African Journal of Microbiology Research Vol. 4 (12), pp. 1251-1256, 18 June, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

Full length Research Paper

An in vitro assessment of inhibitory effect of 16 strains of probiotics on the germination of *Candida albicans*

Huan Tang[1], Jiong Ren[2], Jing Yuan[1], Benhua Zeng[1] and Hong Wei[1]*

[1]Department of Laboratory Animal Science, College of Basic Medical Sciences, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, 400038 Chongqing, P. R. China.
[2]Institute of Combined Injury, State Key Laboratory of Trauma, Burns and Combined Injury, College of Preventive Medicine, Third Military Medical University, 30 Gaotanyan Road, Chongqing, 400038, China

Accepted 31 May, 2010

The purpose of the study was to evaluate the antagonistic activity of probiotics isolated from products to germination of *Candida albicans in vitro*. The spent culture supernatant, live bacteria, heat-killed bacteria of 16 strains of probiotics and main bacterial short-chain fatty acids were applied to inhibit the germination of *C. albicans in vitro* by crystal violet-based germ tube assay. Neutral SCS of all the probiotcis evaluated in this research could decrease germination significantly and live bacteria of *Lactobacillus rhamnosus* LGG, *Lactobacillus plantarum* LA, *Lactobacillus johnsonii* JCM1022, *Bacillus Longum*-2, *Bacillus sub.* and *Bacillus lich* could partially inhibit the conversion of yeast to germ. However, all the heat-killed bacteria failed to control the germ tube formation. Furthermore, only butyric acid blocked the conversion of yeast to hypha among all the SCFAs. These results suggest that *L. rhamnosus* LGG, *L. plantarum* LA and *L. johnsonii* JCM1022, *B.Longum*-2, *Bacillus sub.* and *B. lich* maybe potential strains to use as antifungal drugs and the inhibition seems to have direct correlation to the metabolites butyric acid.

Key words: *Candida albicans,* antifungal drugs, germ tube, probiotics, butyric acid.

INTRODUCTION

Candida albicans is one of the major causes for nosocomial infections. Immune compromised individuals, cancer patients, diabetics and others who have long term use of antibiotics are especially susceptible to candidiasis resulting in high incidence of mortality (Calderone and Fonzi, 2001). *C. albicans* is a dimorphic fungus which can grow in either budding or filamentous form. Several reports show that the conversion of the yeast to the hypha is accompanied by enhancement of the virulence to the host (Teresa et al., 2003) and the initiation of the switch is germ tube formation. Germ tube can promote *C. albicans* adhere to mucosal surface and then penetrate to the epithelium (Kretschmar et al., 1999). In addition, the cells capable of germination can escape from phagocytosis and make direct damage to the tissues (Marcil et al., 2002). So, as a putative virulence factor, germ tube inhibition is now considered to be a target for screening the new antifungal agents (Hawser and Islam, 1999; Brayman and Wilks, 2003). Probiotics, such aslactic acid bacteria, widely used in the food industry, are proved to be beneficial to the patients of digestive diseases and prevent the gastrointestinal bacterial infection (Vieira et al., 2008; Klarin et al., 2008; Lin et al., 2008). Furthermore, *Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus* GG are reported to prevent the candidiasis (Wagner et al., 2000; Manzoni, 2007; Rönnqvist et al., 2007), but the further researches about its mechanism is still absent. The aim of this paper is to investigate the inhibitory effects of 16 strains of probiotics on the germ tube formation of *C. albicans* so as to find some potential alternative therapeutic bacteria. At the same time, the main metabolites short-chain fatty acids (SCFAs) of lactic acid bacteria were used to block of germination which could indicate the possible mechanism of the inhibition.

*Corresponding author. E-mail: weihong63528@163.com. Tel: 86-23-68753882. Fax: 86-23-68752051.

