TRIAL#96: IN VITRO TEST ON THE EFFECT OF THE TYPIFIED LACTIC YEAST (KLUYVEROMYCES MARXIANUS B0399) ON THE DEVELOPMENT OF CANDIDA ALBICANS ATCC10231.

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1.INTRODUCTION

Probiotic organisms are defined by the guidelines of the FAO/WHO (Cordona, Argentina 2001) as living microorganisms which give beneficial effects when taken in an adequate quantity.

Different studies highlight the efficacy of probiotics:
- in the modulation of the immune system
- in the prevention and in the treatment of intestinal dismicrobisms that can provoke diarrhea or syndromes caused by degenerations of inflammatory reactions (e.g. Crohn's disease, irritable bowel syndrome) (Castagliuolo M.S. et al., Gorbach S.L. 2000),
- in the reduction of the development of allergic phenomena such as asthma and eczema in children (Benn C. et al. 2002) if used mothers during pregnancy,
- in the reduction of the risk of infection in the genitourinary tract (Senok A.C. et al. 2005, Reid G. et al. 2003, Reid G. et al. 2004, Reid G. 2001).

The proposed mechanisms of action connected to these effects are quite varied: competition for space and nutrients, activation of the immune system of the host and production of antimicrobic catabolites (short-chained fatty acids and, in particular, lactic acid, hydrogen peroxide…).
It was also demonstrated that many yeasts show a significant partial or total killer-type activity against pathogenic fungi of clinical importance (Sugisaki Y. et al. 1983, Walzer G.M. et al. 1995,Cerikcioglu N. 2003). This activity was attributed to the production of protein-type toxins.

In particular, a possible antimicotic action of Kluyveromyces marxianus B0399 was noted by MIclavez et all. (2006, personal communication) that seem to demonstrate a positive in vivo effect of K. marxianus B0399 on patients suffering from candidiasis. This homofermentative yeast, commonly found in fermented diary products, is utilized as a probiotic in persons affected by intestinal problems deriving from diysbiosis (meteorism, constipation alternating with diarrhea, difficulty in assimilation, etc.) and/or lactose intolerance, taken that this yeast produces the enzyme β galactosidase.

2.MATHERIAL AND METHODS

2.1. Yeast strains and culture media

K. marxianus B0399 was produced and provided by Turval laboratories Ltd. *C. albicans* ATCC 1023 was acquired from Oxoid.
K. marxianus B0399 was maintained on MV1 medium (lactose 2%, hydrolized casein 1%, yeast extract 0,1%, peptone 0,5%, solidified with agar 1.5% when required) while *C. albicans* ATCC was maintained on YEPD (glucose 2%, yeast extract 1%, peptone 2% solidified with agar 1.8% when required).
All strains were maintained at 4 °C and cultivated at 37°C unless otherwise stated.
Agar overlay assays were performed on Chromalbicans agar (Biolife) at pH 6.2.

2.2. Agar overlay assays

"Chromalbicans" agar ("Biolife")enabled phenotypic distinction of the two yeast species. *C. albicans* ATCC 10231 colonies appeared blue, while *K. marxianus* B0399 colonies appeared milky-white. Cell suspensions were inoculated by the spread-plate technique. These procedures and general culturing conditions are the same in all tests unless otherwise specified.
Concentrations of yeast inocula (CFU/mL) were determined by using a Petroff-Hausser counting chamber. All tests were done in triplicate.

TEST A
Test A was designed to assess the inhibition of existing biofilms of *C. albicans* by a *K. marxianus*, probiotic as might occur during an active infection. An overnight culture of *C. albicans* ATCC 10231 grown in YEPD was resuspended in a 0.85% physiological solution (PBS) at a concentration of 3 x 10^5 CFU/mL and 100 µL of the suspension (3 x 10^4 CFU) was spread onto Chromalbicans agar plates. Plates were incubated at 30° C for 12 hours. At the end of the 12-hour incubation, 10 µL of the overnight culture of *K. marxianus* B0399 broth (3 x 10^6 CFU/mL; 3 x 10^4 CFU) was spotted on the plate (Figure 1A) and left to absorb. The Petri dish was incubated at 30°C for 48 hours and then scored for inhibition of *C. albicans*.

A variation of this test was done in the following way: *C. albicans* inoculated, and 12h incubated, Chromalbicans plate (as previously described) was poured with and left to absorb 500 µl of a *K. marxianus* B0399 PBS suspension. Two different concentrations of *K. marxianus* B0399 PBS suspensions were tested, suspension i) 6 x 10^5 CFU/mL and ii) 6 x 10^4 CFU/mL. The plates were then incubated again at 30°C for 48 hours.

