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

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Summary

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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-*

cans 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) 2 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

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 ºC. 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 ºC. 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 10^8 CFU/cm^3 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 delbrieckii* 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 cm^3 skimmed milk + 0.5 cm^3 suspension of the pathogen *Candida albicans* NBIMCC 74 in saline solution (concentration 10^8 CFU.cm^{-3}).
2) *Lactobacillus* or *Bifidobacterium* control – 9.5 cm^3 skimmed milk + 0.5 cm^3 of the 24-hour culture of the respective *Lactobacillus* or *Bifidobacterium* strain (concentration 10^8 CFU.cm^{-3}).
3) Mixture – 9 cm^3 skimmed milk + 0.5 cm^3 of the 24-hour culture of the respective *Lactobacillus* or *Bifidobacterium* strain + 0.5 cm^3 of the suspension of the pathogen *Candida albicans* NBIMCC 74 in saline solution (concentration 10^8 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 measured on 0^{th}, 24^{th}, 48^{th}, 60^{th} and 72^{nd} hour of cultivation, while the number of viable cells – on 0^{th}, 12^{th}, 24^{th}, 36^{th}, 48^{th}, 60^{th} and 72^{nd} hour of incubation.

Plate count method

Serial tenfold dilutions of all samples were made in 0.5% saline solution and used to inoculate triplicate LAPTg10-agar plates for the enumeration of the *Lactobacillus* strain; LBG-agar plates for the enumeration of the pathogen by the spread plate method and solid medium for bifidobacteria by the pour plate method. These were incubated at 37 ±1 °C for 72 hours to determine the bacterial count (Frank & Yousef, 2004). The count of the colonies was then used to estimate the number of bacteria or yeasts in the original sample.

Determination of the titratable acidity

Ten cm^3 of each sample were mixed with 20 cm^3 of distilled water. 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 (°T) corresponds to 1 cm^3 0.1 N NaOH, needed for the neutralisation of an equivalent amount of organic acid, contained in 100 cm^3 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

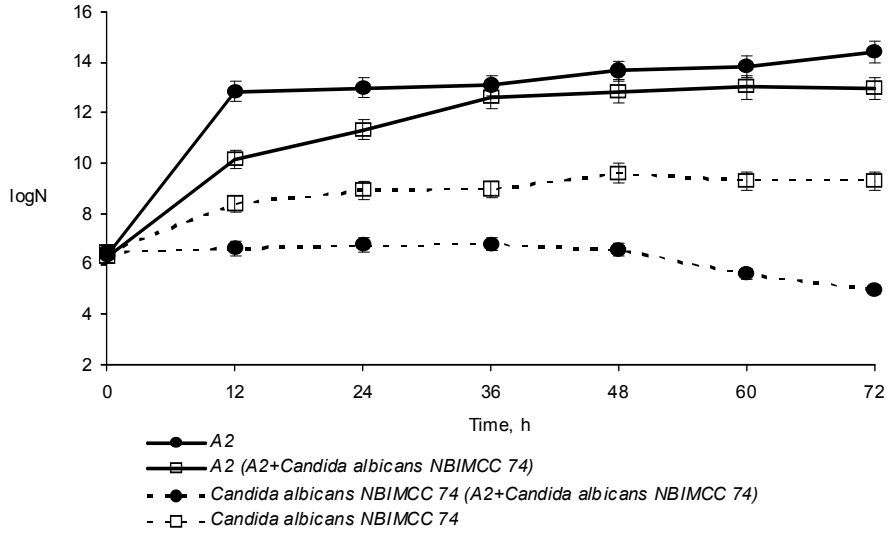

Fig. 1. Survival of *Lactobacillus acidophilus* A2 and *Candida albicans* NBIMCC 74 during either separate cultivation or co-cultivation at 37±1 °C.