MATERIALS AND METHODS

Strains and medium

A quality control strain *C. albicans* ATCC90028 which was recommended by national committee for clinical laboratory standards (NCCLS) was used to test the germination. Cells were grown in yeast extract-peptone-dextrose (YPD) medium for 48 to 72 h at 30°C without shaking. The organisms were washed twice by 1 × PBS and finally resuspended in fetal bovine serum (FBS, TBD) to 5 × 10^6 cells/ml.

16 strains of probiotics were investigated in this study. *L.* rhamnosus ATCC 53103 (L.GG) was generously present; L. johnsonii JCM1022, Lactobacillus reuteri JCM1081, Lactobacillus gasseri JCM1130 were obtained from Japan. Collection of microorganism, L. acidophilus 1.1878 and L. rhamnosus 1.0120 were purchased from China General Microbiological Culture Collection Centre (Beijing, China). Lactobacillus plantarum LA, Lactobacillus bulgaricus LB, Bifidobacterium Longum Bif.I-1, Bifidobacterium Longum Bif. I-2, Bifidobacterium adolescentis Bif. ado, *Enterococcus faecalis (E. faecalis), Enterococcus faecium (E. faecium), Bacillus subtilis (B. sub)* and *Bacillus licheniformis (B. lich)* were all separated from different probiotic products made in China. Lactobacilli were inoculated in MRS (deMan-Rogosa-Sharpe) broth (Oxoid). Bifidobacterium were placed in BSM broth. Both lactobacilli and bifdobacterium grew at 37°C anaerobically and statically. Enterococci and Bacilli grew separately in M17 broth (Difco) and LB broth at 37°C aerobically. Then the fresh cells were diluted to the concentration of 5 × 10^7 and 5 × 10^6 cells/ml in 1×PBS respectively.

Crystal-violet based germ tube assay

The assay was used to measure germ tube formation of *C. albicans* as previous reports (Abe et al., 1994; Brayman and Wilks, 2003). In short, ATCC90028 diluted in FBS was plated in 96-well flat-bottom with the volume of 100 μl and the different additions with the same volume were added before the plate was incubated at 37°C for 2 h to induce germ tube formation. The medium in the wells were discarded when plate was removed from the incubator and then 70% ethanol and the 200 μl 25% sodium dodecyl sulphate (SDS) were used to wash each wells.

After washed by the distilled water for 3 three times, germ tube adhere to the plate bottom were stained by 200 μl 0.1% crystal violet for 10 min. Plate was washed three times additionally by the distilled water, once by 0.25% SDS and three times with water. When the plate air dried, 200 μl of isopropanol-0.04 N HCl and 50 μl 0.25% SDS were then added to elute the crystal violet. A_{590} was determined by a spectrophotometer. The assay was performed in triplicate and repeated. The germination rate was measured as %control= (A_{590} for experimental well- A_{590} for blank well) / (A_{590} for control well- A_{590} for blank well).

Probiotics and SCFAs treatment

Additions in the crystal-violet based germ tube assay mentioned above included the 24 h spend culture suspension (SCS), live bacteria and heat-killed bacteria of 16 strains of probiotcis with the volume 100 μl. Furthermore, the main metabolites of the lactic acid bacteria including lactic acid, acetic acid, propionic acid and butyric acid (all from Sigma) were diluted in 1 × PBS to different concentrations and added to wells, respectively and the wells added equal volume of 1 × PBS was used as the control. In order to avoid effect of pH on germination, all the SCS and the SCFAs were buffered to neutral and filtrated by the 0.22 μm-pore-size filters.

Statistical analysis

All of the data were analysed by student's *t* test (two-tailed, unequal variance) with SPSS 12.0 software. Values of P ≤ 0.05 were considered statistically significant and a P value ≤ 0.01 were considered to be outstanding significant.

RESULTS

Effects of neutral spend culture suspension on germ tube formation

The results showed that all of the probiotics supernatant could inhibit the germination of *C. albicans* significantly (Figure 1), suggesting that some soluble compounds in culture supernatant may be responsible for the inhibition. It should be noted that the addition of 24 h cultures of LA and JCM 1022 almost completely inhibited germination (about 90% inhibition) and the inhibition effect was better than a novel probiotic LGG.