TEST C
A Chromalbicans agar plate was inoculated using the spread-plate technique with a 100 µL PBS suspension of *K. marxianus* B0399 (3 x 10^6 CFU/mL; 3 x 10^5 CFU) and incubated at 30 °C for 12 hours. The plate was then poured and left to absorb 500 µl of a *C. albicans* ATCC 10231 PBS suspension (6 x 10^4 CFU/mL; 3 x 10^4 CFU). The plate was incubated again at 30° C for 48 hours.

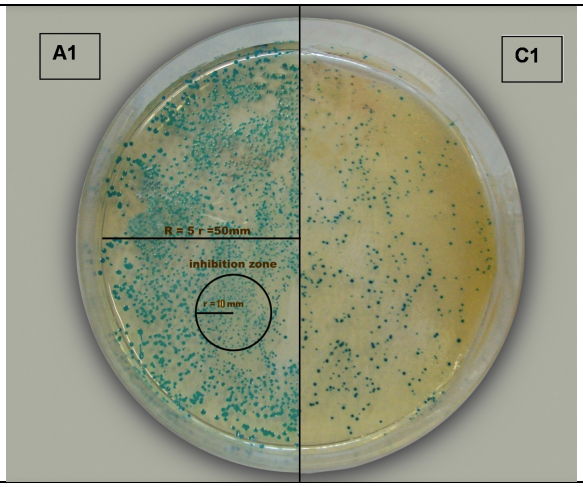
TEST D
YEPD broth was simultaneously inoculated with the equal amounts of *C. albicans* ATCC 10231 and *K. marxianus* B0399 overnight liquid cultures. The final concentration of albicans ATCC 10231 and *K. marxianus* B0399 in the suspension was 3 x 10^4 CFU/mL and 3 x10^5 CFU/mL, respectively. The resulting *K. marxianus*/*C. albicans* microbial suspension was then incubated at 30°C for 48 hours. After the incubation, 100 µL of the *K. marxianus*/*C. albicans* broth was plated on Chromalbicans agar and incubated at 30°C for 48 hours.

TEST E
100 µl of mixed, *K. marxianus*/*C. albicans*, YEPD culture, inoculated like in the previous test, was immediately plated on a Chromalbicans agar Petri dish (without previous incubation). The Petri dish was incubated at 37°C for 48 hours.

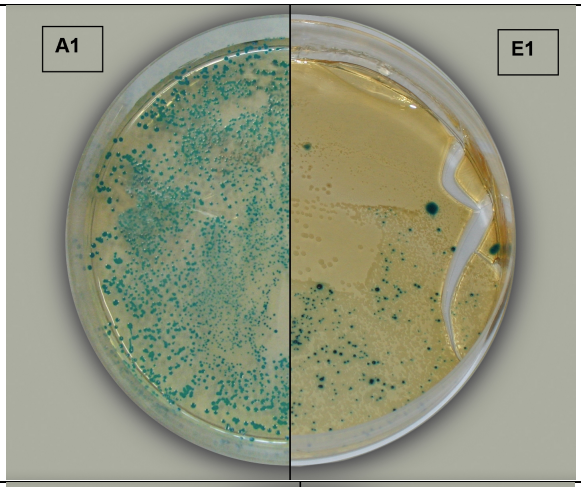
3. RESULTS AND DISCUSSION

The surface activity of *K. marxianus* B0399 against *Candida albicans* was observed by several modified (A-F) agar overlay tests, set up as explained previously in the text, in order to better mimic the conditions of vaginal mucosa.
The results from Test A were compared with the results of the other 3 tests (Fig.1a-c).

a)



b)



c)