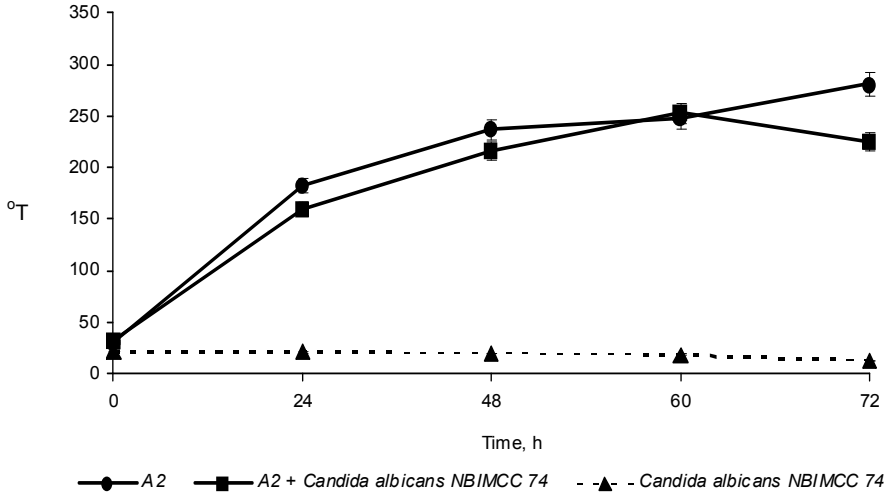

Fig. 2. Change in the titratable acidity (°T) of the medium during separate cultivation of *Lactobacillus acidophilus* A2 and *Candida albicans* NBIMCC 74 and during co-cultivation of the two microorganisms at 37±1°C.

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} CFU.cm^{-3}), while the concentration of *C. albicans* NBIMCC 74 reached 2×10^{9} 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-*

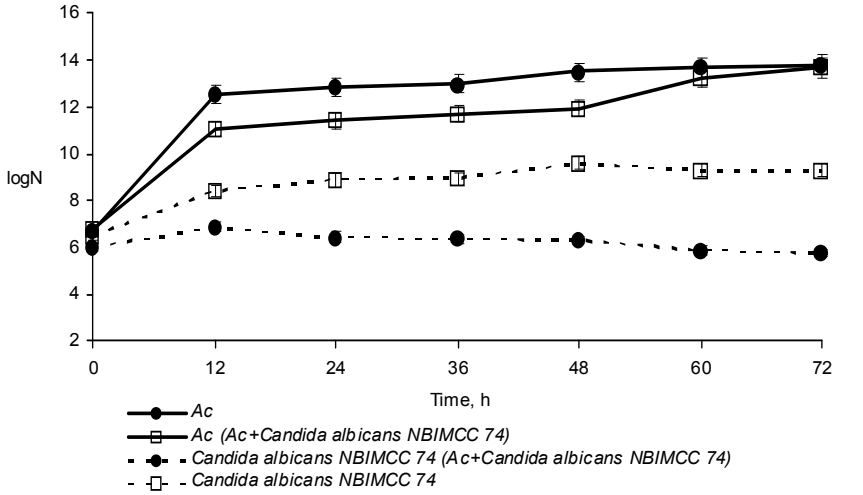

Fig. 3. Survival of *Lactobacillus acidophilus* Ac and *Candida albicans* NBIMCC 74 during either separate cultivation or co-cultivation at 37±1°C.