Effect of live bacteria on germ tube formation

Figure 2 indicated the germination of *C. albicans* when different concentration ratios of live probiotics to *C. albicans* were incubated within the same well. Results showed that antimicrobial activities against *C. albicans* of 16 strains displayed diversity. Only 6 strains of probiotics could repress the germination significantly, which including L.GG, LA, JCM1022, Bif.I-1, *B. sub* and *B. lich* (Figure 2a). However, the inhibitory effects were eliminated following the ratio decline and only L.GG, *B. sub* and *B. lich* still appeared the significant ability to inhibit the *C. albicans* morphogenesis (Figure 2b).

Effect of heat-killed bacteria on germination

All the probiotics observed in our study were incapable to block the transition after thermal inactivation at 80°C for 30 min (Data not show).

Effect of short-chain fatty acids on germination

Figure 3 showed that among the SCFAs used in the test only butyric acid could suppress the germination of *C. albicans* after buffered to neutral and the inhibitory effect seemed to be dose-related. Butyric acid could block the germ tube formation with the concentration above 25 mM.

DISCUSSION

In vitro, some substances produced by specific probiotics strains have been found to exert an inhibitory effect upon *C. albicans* (Osset et al., 2003; Strus et al., 2005; Suido and Miyao, 2008). Our results got from bacteria-free SCS

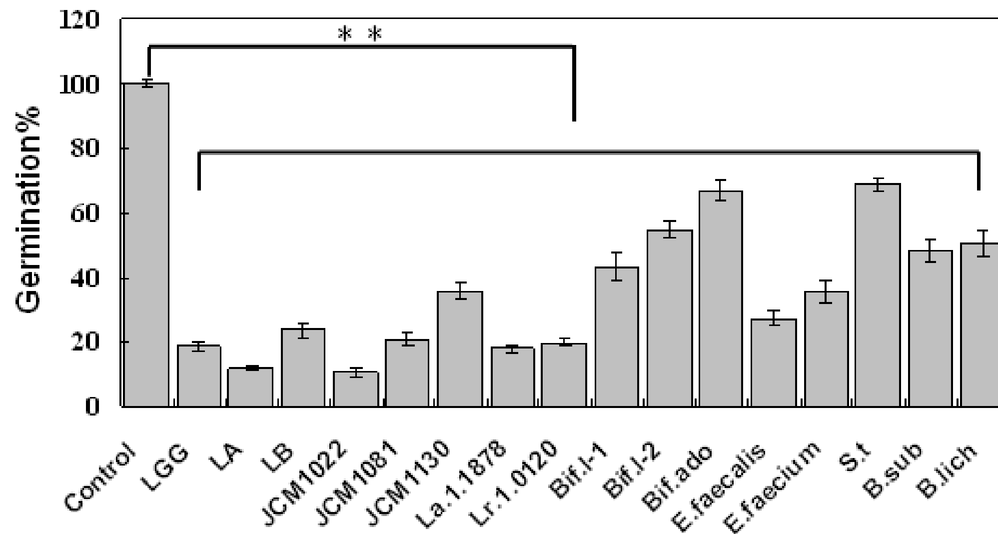

Figure 1. Effect of SCS (pH = 7.4) of probiotics on CA germ tube formation. 24 h SCS of probiotics were collected by centrifuging at 10,000*g* for 30 min at 4℃ and all the SCS were buffered to neutral and filtrated by the 0.22 μm-pore-size filters unit (Millipore, Molsheim, France) after being centrifuged. 100 μl of SCS were added to the 96-well flat-bottom plate as the additions respectively. In the control wells, SCS were replaced by PBS and the final concentration of FBS in all the wells were 50%.All value were reported as mean ± S.D, **, P ≤ 0.01 VS control. The assay was performed in triplicate and repeated.

