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Preliminary probiotic selection of dairy and human yeast strains

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Probiotics are bacteria or yeast cells that, after delivery into the gastrointestinal tract, exert beneficial effects to the host. Despite the occurrence of yeasts in many dairy related products and in the human gastrointestinal tract, their potential role as probiotics has been overlooked. In this study we evaluated the *in vitro* probiotic properties of yeast strains isolated from feta cheese or infants' gastrointestinal tract. All strains tested (except one strain) contained no plasmid DNA and exhibited *in vitro* probiotic properties such as acid and bile tolerance, adhesion to Caco-2 cells, cholesterol removal ability and immunostimulatory activity. Thus, these are potential candidate strains to be used as gene delivery and expression systems for therapeutic or vaccination purposes as well as probiotics with the ability to decrease cholesterol levels. Moreover, we showed that besides the strains of the *Saccharomyces* genus other yeast species such as *Kluyveromyces lactis* may also be considered as presumptive probiotics. However, despite the above promising results, additional experimentation is required to examine the effects of these yeast strains in the gastrointestinal tract after their oral administration.

Key words: adherence, cholesterol reduction, mitogenic activity, probiotic, yeasts.

INTRODUCTION

Several non-pathogenic microorganisms, which are classified in the status of Generally Recognized As Safe (GRAS), are used as probiotics, as live bacterial vaccine or as gene delivery vectors (Steidler, 2003; Braat *et al.*, 2006). In recent years, much attention has been paid to the design of functional foods that contain probiotic microbial strains responsible for beneficial health effects on the host. Probiotics are living microorganisms which, upon ingestion in adequate amounts, confer health benefits to the host (FAO/WHO, 2002). The local or systemic health benefits of probiotics are achieved by bacterial antagonism that improves the intestinal function or microflora composition, and by immunomodulation, which help the healthy host to maintain a "physiological state of inflammation" in the intestine or to control several infectious, inflammatory and immunologic reactions (Maldonado Galdeano *et al.*, 2007).

Potential probiotic strains have to bear specific characteristics such as the ability to modulate immune responses and the antimicrobial activity (Ouwehand *et al.*, 1999; Tuomola *et al.*, 2001; FAO/WHO, 2002). In addition, probiotic strains should be safe and metabolically active within the gastrointestinal tract, resist gastric acid and bile and should be able to colonize the intestine, at least temporarily, by adhering to the intestinal epithelium. Control of serum cholesterol may also be considered among probiotic criteria. Probiotic effects are strain specific and may vary according to the properties of an individual strain. Moreover, *in vivo* testing is expensive and time con-

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suming and requires approval by ethical committees. Therefore, reliable *in vitro* systems which reflect specific effects and allow the selection of promising strains among numerous microorganisms have been developed and used (Tuomola *et al.*, 2001; FAO/WHO, 2002).

The most extensively studied probiotics belong to the genera *Lactobacillus* and *Bifidiobacterium* (Borriello *et al.*, 2003; Tuohy *et al.*, 2003). Despite the occurrence of yeasts in many dairy related products (Fleet, 1990; Jakobsen & Narvhus, 1996) and in the human gastrointestinal tract (Knoke, 1999; Czerucka *et al.*, 2007), their potential as probiotics has been overlooked. *Saccharomyces boulardii* and brewer's yeast are practically the only yeasts commercialized as probiotic in human medicine (Sargent & Wickens, 2004; Czerucka *et al.*, 2007). In addition, several yeast strains such as *Kluyveromyces lactis*, *K. marxianus*, *Isaatchenkia orientalis*, *S. cerevisiae* and *Debaryomyces hansenii* have shown antifungal, antibacterial, antiinflammatory and antitumoral activity (Oh *et al.*, 2002; Diniz *et al.*, 2003; Lopitz-Otsoa *et al.*, 2006).

Other microorganisms were also evaluated for their potential use as live recombinant vaccines (Medina & Guzman, 2001; Fernandez-Arenas *et al.*, 2004; Upadhyaya & Manjunath, 2009). These include non-colonizing and non-pathogenic "harmless" microorganisms that exist in foods and commensal microorganisms members of the human microflora. The immuno-adjuvant activity of these microorganisms is based on their capacity to activate innate as well as adaptive immunity including production of chemokines and cytokines.