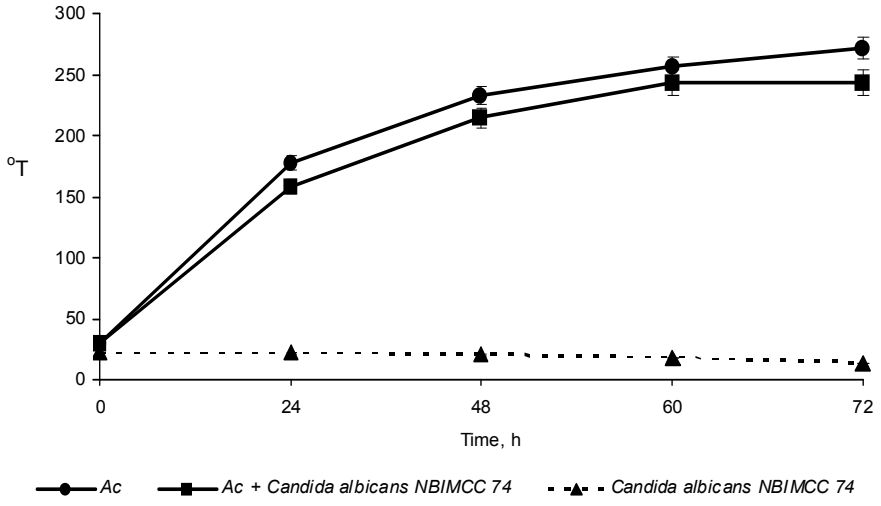

Fig. 4. Change in the titratable acidity (°T) of the medium during separate cultivation of *Lactobacillus acidophilus* Ac and *Candida albicans* NBIMCC 74 and during co-cultivation of the two microorganisms at 37±1°C.

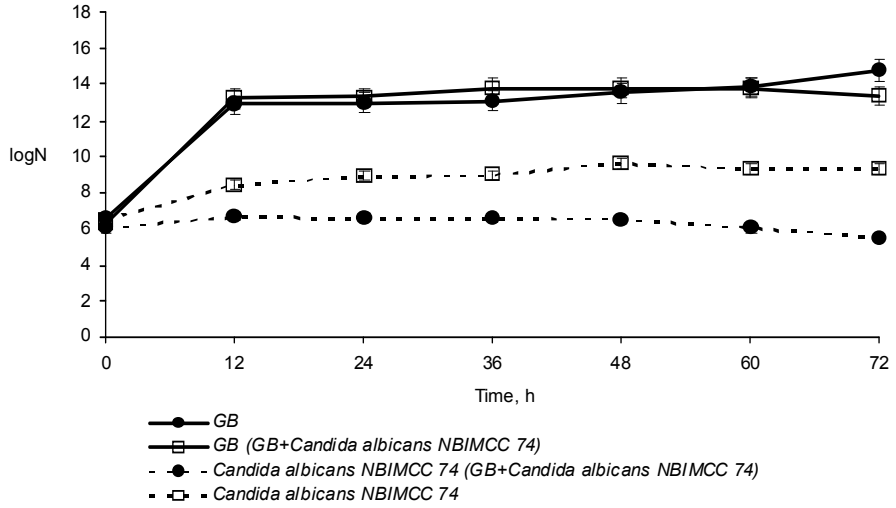

Fig. 5. Survival of *Lactobacillus delbrueckii* ssp. *bulgaricus* GB and *Candida albicans* NBIMCC 74 during either separate cultivation or co-cultivation at 37±1°C.

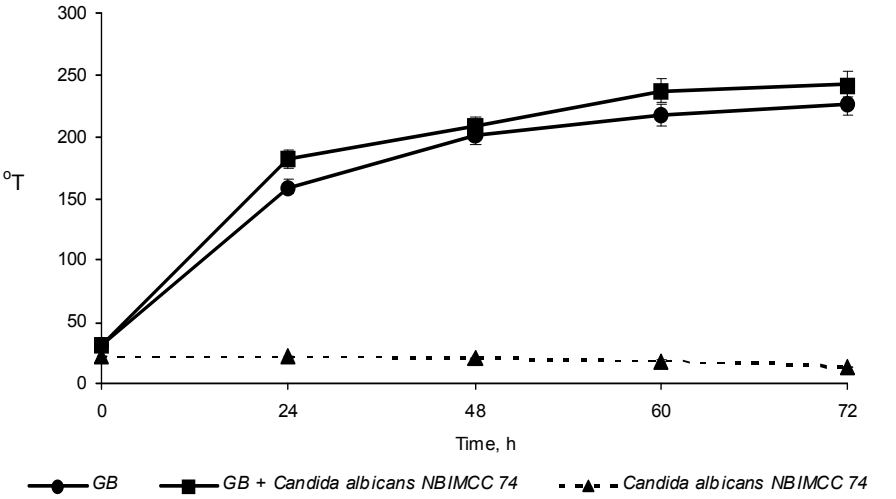

Fig. 6. Change in the titratable acidity (°T) of the medium during separate cultivation of *Lactobacillus delbrueckii* ssp. *bulgaricus* GB and *Candida albicans* NBIMCC 74 and during co-cultivation of the two microorganisms at 37±1°C.