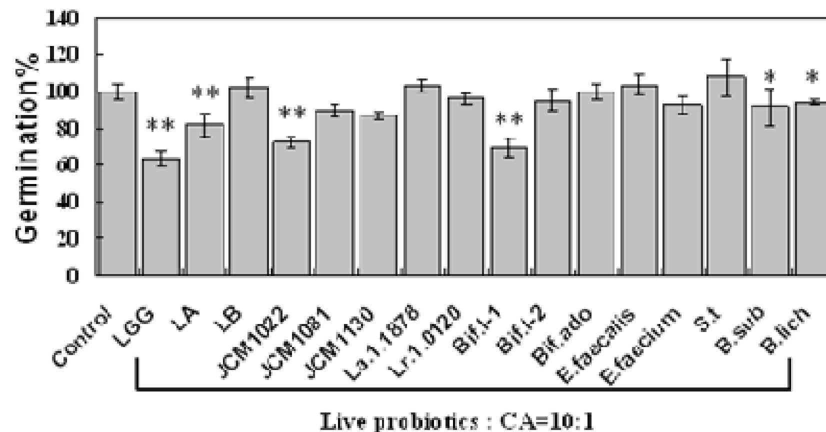

Figure 2a. Effect of live bacteria of probiotics on CA germ tube formation. The fresh cells of probiotics were washed three times and suspended in 1 × phosphate-buffered saline to 5 × 10^7 cells ml^{-1}. The suspensions were added to the 96 well flat-bottom plate contained ATCC 90028 as the additions respectively. 1 × PBS were added to the control wells as conditions and the final concentration of FBS in all the wells were 50%. All value were reported as mean ± S.D, *: P ≤ 0.05 VS control, **: P ≤ 0.01 VS control. The assay was performed in triplicate and repeated.

and four main SCFAs with different concentrations reinforced that the active metabolites may contribute to the inhibition of the switch.

Butyric acid could block the germ tube formation with the concentration above 25 mM, which was closed to normal concentration of butyric acid in human large intestine detected in the early research (Cummings et al., 1984). Though lactic acid was the most important metabolites of lactic acid bacteria, our results suggested that it was not the primary determinant to the inhibition on *C. albicans* morphogenesis. In addition, all the media we incubated in the platform were buffered to pH 7.4 and a

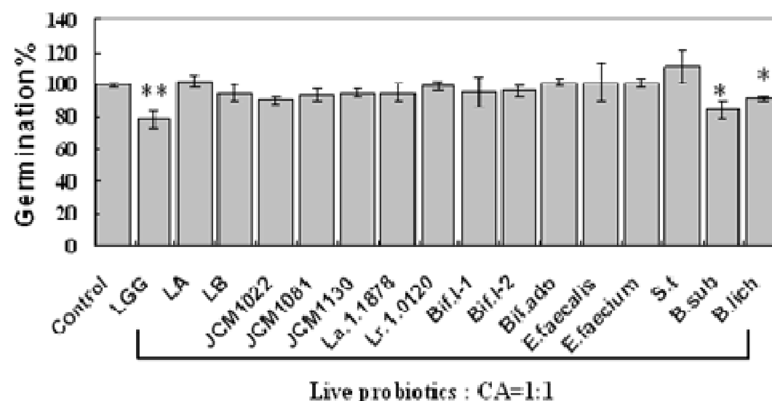

Figure 2b. Effect of live bacteria of probiotics on CA germ tube formation. The fresh cells of probiotics were washed three times and suspended in 1×phosphate-buffered saline to 5 × 10^6 cells ml^{-1}. The suspensions were added to the 96 well flat-bottom plate contained ATCC 90028 as the additions respectively. 1 × PBS were added to the control wells as conditions and the final concentration of FBS in all the wells were 50%. All value were reported as mean ± S.D, *: P ≤ 0.05 VS control,**: P ≤ 0.01 VS control. The assay was performed in triplicate and repeated.

