IN VITRO INHIBITORY ACTIVITY OF BIFIDOBACTERIUM AND LACTOBACILLUS STRAINS AGAINST CANDIDA ALBICANS

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Summary


One of the requirements to probiotic cultures is to exhibit high antimicrobial activity against pathogenic microorganisms. The aim of this work was to determine the degree of suppression of Candida albicans by strains lactobacilli and bifidobacteria of human origin. The antifungal activity of four strains of lactobacilli and bifidobacteria of human origin – Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum sp. 4 – against Candida albicans NBIMCC 74 by co-cultivation under static conditions at 37°C in skimmed milk was studied. Despite the high concentration of viable cells of lactobacilli or bifidobacteria, all four strains restrained the growth of the pathogen (biostatic action) and by the 72nd hour of co-cultivation the pathogen retained high concentration of viable cells. The measured high titratable acidity during the co-cultivation of lactobacilli or bifidobacteria and the pathogen did not affect the growth of the pathogen. The performed studies are of potential importance in the treatment of candidiasis in humans and animals. The tested strains are a primary basis for the creation of probiotics for the treatment of these serious diseases.

Key words: antifungal activity, bifidobacteria, Candida albicans, joint cultivation, lactobacilli, probiotic

INTRODUCTION

The yeast species Candida is a frequently encountered opportunistic pathogen in humans and can be isolated from 50% to 60% of the oral cavities of healthy adults. It also colonises the intestinal and vaginal epithelia (Samaranayake, 2002).

Candida albicans is a member of the indigenous microflora of the gastrointestinal (GI) tract and mucocutaneous membranes in healthy humans. Amongst Candida species, C. albicans is the most commonly isolated species from the oral cavity and is responsible for most superficial and systemic fungal infections (Odds, 1994). It is frequently involved in complicated systemic infections and mortality in patients undergoing chemotherapy for cancer (Eras et al., 1972; Bodey, 1984), immunosuppressive therapy (Myerowitz et al., 1977), or prolonged antibiotic therapy (Verghese et al., 1988). For example, nearly 90% of AIDS patients are infected with C. albicans (McCarthy, 1992). In addition, C. albi-
C. albicans was proposed to play a role in some cases of atopic diseases (Gumowski et al., 1987; Savolainen et al., 1993). Previous reports showed that antifungal drug therapy decreased both clinical scores and serum IgE levels in patients with atopic dermatitis (AD) who displayed IgE-mediated hypersensitivity to C. albicans (Bäck et al., 1995; Morita et al., 1999). C. albicans is rarely found in skin cultures (Aly et al., 1977; Keswick et al., 1987), but is frequently detected in faecal cultures of AD patients (Buslau et al., 1990). Although these findings suggest a relation between GI candidiasis and allergic diseases such as AD, there are few experimental studies supporting this idea (Yamaguchi et al., 2005).

Probiotics are live microorganisms that confer beneficial effects on the health of the host when administered in appropriate amounts (Kalliomaki et al., 2001; Brown & Valiere, 2004). Microorganisms involved in the composition of probiotics must have the following characteristics: 1) to be part of the natural microflora of humans and animals; 2) to have the ability to adhere to epithelial cells or cell lines; 3) to be able to survive in the environment of the stomach and the intestines, i.e. to survive under conditions of acidic pH in the stomach and to be resistant to the action of bile; 4) to be able to reproduce in the GI tract; 5) to allow industrial cultivation during which they accumulate high concentrations of viable cells; 6) to possess antimicrobial activity against conditionally pathogenic, carcinogenic and pathogenic microorganisms; 7) to produce antimicrobial substances; 8) to modulate the immune response and 9) to be safe for clinical and food applications. The studies of Saxelin et al., (1996 a,b), Donohue & Salminen (1996) and Salminen et al., (1998) demonstrate the safety of lactic acid bacteria and bifidobacteria. The strains belonging to the genera Lactobacillus, Lactococcus and Bifidobacterium bifidum are most often assigned a GRAS status.