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 1×10^5 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 6×10^5 CFU.cm^{-3} by the 72nd 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 – 3×10^5 CFU.cm^{-3} by the 72nd 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 72nd hour at 1×10^6 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 72nd 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

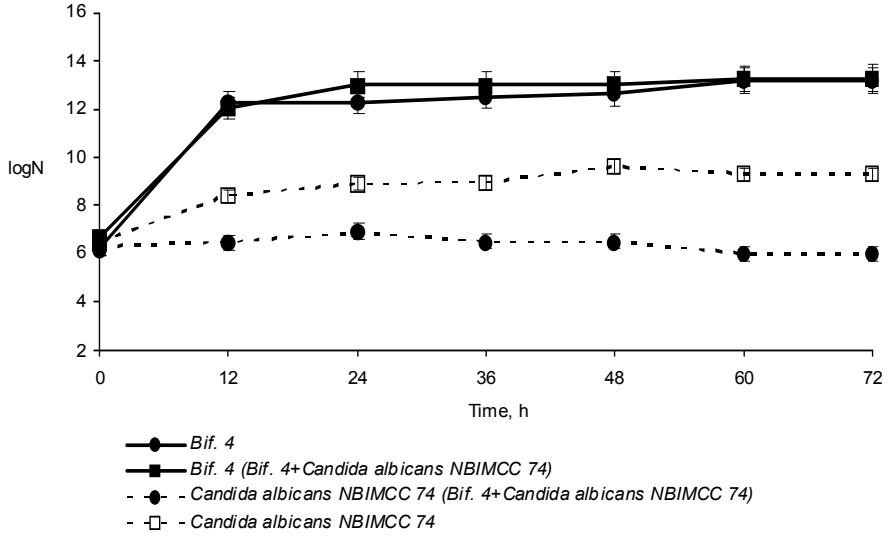

Fig. 7. Survival of *Bifidobacterium bifidum* Bif. 4 and *Candida albicans* NBIMCC 74 during during either separate cultivation or co-cultivation at 37±1 °C.

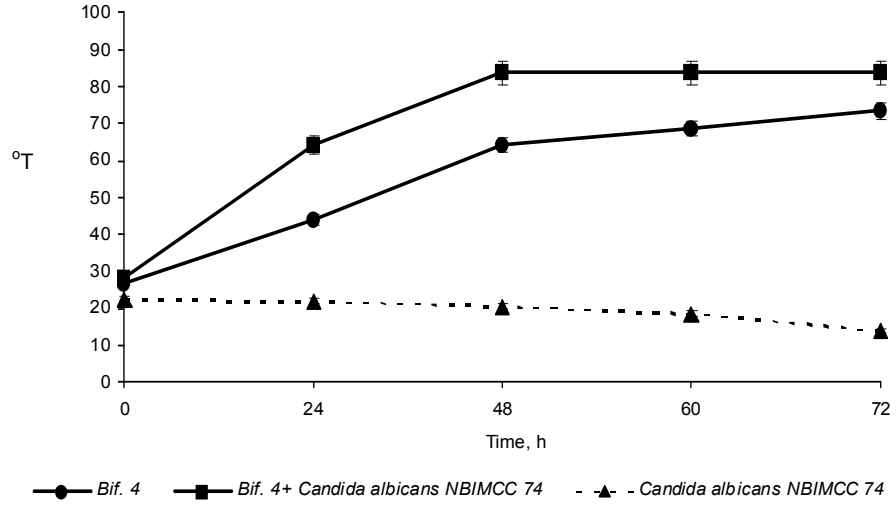

Fig. 8. Change in the titratable acidity (°T) of the medium during separate cultivation of *Bifidobacterium bifidum* Bif. 4 and *Candida albicans* NBIMCC 74 and during co-cultivation of the two microorganisms at 37±1°C.

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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