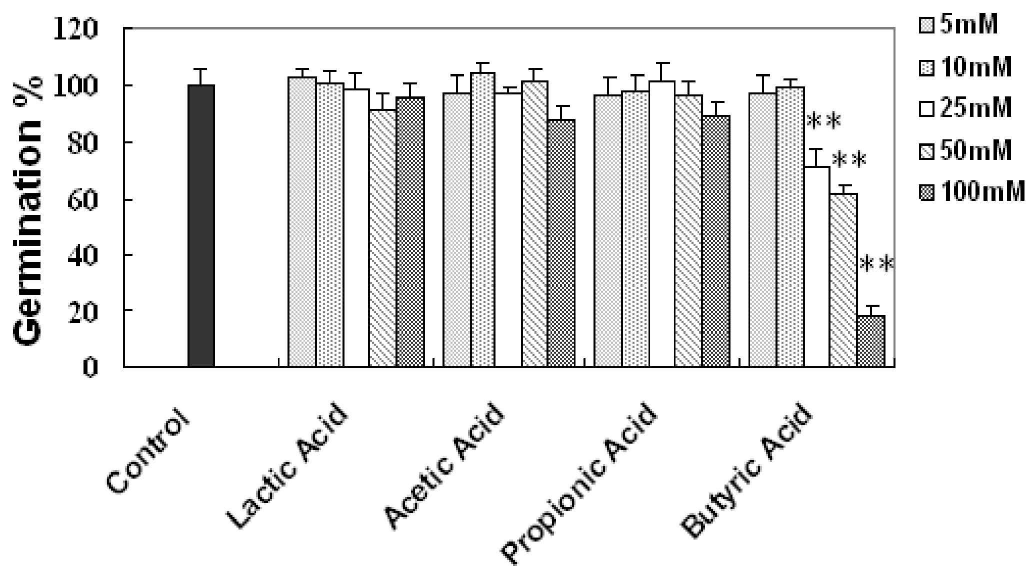

Figure 3. Effect of SCFAs on CA germ tube formation. The SCFAs with different concentration were added to the 96-well flat-bottom plate contained ATCC 90028 as the additions respectively. 1×PBS were added to the control wells as conditions and the final concentration of FBS in all the wells were 50%. All value were reported as mean±S.D; *, P ≤ 0.05 VS control. **, P ≤ 0.01 VS control. The assay was performed in triplicate and repeated.All value were reported as mean±S.D; *, *P* ≤0.05 VS control. **, P ≤ 0.01 VS control. The assay was performed in triplicate and repeated.

noticeable decline of germination rate still represented. Meanwhile, neutralizing the butyric acid did not cause the disappearance of inhibitory effect, suggesting that the inhibition could have been predominantly due to acid per sec instead of pH. The mechanism of inhibition by butyric acid remained unclear and previous researches supposed butyric acid might interfere with cytoskeletal assembly and then the morphogenesis could be blocked (Hoberg and Cihlar, 1983). Related researches considered that in the switching process of *C. albicans,* the acetylation of histones played an important role while butyrate just one of the inhibitors of histone deacetylase

(Klar et al., 2001; Smith and Edlind, 2002).

Furthermore, in an immunodeficient mouse model of gastrointestinal candidiasis, the presence of probiotic bacteria including *L. acidophilus, L. reuteri, L. casei* GG and Bifidobacterium animalis can prolong the survival of the *C. albicans* infected mice (Wagner et al., 1997). At the same time, some candidiasis clinical studies showed that oral probiotics could enhance the clearance of *C. albicans* from patients' intestine and vagina (Manzoni et al., 2006; Manzoni, 2007). Evidences mentioned above suggested that live bacteria of probiotics were effective in preventing candida gut colonization and systemic dissemination. Although many reports proved that lactobacilli and bifidobacterium exerted excellent ability of adhesion to the intestinal mucosa (Bernet et al., 1994; Hooper and Gordoon, 2001), they did not adhere to the tissue culture plastic used in our study. Furthermore, *B. sub* and *B. lich* showed outstanding inhibition to the germination, but almost no evidence supported that *Bacilli* process the antimicrobial function by adhesion. So, the antagonism of live probiotics to the *C. albicans* appeared due to the production of active metabolites rather than the competitive exclusion.

