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SCREENING AMONG LACTIC ACID BACTERIA ISOLATED FROM NATURAL SOURCES FOR THEIR ANTI-*CANDIDA* IHIBITORY ACTIVITY

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Abstract

Candida, known as an "opportunistic" human pathogen, has coevolved with humans by persisting in mucosa and on the skin. The mechanism and factors determining the transformation of the "conolization" status into disease status is still under research. However, new preventive solutions for such events are under investigation. In our study we have tried to prove that some lactic acid bacteria (LAB), isolated from natural fermented food sources (Kombucha and fermented cocoa beans) have inhibitory effect on different species of Candida with different pathogenic potential (C. albicans, C. parapsilosis, C. krusei and C. guilliermondii). The inhibitory tests employed two different methods (transversal and radial strike). Among nine LAB strains isolated from cocoa beans fermentation, two have significant inhibitory activity against Candida parapsilosis. In the case of Kombucha source, two out of seven LAB strains have significant inhibitory activity against Candida albicans, Candida krusei and Candida parapsilosis; low level inhibition has been noticed against Candida guilliermondii. When mixing different combination of LAB from both natural sources, the anti-Candida effect was neither augmented, nor decreased

Key words*: lactic acid bacteria, Kombucha, cocoa fermentation, anti-Candida.*

INTRODUCTION

Human microbiota is complex, within which includes both commensal microorganisms, and pathogenic or facultative pathogenic microorganisms. *Candida* species strains is an example of microorganisms present in human microbiota which, in terms of balance of microflora of the human individual, it does not pose any health problems (Ionescu et. al., 2013). However, in cases of mass propagation, the genus *Candida* can produce local infections such as vaginal infectionsor even infections throughout the body, called candidaemia. The main the main species causing candidiasis is *C. albicans* (Silva et al., 2016), followed by *Candida parapsilosis Candida glabrata*, *Candida tropicalis* and *Candida krusei*, representing over 90% of cases of invasive infection caused by the genus *Candida* (Sardi et al., 2013; Pappas et al., 2015). In an article published in 2014 by Yapar N., citing CDC and the National Healthcare Safety Network, he states that the genus *Candida* is in the fifth place in the above hospital-acquired and in fourth place in the case of bloodstream. The use of various antifungal drugs such as azoles (fluconazole or ketoconazole), echinocandins (micafungin, caspofungin), amphotericin B or nucleoside analogues (Spampinato & Leonardi, 2013) have increased, in recent years, Candida's resistance to these treatments. difficult to treat certain types of candidiasis (Sanguinetti et al., 2015). Therefore, research and development of alternative cures for the treatment of candidiasis is a new trend in modern medicine (Vamanu & Voica, 2017). A good research path is represented by probiotics worth mentioning being genus *Lactobacillus*, the genus *Bifidobacterium*, the genus *Bacillus*, *Saccharomyces cerevisiae* (Silva et al., 2016).

All of the above lead researchers to conclude that the use of probiotics in fighting candidiasis may be the best solution in current medical conditions.

The context the aim of of the study was to characterize functional strains of microorganisms with anti-microbial activity and their recovery in the form of products with mixed anti-microbial and probiotic activity.

In the present work, anti-*Candida* activity of different lactic acid bacteria originated from different natural sources were studied: strains of lactic bacteria isolated from fermented cocoa

beans and strains of lactic bacteria isolated from Kombucha SCOBY.
Strains of lactic bacteria isolated from cocoa beans and isolated ones from kombucha are presented in Table 2.

MATERIALS AND METHODS

The *Candida* species used to carry out the experiments and on which the anti-*Candida* activity was carried out are part of the collection of the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest and are presented in Table 1.