The spectrum of probiotic action is expressed in a wide range of food, physiological and antimicrobial effects (Agerholm-Larsen et al., 2000; Nomoto, 2005). The beneficial effects include improvement of the health in gastro-intestinal infections, reduction of the serum cholesterol levels, protection of the immune system, inhibition of infection caused by Helicobacter pylori, Crohn's disease, restoration of the microflora in the stomach and intestines after antibiotic treatment, anticancer properties, antimutagenic effect, antidiarrhoeal properties etc. (Imasse et al., 2007; Shah, 2007).

One of the requirements to the probiotic cultures is a high antimicrobial activity against pathogenic microorganisms. Candidiasis is a disease that requires prolonged treatment, which involves the use of both antifungal antibiotics and probiotics. The bacterial microbiota of the stomach is vital in promoting gastric colonisation resistance against the opportunistic pathogen C. albicans. The stomach is a preferential niche for Candida colonisation as gastrectomised rodents are unable to support Candida colonisation (Artwohl et al., 1888). Within the stomach, Lactobacillus, an important contributor to host health, can prevent colonisation of Candida through displacement of yeast from the epithelial layer of the stomach. Previous studies demonstrated that penicillin treatment reduces Lactobacillus populations and promotes yeast colonisation of the gastric epithelium (Savage, 1969). Furthermore, in vitro assays have found that Lactobacillus spp. can significantly
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inhibit C. albicans germ tube formation (Noverr & Huffnagle, 2004). The ability of Lactobacillus to displace Candida from the epithelial layer of the stomach, inhibit hyphal invasion (Savage, 1969), and prevent germ tube formation (Noverr & Huffnagle, 2004; Noverr et al., 2004) has been also demonstrated.

Several studies have assessed the efficacy of probiotics for prophylaxis and therapy of C. albicans infections (Hilton et al. 1992; Berg et al., 1993; De Petrino et al., 1995; Satonaka et al., 1996). Vaginitis in apparently healthy women can be caused by C. albicans, and the ingestion of yogurt containing Lactobacillus acidophilus has been reported to reduce the occurrence of recurrent vaginal candidiasis (Hilton et al., 1992). Laboratory animal studies also suggest that probiotics may be useful for the prevention of candidiasis. Mice immunosuppressed with corticoid drugs recovered more quickly from orogastric candidiasis when they were fed cultures of L. acidophilus, L. casei and L. delbrueckii prior to oral C. albicans challenge (De Petrino et al., 1995). Oral administration of heat-killed Enterococcus faecalis prior to oral and systemic infection of cyclophosphamide-treated mice with C. albicans prolonged their survival (Satonaka et al., 1996).

The aim of the present work was to determine the degree of inhibition of Candida albicans by lactobacilli and bifidobacteria of human origin by joint cultivation of the respective Lactobacillus or Bifidobacterium strain and the pathogen.

MATERIALS AND METHODS

Strains and media
The strains used in the present research were: Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium sp. Bif. 4 of human origin. The four strains of the genus Lactobacillus were isolated by the authors and currently included in the collection of microorganisms of the Department of Microbiology at the University of Food Technologies, Plovdiv, Bulgaria. The studied strains were cultivated in a liquid medium (skimmed milk) at 37 °C. They were isolated from a single colony and cultivated in skimmed milk until coagulation, and stored as a stock-culture in skimmed milk with 5% v/v sucrose at −20 °C. The strains have been identified to species category by physiological, biochemical and molecular-genetic methods (unpublished data).

Candida albicans NBIMCC 74 was delivered by the NBIMCC, Sofia, Bulgaria.

The used media were:
- Sterile skimmed milk with titratable acidity 16–18ºT;
- Saline solution;
- LAPTg10-broth;
- LAPTg10-agar;
- LBG-agar;
- Solid medium for bifidobacteria.

Preparation of Candida suspensions
Prior to the experiment, Candida albicans NBIMCC 74 was cultured at 37 °C for 18 h on Sabouraud’s dextrose agar (SDA), and a loopful of growth was inoculated into saline solution to 10^7 CFU/cm^2 by optical density adjustment.