It has been claimed that some heat-killed lactobacilli could protect immunodeficient mice from orogastric candidiasis (Wagner et al., 2000). All the probiotics observed in our study were incapable to block the transition after thermal inactivation at 80°C for 30 min. This result reinforced that active compound or metabolically active bacteria were responsible for the blocking of the morphological switch of *C. albicans*.

Researches about discovering antifungal substances with high performance and fewer side effects were very prevalent just now. Plant oils (Devkatte et al., 2005), LAAE-14 (Lucas et al., 2004) and *Streblus asper* leaf-extract (Taweechaisupapong et al., 2005) etc. were all considered to be the potential candidates. Compared with the xenobiotics, probiotics are one part of the indigenous microflora in human or animals' intestine, so they are more suitable to use as antifungal drugs**.**

In summary, data present in our study demonstrated that live probiotics including LGG, LA, JCM1022, B.Longum-2, *Bacillus sub*. and *B. lich* could inhibit the switch of *C. albicans* from bud to filament *in vitro* and the inhibition was likely associated with the production of the butyric acid by live bacterium. This might represent some candidate probiotic strains toward the development of anti-fungal drugs and provide further research clues of the antagonistic mechanisms.

ACKNOWLEDGEMENTS

We thank Dr. Lee Yuan-kun of National University of Singapore for presenting the *Lactobacillus rhamnosus* L.GG. The project was sponsored by National Basic Research Program of China (NO. 2007CB513007), National High Technology Research and Development Program of China (NO.2007AA10Z356).

REFERENCES

Abe S, Satoh T, Tokuda Y, Tansho S, Yamaguchi H (1994). A rapid colorimetric assay for determination of leukocyte-mediated inhibition of mycelial growth of *Candida albicans*. Microbio. Immunol., 38: 385-388.

Bernet MF, Brassart D, Neeser JR, Servin AL (1994). *Lactobacillus acidophilus* LA1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. Gut*.,* 35: 483-489.

Brayman TG, Wilks JW (2003). Sensitive Assay for antifungal activity of glucan synthase inhibitors that uses germ tube formation in *Candida albicans* as an end point. Antimicrob. Agents. Chemother., 47: 3305-3310.

Calderone RA, Fonzi WA (2001).Virulence factors of *Candida albicans*. Trends. Microbiol., 9: 327-335.

Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT (1987).Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut*.,* 28: 1221-1227.

Davis D (2003). Adaptation to environmental pH in *Candida albicans* and its relation to pathogenesis. Curr. Genet., 44: 1-7.

Devkatte AN, Zore GB, Karuppayil SM (2005). Potential of plant oils as inhibitors of *Candida albicans* growth. *FEMS* Yeast. Res., 5: 867-873

Hawser S, Islam K (1999). Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of *Candida albicans*. J. Antimicrob. Chemother., 43: 411-413

Hoberg KA, Cihlar RL, Calderone RA (1983).Inhibitory effect of cerulenin and sodium butyrate on germination of *Candida albicans*. Antimicrob Agents. Chemother., 24: 401-408

Hooper LV, Gordon JI (2001). Commensal host-bacterial relationships in the Gut. Sci., 292: 1115-1118.

Klar AJ, Srikantha T, Soll DR (2001). A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen *Candida* albicans. Gen., 158: 919-924.

Klarin B, Wullt M, Palmquist I, Molin G, Larsson A, Jeppsson B (2008). *Lactobacillus plantarum* 299v reduces colonisation of *Clostridium difficile* in critically ill patients treated with antibiotics. Acta. Anaesthesiol. Scand., 52: 1096-1102.