Table 1. Strains of *Candida* used

No.	**Strain**
1.	*Candida albicans* ATCC 10231
2.	*Candida guillermondii* MI 40
3.	*Candida krusei* 2016 MI 41
4.	*Candida parapsilosis* ATCC 20019

Table 2. Strains of lactic bacteria used

No.	Code	Species
Isolated from cocoa beans		
1.	Ped-3	*Enterococcus faecium*
2.	Ped-2	*Weissella cibaria*
3.	Lab 11.2	*Enterococcus faecium*
4.	Lab 11.1	*Lactobacillus farciminis*
5.	A19	*Weisella cibaria*
6.	Lab 9	*Pediococcus pentosaceus*
7.	Lab 10	*Weisella cibaria*
8.	A21	not identified
Isolated from fermented tea (kombucha)		
1.	S1	*Pediococcus pentosaceus*
2.	S2	*Pediococcus pentosaceus*
3.	S3	*Pediococcus pentosaceus*
4.	L3	*Pediococcus pentosaceus*
5.	L5	*Pediococcus acidilactici*
6.	F1	nd
7.	F2	nd

The culture medium used to cultivate the lactic bacteria was MRS at pH 6.5 ± 0.2.
The lactic bacteria strains source was the frozen stock belonging to the Microbiology Laboratory of the Faculty of Biotechnologies of the University of Agronomic Sciences and Veterinary Medicine of Bucharest. Each strain was cultivated as follows: 200 µL of bacterial suspension were taken from the Eppendorf tube containing the strain and were placed in a test tube with 10 mL of MRS medium and cultivate at 37ºC for 24 hours. From this parent culture, a dilution was performed: 1 mL of bacterial suspension was sown in test tubes with 10 mL of MRS culture medium. The revitalization method is described in the literature (Terpou et al., 2019), but has been slightly modified.
The inhibitory tests employed two different methods: transversal and radial strike. There are data in the literature that propose the two methods for testing the antimicrobial activity of lactic bacteria for rapid test results and efficient results. The good results seem to be due to the production of metabolites and good diffusion of metabolites produced by the lactic bacteria tested by the two methods (Coman et al., 2014; Balouiri et al., 2016).
The strains of lactic bacteria were inline seeded on Petri dishes with MRS medium. They were allowed to grow at 27°C for 48 hours and subsequently were perpendicularly inoculated to the *Candida* strains (*C. albicans*, *C. guillermondii*, *C. krusei*, *C. parapsilosis*).
In the case of the radial streak method, 10 µl of LAB suspension was inoculated on spot in Petri dishes containing MRS solid culture medium. After a 48 h incubation at 37°C, the plates radially incoluated with suspension of *Candida* species. After other 24 h incubation at 37°C, antimicrobial activity was observed. All tests have been done in triplication. Inhibitory activity was calculated by decreasing the diameter of the circle of the spread area of the lactic bacterium strain from the diameter of the observed inhibition zone.
In the case of the transversal method, the inhibitory activity was calculated by measuring the inhibition area observed between lactic bacteria and the pathogenic strain.
The study on the antimicrobial activity was followed by testing the inhibitory activity of mixtures of lactic strains by modified diffusion method (Olaru & Popa, 2019). Each strain was cultivated as follows: 200 µL of bacterial suspension were taken from the Eppendorf tube containing the strain and were placed in a test tube with 10 mL of MRS medium. From this parent culture, a dilution was done: 1 mL of bacterial suspension was sown in test tubes with 10 mL of MRS medium. After incubation at 37°C for 24 hours, they were used according to the modified diffusion method.
Each potential probiotic strain was inoculated in 10 µL volume spots and incubated for 48h at

37°C. The cultivation of lactic strains was carried out both individually and in 1:1 mixture with suspension 10^7 UFC/mL. Pathogenic reference strains were inoculated in an MRS 1% agar environment, melted and maintained at 45°C. After pouring over the spots containing the developed lactic strains, they were incubated at 37°C for 24 h. After incubation, the inhibition region was observed. It was worked in duplicate, using both MRS 1% agar with Tween 80.

RESULTS AND DISCUSSIONS

The inhibitory activity of LAB strains isolated from cocoa beans can be observed in Figure 1, while Tables 3 and 4 present the measurement results of the inhibitory activity on *Candida* strains for lactic bacteria isolated from cocoa beans.