Determination of the antimicrobial activity of lactobacilli and bifidobacteria against the pathogen Candida albicans NBIMCC 74
To determine the antimicrobial activity of Lactobacillus acidophilus A2, Lactoba-
cillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum Bif. 4 against Candida albicans NBIMCC 74, 24-hour cultures of the lactobacilli or bifidobacteria strains developed in skimmed milk were used. The following experimental design was applied:
1) pathogen control – 9.5 cm³ skimmed milk + 0.5 cm³ suspension of the pathogen Candida albicans NBIMCC 74 in saline solution (concentration 10⁸ CFU.cm⁻³).
2) Lactobacillus or Bifidobacterium control – 9.5 cm³ skimmed milk + 0.5 cm³ of the 24-hour culture of the respective Lactobacillus or Bifidobacterium strain (concentration 10⁸ CFU.cm⁻³).
3) Mixture – 9 cm³ skimmed milk + 0.5 cm³ of the 24-hour culture of the respective Lactobacillus or Bifidobacterium strain + 0.5 cm³ of the suspension of the pathogen Candida albicans NBIMCC 74 in saline solution (concentration 10⁸ CFU.cm⁻³).

All samples were incubated under static conditions in a thermostat at 37±1 °C for 72 hours, collecting samples at 0, 12, 24, 36, 48, 60 and 72 hours of incubation. The changes in the titratable acidity and the concentration of viable cells of the pathogen and of the respective Lactobacillus or the Bifidobacterium strains were monitored. The titratable acidity was determined by titration of each sample with 0.1 N NaOH using phenolphthalein as an indicator until the appearance of a pale pink colour persisting over 1 min. One Torner degree (°Т) corresponds to 1 cm³ 0.1 N NaOH, needed for the neutralisation of an equivalent amount of organic acid, contained in 100 cm³ of the cultural medium (Denkova, 2005).

Statistical analysis

Data from triplicate experiments were processed using MS Office Excel 2003 software, at level of significance of P<0.05.

RESULTS

The degree of inhibition of Candida albicans NBIMCC 74 by strains of bifidobacteria and lactobacilli of human origin – Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum Bif. 4 – was determined by joint cultivation of the lactobacilli or bifidobacteria and the pathogen under static conditions at 37±1°C. The changes in the number of viable cells (Fig. 1, 3, 5 and 7) of the Lactobacillus or the Bifidobacterium strain and of the pathogen in the respective controls and in the mixtures, as
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During the separate cultivation each of the tested strains of lactobacilli and bifidobacteria – Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum Bif. 4 –

well as in the titratable acidity were traced (Fig. 2, 4, 6 and 8).

During the separate cultivation each of the tested strains of lactobacilli and bifidobacteria – Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum Bif. 4 –
accumulated a high concentration of viable cells after 72 h of incubation (about $10^{14} \text{CFU.cm}^{-3}$), while the concentration of *C. albicans* NBIMCC 74 reached $2 \times 10^9 \text{CFU.cm}^{-3}$. In separate cultivation the titratable acidity reaches 73.47 °T for *bifidobacteria* and 227.39 °T–281.33 °T for the *Lactobacillus* strains, while the titratable acidity of *Candida albicans* NBIMCC 74 control decreased during separate cultivation of the strain.

During joint cultivation of *Lacto-

![Fig. 3. Survival of Lactobacillus acidophilus Ac and Candida albicans NBIMCC 74 during either separate cultivation or co-cultivation at 37±1°C.](image)

![Fig. 4. Change in the titratable acidity (°T) of the medium during separate cultivation of Lactobacillus acidophilus Ac and Candida albicans NBIMCC 74 and during co-cultivation of the two microorganisms at 37±1°C.](image)
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bacillus acidophilus A2 and Candida albicans NBIMCC 74, an increase in the concentration of viable cells of both microorganisms during the first 36 h was observed, after which the concentration of viable lactobacilli cells continued to increase, while that of the pathogen was slightly reduced and by the 72nd h the concentration of viable C. albicans cells was $1 \times 10^5$ CFU.cm$^{-3}$ (Fig. 1).
The pattern of inhibitory activity of *Lactobacillus acidophilus* Ac against *C. albicans* NBIMCC 74 was exhibited though an increase in the concentration of viable cells of *Lactobacillus acidophilus* Ac and *C. albicans* NBIMCC 74 during the first 12 h, then the concentration of viable lactobacilli cells continued to increase, while that of the pathogen was slightly reduced to attain $6 \times 10^5$ CFU.cm$^{-3}$ by the 72$^{nd}$ hour (Fig. 3).