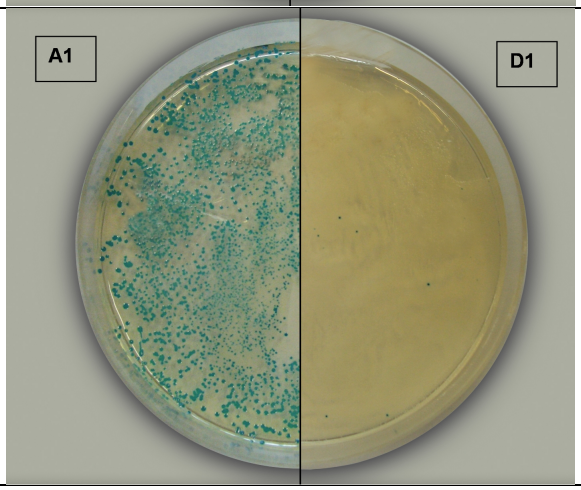


Fig. 1. **Agar overlay assay**

The left half of figure 1a, indicated as A1, demonstrated that in the circular area spotted with the *K. marxianus* B0399 suspension, indicated with the radius "r", colonies of *C. albicans* ATCC 10231 grew smaller in size (fig.1a - A 1) compared to the colonies grown outside of the *K. marxianus* B0399 zone (larger *C. albicans* colonies, numerous and evident on the rest of the agar surface area indicated with the radius "R"). The morphology of the two types of colonies was identical. With respect to the surface densities (CFU/mm^2) of the two yeast strains inside of the area of interference, the concentration (surface density) of *K. marxianus* B0399 was 10-fold higher than the one of *C. albicans*. Precisely 3 x 10^3 CFU/mm^2 (10 x 10^{-3} mL x 3 x 10^6 CFU/mL divided by 10 mm^2) for *K. marxianus* and 3 x 10^2 CFU/mm^2 for *C. albicans* (100 x 10^{-3} mL x 3 x 10^5 CFU/mL divided by 100 mm^2).

The 10-fold higher surface density/concentration of the "killer" strain seems to be a relevant determinant of the inhibition since the "small colony phenotype" of the target strain was repeated in the variation of the test A in which 500 µl of a 6 x 10^5 CFU/mL *K. marxianus* PBS suspension was poured over the whole plate in order to obtain a 10-fold higher surface density with respect to the target strain; conversely the "small colony phenotype" was lacking when 6 x 10^4 CFU/mL *K. marxianus* PBS suspension was used and the surface densities of the two strains were equal (data not shown).

As shown in the figure 1a - right half ("C1" on the fig.1a) and figure 1b - right side ("E1" on the fig.1b), stronger inhibition phenotype was obtained with the tests C and E where the "killer" strain was plated either before (test C) or simultaneously (test E) with the target strain. The relative concentrations of the two strains as well as the absolute cell number plated were kept the same as in test A. This allows us to conclude that the evident stronger *Candida* growth inhibition is correlated with the timing at which the "killer" and target strains come into contact with each other.

The strongest inhibition, however, was obtained when the "killer" and target strains were first incubated together in liquid media and then plated on Chromalbicans agar – test D (fig.1c-right

half, denominated “D1” on the figure). The relative concentrations of the two strains, as well as the absolute cell number plated, were kept the same as those in test A. This might indicate that the strongest inhibition phenotype (small and isolated *C. albicans* ATCC 10231 developed with difficulty) is a direct consequence of longer inhibition time (two days in liquid medium plus two days on the agar plate) and/or production of the putative bioactive molecule (“killer toxin”) released by the “killer strain”, *K. marxianus* B0399 and dispersed in the liquid medium.

4.CONCLUSIONS

Several variations of “agar overlay” assay, designated as test A-D in the text, were designed in order to provide insight into the antagonistic capacity of *K. marxianus* B0399 strain against *C. albicans* as well as into the physiological conditions required for maximal anti-*Candida* activity by *K. marxianus* B0399. All the tests revealed that the growth of C. albicans ATCC 10231 colonies in the presence of K. Marxianus B0399 was negatively influenced by the presence of K. marxianues B0399. Candida colonies appeared inferior in diameter compared to those grown in the absence of the probiotic yeast (test A).
It can also be observed that K. Marxianus B0399 has the capability to inhibit the development of the C. albicans ATCC 10231 colonies when K. Marxianus B0399 is inoculated preemptively in regards to Candida -- test D, C and E).

Test A mimics the situation where Candida colonizes intestinal/vaginal mucosa in the absence of *K. marxianus*, where it develops without any interference or inhibition of the latest. The successive administration of *K. marxianus* should have restrained the growth of the pathogen; Similarly to this assumption, after addition of *K. marxianus* and co-incubation of the two strains, *Candida* colonies regressed and their diameter minimized.
Test C mimics the situation where *K. marxianus* first colonizes the intestinal/vaginal mucosa (“prevention - type utilisation”) and competitively hinders *Candida* colonisation (small and isolated colonies in our *in vitro* model).
Test E mimics the situation where two yeasts reach the intestinal/vaginal mucosa at the same time; the one with better adhering capacity would develop on the expense of the other yeast.
Finally, Test D represents a situation where the two yeasts reach the intestinal mucosa simultaneously after previous contact – it might be the “direct” contact between K.marxianus and C.albicans that have led to almost complete inhibition of Candida growth whose colonies appeared small and distant between them.

Considering that the morphology of the C. albicans ATCC 10231 colonies does not change in the presence of K. Marxianus B0399, it is presumed that there are no variations of the superficial structure of the Candida cellular wall, but that there are other mechanisms that intervene to alter the development. The possible mechanisms of action connected to anti-*Candida* effects could be quite varied going from competition for space and nutrient, over killer toxin production. The results show an effect of inhibition of K. marxianux B0399 against C. albicans ATCC 10231, but the mechanisms of action that pertain to these observations are unclear and therefore more examination is necessary.