The data indicates a good inhibition of *Candida* strains by Lab 11.1 (*Lactobacillus farciminis*), Lab 9 (*Pediococcus pentosaceus*) and A19 (*Weisella cibaria*) confirmed by both used methids, radial and transversal. In the case of Lab 11.1 (*Lactobacillus farciminis*) the highest inhibition activity was noticed against *C.parapsilosis*, followed by *C. guiliermondii* and *C. albincans*. No inhibitory activity was observed against *C. krusei.*

In the case of Lab 9 (*Pediococcus pentosaceus*) the inhibitory activity was almost the same against *C. parapsilosis* and *C. guilliermondii*, while low inhibition was obtained against *C. albicans.* A different situation was found in the case of A19 (*Weisella cibaria*); the highest inhibitoy activity was found against *C. albicans*; however, the results obtained by both tested method are only partially reproductibl, which is athe same in the case of the non-identified strain A21.

Among all *Candida* specie, the strain *Candida krusei* 2016 MI 41 shows resistance to all strains of lactic bacteria isolated from cocoa beans, while *Candida parapsilosis* ATCC 20019 has the highest sensitivity to lactic bacteria isolated from cocoa beans.

Aspects of the inhibitory activity of Kombucha LAB strains can be observed in Figure 2, while the measurements results are in Tables 5 and 6.

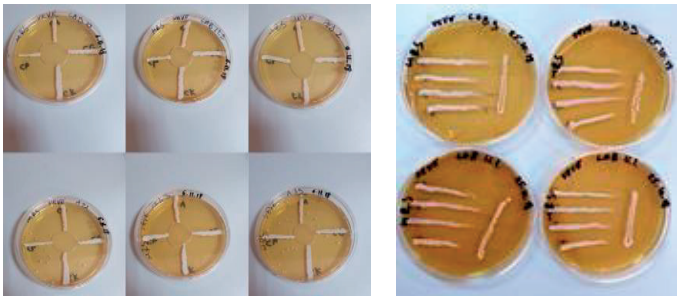

Figure 1. Inhibitory activity of lactic bacteria isolated from cocoa beans on *Candida* species tested by radial streak method (left) and of Lab 9 and Lab 11.1 tested by transversal method (right)

Table 3. Calculation of the inhibition zone of lactic bacteria isolated from cocoa beans on pathogenic strains tested by radial streak method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
Lab 11.1	2.00±0.00	9.5±0.70	nd	11.5±4.94
Lab 11.2	nd	nd	nd	nd
Lab 10	nd	nd	nd	nd
Lab 9	1.00±1.41	2.5±3.53	nd	4±5.65
Ped 2	nd	Nd	nd	nd
Ped 3	nd	nd	nd	nd
A19	8.5±12.0	5±7.07	nd	2.5±3.53
A21	nd	2.5±3.53	nd	2.5±3.53

*Inhibition area measured in mm

Table 4. Calculation of the inhibition zone of lactic bacteria isolated from cocoa beans on pathogenic strains tested by transversal method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
Lab 11.1	3±0	7.5±0.70	1±11.3	9.5±3.55
Lab 11.2	nd	nd	nd	nd
Lab 10	nd	nd	nd	nd
Lab 9	1±0.2	5.5±4.0	0.5±10.2	7±4.22
Ped 2	nd	nd	nd	nd
Ped 3	nd	Nd	nd	1±0.7
A19	1.5±13.0	0.5±4.27	nd	nd
A21	nd	nd	nd	nd

*Inhibition area measured in mm

Table 5. Calculation of the inhibition zone of lactic bacteria isolated from Kombucha on pathogenic strains tested by radial streak method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
S1	nd	4±0.82	6±1.41	5.5±0.94
S2	nd	5.5±0.47	4.5±0.47	3.5±0.94
S3	nd	4±0.82	6.5±0.94	3.5±0.94
L3	13.5±2.87	5.5±4.0	10±0.82	11.5±2.05
L5	17±1.41	14.5±0.94	14±3.56	4.5±0.47
F1	3.5±2.87	5±0.82	4.5±0.94	5.5±4.03
F2	nd	4.5±0.94	5±0	4.5±0.94

