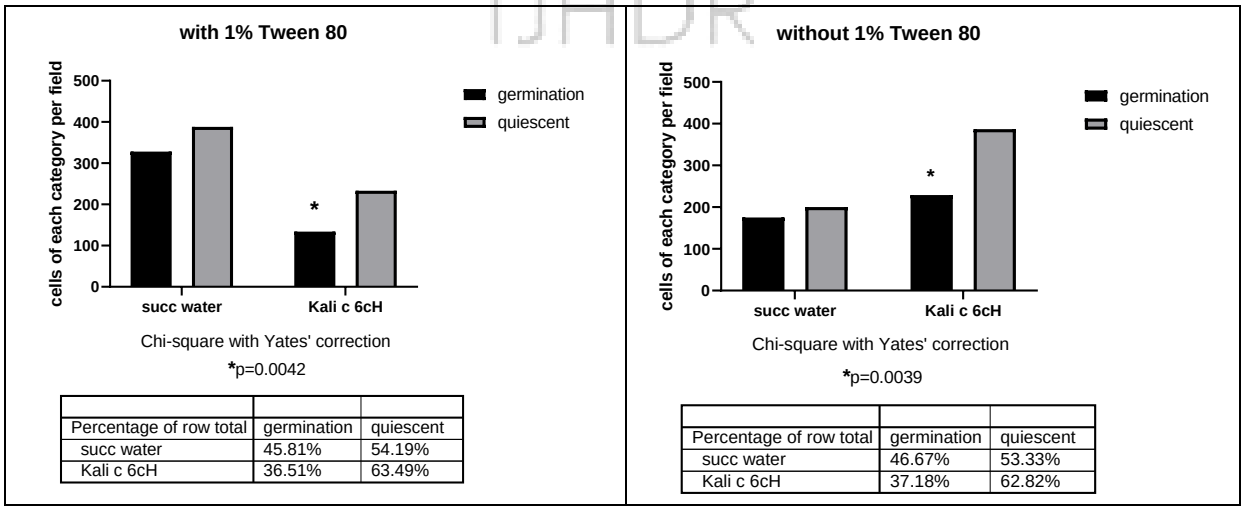


Figure 1. CFU counting *Malassezia pachydermatis* cultures on Sabouraud agar medium with or without 1% Tween 80, after the treatment with homeopathic medicines and controls (*Ethilicum* 2cH, succussed water and water). One-way ANOVA followed by Tukey test. The statistical parameters are indicated at the bottom of the graphics. No statistical significance was identified among the groups. Treatments are shown according to the code (A – F).



Percentage of row total	germination	quiescent
succ water	45.81%	54.19%
Kali c 6cH	36.51%	63.49%

Percentage of row total	germination	quiescent
succ water	46.67%	53.33%
Kali c 6cH	37.18%	62.82%

Figure 2. (A) Frequency of quiescent and germinative forms of *Malassezia pachydermatis* treated with *Kali carbonicum* 6cH or succussed water; (A) in a medium containing 1% Tween 80, (B) in a medium containing no Tween 80. X^2 with Yates correction, (A) p=0.0042; (B) p=0.0039.

On the other hand, the cytomorphological analysis showed that the treatment of yeasts with *Kali carbonicum* 6cH produced inhibition of 69 and 74% in the germination ratio, without and with 1% Tween 80 respectively, being these differences statistically significant in relation to the control - succussed water (Figure 2). The other medicines presented about 40% of germination ratio reduction regarding the same control (Table 1).

Table 1. The germination / quiescent ratio of *Malassezia pachydermatis* was analyzed by differential counting from smears stained by Gram´s method under different treatments *in vitro*. Samples harvested from medium prepared with or without 1% Tween 80.