Kretschmar M, Hube B, Bertsch T, Sanglard D, Merker R, Schröder M, Hof, H, Nichterlein T (1999). Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis,. Infect. Immun., 67: 6637-6642.

Lin CK, Tsai HC, Lin PP, Tsen HY, Tsai CC (2008).*Lactobacillus acidophilus* LAP5 able to inhibit the *Salmonella choleraesuis* invasion to the human Caco-2 epithelial cell. Anaerobe., 14: 251-255.

Lucas R, Villamón E, Payá M, Alves M, Olmo E, del, Gozalbo,D, Gil ML (2004). LAAE-14, a new anti-inflammatory drug, increases the survival of *Candida albicans*-inoculated mice. FEMS. Immunol. Med. Microbiol., 40: 239-242.

Manzoni P (2007). Use of *Lactobacillus casei subspecies Rhamnosus* GG and gastrointestinal colonization by Candida species in preterm neonates, Supp. J. Pediatr. Gastroenterol. Nutr., 45(3): 190-194.

Manzoni P, Mostert M, Leonessa ML, Priolo C, Farina D, Monetti C, Latino MA, Gomirato G (2006). Oral supplementation with *Lactobacillus casei subspecies rhamnosus* prevents enteric colonization by Candida species in preterm neonates: a randomized study. Clin. Infect. Dis., 42: 1735-1742.

Marcil A, Harcus D, Thomas DY*,* Whiteway M (2002). *Candida albicans* killing by RAW 264.7 mouse macrophage cells: effects of Candida genotype, infection ratios, and gamma interferon treatment. Infect. Immun., 70: 6319-6329.

Osset J, Garcia E, Bartolome RM, Andreu A (2001). Role of *Lactobacillus* as protector against vaginal candidiasis. Med. Clin., 117: 285-288.

Rönnqvist D, Forsgren-Brusk U, Husmark U, Grahn-Håkansson E (2007). *Lactobacillus fermentum* Ess-1 with unique growth inhibition of vulvo-vaginal candidiasis pathogens, J. Med. Microbiol*.,* 56: 1500-1504

Smith WL, Edlind TD (2002). Histone deacetylase inhibitors enhance

Candida albicans sensitivity to azoles and related antifungals: correlation with reduction in CDR and ERG upregulation. *Antimicrob* Agents. Chemother., 46: 3532-3539.

Strus M, Brzychczy-Wloch M, Kucharska A, Gosiewski T, Heczko PB (2005). Inhibitory activity of vaginal Lactobacillus bacteria on yeasts causing vulvovaginal candidiasis, Med. Dosw. Mikrobiol., 57: 7-17.

Suido H, Miyao M (2008). *Bifidobacterium longum-fermented broccoli* supernatant inhibited the growth of *Candida albicans* and some pathogenic bacteria in vitro Biocontrol. Sci., 13: 41-48.

Teresa B, Barbara B, Klaus S, Joachim M (2003). Calcineurin is essential for virulence in *Candida albicans*, Infect. Immun., 71: 5344-5354.

Taweechaisupapong S, Choopan T, Singhara S, Chatrchaiwiwatana S, Wongkham S (2005). In vitro inhibitory effect of Streblus asper leaf-extract on adhesion of *Candida albicans* to human buccal epithelial cells. J. Ethnopharmacol., 96: 221-226.

Vieira LQ, dos Santos LM, Neumann E, da Silva AP, Moura LN, Nicoli JR (2008) Probiotics protect mice against experimental infections, Supp. J. Clin. Gastroenterol., 42(3): 168-169.

Wagner RD, Pierson C, Warner T, Dohnalek M, Farmer J, Roberts L, Hilty M, Balish E (1997). Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. Infect. Immun., 65: 4165-4172.

Wagner RD, Pierson C, Warne,r T, Dohnalek M, Hilty M, Balish E (2000). Probiotic effects of feeding heat-killed *Lactobacillus acidophils* and *Lactobacillus casei* to *Candida albicans*-colonized immunodeficient miceM. J. Food. Prot., 63: 638-644.