The joint cultivation of *Lactobacillus delbrueckii* ssp. *bulgaricus* GB and *Candida albicans* NBIMCC 74 was characterised by maintaining high concentration of living cells of the pathogen until the end of the process – $3 \times 10^5$ CFU.cm$^{-3}$ by the 72$^{nd}$ h, regardless of the increase in the concentration of viable cells of *L. delbrueckii* ssp. *bulgaricus* GB (Fig. 5).

The concentrations of viable cells of *Bifidobacterium bifidum* Bif. 4 and of the pathogen increased during the entire incubation. The number of viable cells of the pathogen remained high until the 72$^{nd}$ hour at $1 \times 10^6$ CFU.cm$^{-3}$ (Fig. 7).

The titratable acidity of the pathogen control was significantly lower than that of the lactobacilli control and each of the mixtures (Fig. 2, 4, 6 and 8). *Lactobacillus acidophilus* A2, *Lactobacillus acidophilus* Ac, *Lactobacillus delbrueckii* ssp. *bulgaricus* GB and *Bifidobacterium* sp. Bif. 4 exerted a biostatic effect on the growth of the pathogen due to the accumulation of lactic and other organic acids which leads to lowering the pH of the medium increasing the titratable acidity in the mixtures. However, by the 72$^{nd}$ h, the viability of *C. albicans* was preserved (Fig. 1, 3, 5 and 7).

**DISCUSSION**

Despite numerous therapeutic improvements, especially in the field of antibiotic
therapy, gastrointestinal infections and their consequences remain a major clinical problem. In addition, there has been a dramatic increase in the incidence of antibiotic-resistant microbial pathogens. There is a concern that the industry will no longer be able to develop effective antibiotics at a rate sufficient to compete with the development of microbial resistance to older antibiotics. These factors have renewed the interest in the possibility of deliberately feeding beneficial microorganisms to humans as an alternative to antibiotic therapy in gastrointestinal disorders. Probiotics are also an attractive treatment alternative as they allow avoiding antibiotics, including antifungals, which further delay recolonisation by normal colonic flora (Rolfe, 2000).

The mechanisms underlying the inhibitory activity of lactic acid bacteria and bifidobacteria against pathogens, including *C. albicans* appear to be multifaceted, including the production of hydrogen peroxide, lactic acid, and antibacterial compounds such as bacteriocins or bacteriocin-like molecules, nonbacteriocin molecules, and non-lactic acid molecules (McGroarty & Reid, 1988; Servin, 2004; Kaewsrichan et al., 2006). For example, hydrogen peroxide-producing *Lactobacillus* strains exhibited an antagonistic effect against *C. albicans* (Kaewsrichan et al., 2006).

The obtained results confirm the results of Simsek et al. (2006) and Cizeikiene et al. (2013) that lactic acid bacteria and bifidobacteria exert a biostatic effect on the growth of yeasts including *C. albicans*. This is of particular importance for the combined therapy of candidiases.

In conclusion, despite the reported high titratable acidity during joint cultivation of the strains of lactobacilli or bifidobacteria and *C. albicans* NBIMCC 74, the growth of the pathogen was influenced slightly. Anyway, the performed studies are of potential importance for the treat-
ment of candidiasis in people and animals. The studied strains of lactobacilli and bifidobacteria of human origin (Lactobacillus acidophilus A2, Lactobacillus acidophilus Ac, Lactobacillus delbrueckii ssp. bulgaricus GB and Bifidobacterium bifidum Bif. 4) can be considered promising as a basis for the development of probiotics as part of the comprehensive treatment of these serious diseases, due to the more unfavourable conditions for development of C. albicans.

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