Figure 2. Inhibitory activity of lactic bacteria isolated from Kombucha on *Candida* species tested by radial streak method (left) and of L3 and L5 strains by transversal method

All strains of isolated lactic bacteria in kombucha shows anti-*Candida* activity. Although the transversal method mean inhibition is high for most lactic strains, the average standard deviation is high. Clearer results were obtained by radial streak method. Strains of lactic bacteria L3 (*Pediococcus pentosaceus*) and L5 (*Pediococccus acidilactici*) had the most promising results, inhibiting in varying proportions all tested pathogens. L3 presented a high inhibitory action against *C. albicans*, *C. krusei* and *C. parapsilosis* and lower against *C. guilliermondii*. L5 presented a high inhibitory action against *C. albicans*, *C. krusei* and *C. guilliermondii* and lower against *C. parapsilosis*. Following the preliminary results on the inhibitory activity of individual strains, in the next step have been tested the anti-*Candida* potential of mixed suspensions of positive tested Lab strains. Different combinations of L3 (*Pediococcus pentosaceus*), L5 (*Pediococcus acidilactici*) and Lab 11.1 (*Lactobacillus farciminis*) have been employed in a 1:1 ratio: L3 + L5, L3 + Lab 11.1, L5 + Lab 11.1 and Lab 11.1 + L3 + L5 (1:1:1). As seen in Figure 3, the inhibitory activity is kept in different mixture, but the inhibition zone (halo formation) of the single strains is not different than the mixture. By spot method, the highest inhibition activity, in single or mixed strains, was noticed in the case of *C. guilliermondii*, followed equally by *C. parapsilosis* and *C. albicans.*

Our results are confirming and/or completing other reports on anti-*Candida* activity of LAB strains. For instance, strains of *Pediococcus acidilactici* and *Pediococcus pentosaceus*, isolated from honey, have been reported to have significant anti-*Candida* activity (Bulgasem et al., 2013) against *C. krusei*, *C. glabrata* and *C. albicans.* Also, Kim and Kang (2019) have reported that ell-free supernatants of a probiotic *Pediococcus acidilactici* isolated from malt has inhibitory activity on *C. albicans.* Such strains, of *Pediococcus acidilactici* have been recently reported to be used as bread preservative (Bustos et al., 2018). No clear data, on our knowledge, have been reported in regard to *Lactobacillus farciminis* potential to inhibit the *Candida* and candidiasis.

Table 6. Calculation of the inhibition zone of lactic bacteria isolated from kombucha on pathogenic strains tested by transversal method

Lactic bacteria strain	*Candida albicans*	*Candida guilliermondii*	*Candida krusei*	*Candida parapsilosis*
S1	6.5±0.94	5.5±2.49	5.5±1.41	4±6.48
S2	8±4.32	6.5±2.62	12±0.82	3.5±2.05
S3	8±2.87	5.5±2.87	8±5.89	4±2.94
L3	7±1.41	7.5±0.94	15.5±1.89	8.5±1.25
L5	9±0	6.5±1.25	16±2.83	5±2.45
F1	8.5±2.36	5.5±2.49	9±6.48	5.5±4.11
F2	10.5±5.44	5.5±5.19	7±9.90	12.5±11.59

*Inhibition area measured in mm

Figure 5. Inhibitory activity of LAB mixtures on *C. albicans*, *C. guilliermondii*, *C. parapsilosis* and *C. krusei* (from left to right)

CONCLUSIONS

Different LAB strains isolated from fermented cocoa beans and microbial consortium of Kombucha beverages have tested for their anti-*Candida* activity.

Significant inhibition was shown by strains belonging to the following species *Lactobacillus farciminis, Pediococcus pentosaceus, Pediococcus acidilactici* and *Weisella cibaria*.

Mixed supernatant of cultivated LAB haven't increased the inhibitory activity and the reasons are to be discussed and found.

The most inhibited specie among the tested one, were, in order of their inhibition, *C. guilleirmondii, C. parapsilosis* and *C. krusei*.

The LAB strains will be further tested for their probiotic potential as well as for their antioxidative activity and will be subject of new pharmaceutical/cosmetic novel products development.

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