Samples WITH 1% Tween 80		**Germination**	**Quiescent**	**Germination / quiescent ratio**
A	***Kali carbonicum* 6cH**	134	233	73.88%
B	**Ethanol 10% 2cH**	240	259	7.92%
C	***Dolichos pruriens* 6cH**	277	301	8.66%
D	***Sulphur* 6cH**	344	442	28.49%
E	**Succussed water**	328	388	18.29%
F	**Water**	687	951	38.43%

Samples WITHOUT 1% Tween 80		**Germination**	**Quiescent**	**Germination / quiescent ratio**
A	***Kali carbonicum* 6cH**	229	387	69.00%
B	**Ethanol 10% 2cH**	336	415	23.51%
C	***Dolichos pruriens* 6cH**	343	422	23.03%
D	***Sulphur* 6cH**	2077	2482	19.50%
E	**Succussed water**	175	200	14.29%
F	**Water**	1793	1898	5.86%

Discussion

The resistance of fungi to conventional antimicrobial medicines has been a serious concern for public health (Angileri *et al*., 2019; Kim *et al*., 2018; Park *et al*., 2020), and homeopathy has been seen as a possible resource for small animal practice (Ahmad *et al.*, 2018; Nagai *et al*., 2019). This study aimed to contribute to this field by screening homeopathic medicines using an *in vitro* model on *Malassezia pachydermatis* single culture.

The medicines used in the study were already described as therapeutic possibilities for *M. pachydermatis* otitis (Delavechia *et al.*, 2011; Figueiredo, 2016), but this is the first attempt to

demonstrate its effect directly on the microorganisms, with no participation of the host. This approach opens the possibility of using homeopathy as a topic treatment, to be used alone or in association with other systemic therapies (homeopathic or not). This approach must be evaluated in clinical studies in the future.

In vitro studies using homeopathic products have been described in the literature, for example, in the control of resistant bacterial growth when specific homeopathic formulations, such as nosode or Belladonna 30cH, and antibiotics are used together, resulting in the recovery of bacterial sensitivity to the antimicrobial agent (Passeti *et al.*, 2017). Homeopathy has helped mitigate the harmful effects of Agricole defensives in agriculture while controlling plant infections (Damin, 2013; Rissato *et al.,* 2016; Costa, 2019).

Regarding *Malassezia Pachydermatis* infection, the primary therapeutic resource has been Itraconazole (Sfaciotte *et al*., 2015); however, resistance to this agent has been reported (Borman *et al*., 2019). Being homeopathy a therapeutic tool able to work by different mechanisms (regarding those known for classical pharmacology), the possibility of controlling microorganisms' growth with less selective pressure has been questioned (Bonamin, 2019). The present study corroborates this perspective, although homeopathic medicines' exact mechanisms of action are not yet elucidated (Waisse, 2017).

The results presented herein indicate that the best method to identify the effects of homeopathic medicines on yeast growth was the morphological analysis of the microorganisms using the establishment of germination rate (Lázaro *et al*., 2008), independent of the medium condition (with or without 1% Tween 80). The evaluation of colony growth was not sensitive enough, although it is a standard protocol to study the effect of classical allopathic products. The main reason for this discrepancy might be the way of action. Homeopathy cannot kill microorganisms in the sense of having an antimicrobial effect. Instead, it can control the growth rate of individuals in such a manner that could facilitate the adaptation between host and parasite to a nonharmful level, as seen in previous results with other kinds of microorganisms *in vivo* and *in vitro* (Bonamin *et al*., 2013; Coelho *et al*., 2017; Santana *et al*., 2014; 2017; Nagai *et al*., 2019).

This original contribution, presented here as a short communication, allows readers to open a discussion on how *in vitro* simple tests can be helpful as a first step to guide future clinical studies on the various possibilities of homeopathy use in small animal practice.

Conclusion

The treatment *in vitro* of *Malassezia pachydermatis* with *Kali carbonicum* 6cH effectively controlled its growth, independent of the medium condition (with or without 1% Tween 80), whose conditions can favor yeast growth.

More studies *in vitro* are needed to improve the understanding of microorganisms' response to homeopathic stimuli so that better therapeutic protocols can be proposed.

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