In previous study, several *Candida albicans*, *C. parapsilosis*, *D. hansenii*, *I. orientalis*, *K. lactis*, *S. cerevisiae* and *S. boulardii* yeast strains were isolated from infants' gastrointestinal tract (faeces) or feta cheese (Tzanetakis *et al.*, 1996; Andrighetto *et al.*, 2000; Psomas *et al.*, 2001). The investigation of some of the above strains revealed that the *S. cerevisiae* 832 and *S. boulardii* KK1 strains are potential presumptive probiotics as they exhibited *in vitro* probiotic properties such as acid and bile tolerance and cholesterol removal ability (Psomas *et al.*, 2003). In this study, the *in vitro* probiotic properties of 16 of the above strains, as well as four reference strains, were further investigated including acid and bile tolerance, cholesterol removal ability, antibacterial activity, Caco-2 cell line adhesive capacity as well as their *in vitro* immunostimulatory activity on mouse and rat splenocytes.

MATERIALS AND METHODS

Yeast strains

The yeast strains (Table 1) were obtained from the collection of the Laboratory of Food Microbiology and Hygiene, Aristotle University of Thessaloniki. They were isolated from feta cheese and infants' gastrointestinal tract (Tzanetakis *et al.*, 1996; Andrighetto *et al.*, 2000; Psomas *et al.*, 2001). Two of them, the strains *S. cerevisiae* 832 and *S. boulardii* KK1 were previously characterized as promising probiotics (Psomas *et al.*, 2003) since they possessed *in vitro* probiotic properties including acid and bile tolerance and cholesterol removal ability. The probiotic strain *Saccharomyces boulardii* UL (Ultra Levure, Biocodex, France) was also included in our experiments as positive control. The strains *Saccharomyces cerevisiae* NCYC167, *Debaryomyces hansenii* NCYC9 (NCYC – National Collection of Yeast Cultures, Norwich, UK) and *Candida parapsilosis* CBS1954 (CBS – Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands) were included as control strains.

TABLE 1. Yeast strains included in this study and their origin

Origin	Species[1]	Strain
Feta cheese	*Saccharomyces cerevisiae*	746 832 840α 952 982
Infants' faeces	*Saccharomyces boulardii*	KK1
Ultra Levure	*Saccharomyces boulardii*	UL
Feta cheese	*Kluyveromyces lactis*	570 630
Feta cheese	*Debaryomyces hansenii*	414
Infants' faeces	*Candida albicans*	KK2.1 KK2.5 KK3.1 KK4.1
	Candida parapsilosis	KK6.5 KK6P
	Issatchenkia orientalis	KK5Y3
Reference strains	*Saccharomyces cerevisiae*	NCYC167
	Debaryomyces hansenii	NCYC9
	Candida parapsilosis	CBS1954

[1] Yeast identification according to Tzanetakis *et al.* (1996), Andrighetto *et al.* (2000) & Kourelis A. (unpublished data)

Culture conditions and preparation of yeast extracts

Yeasts were grown in Yeast Extract Glucose Peptone (YEGP) broth [2% (w/v) glucose, 0.5% (w/v) yeast extract, 1% (w/v) peptone] at 30˚C for 20-24 hrs. A YEGP agar slope [2% (w/v) glucose, 0·5% (w/v) yeast extract, 1% (w/v) peptone, 1.5% (w/v) agar] was used for the maintenance of the cultures at 4˚C. All strains were subcultured at least three times prior to experimental use.

Precultured yeasts ($\sim 3 \times 10^9$ CFU) were washed three times with phosphate buffered saline (PBS) and resuspended in RPMI-1640 medium containing 2 mM L-glutamine and 24 mM $NaHCO_3$. The suspensions were then autoclaved (121˚C, 1 Atm) for 15 min to break down the cells. Yeast extracts were harvested from the autoclaved yeast suspensions by centrifugation at 1500×g for 10 min. To remove viable and non-viable intact yeast cells each yeast extract suspension was finally filtered through a 0.22 μm pore filter.

Effects of low pH and bile on viability and growth of yeast strains

Growth at low pH was assessed by transferring 0.5 ml of 6 McFarland turbidity of yeast-Ringer suspension in 6 ml PBS pH 3.0 (HCl adjusted) and incubation at 37˚C for 3 hrs (Conway *et al.*, 1987). Viable cells were enumerated in YEGP agar plates after incubation at 30˚C for 48 hrs. Each test was performed three times.

Growth in the presence of bile salts was evaluated by preparing decimal dilutions of a 6 McFarland turbidity yeast-Ringer suspension in YEGP plates containing 0%, 0.15%, 0.3% and 0.5 % (w/v) Oxgall Dehydrated Bile (BBL) as described previously (Klaenhammer & Kleeman, 1981). Yeast colonies were enumerated after incubation of the agar plates at 37˚C for 48 hrs. Each test was performed three times.

Antimicrobial activity

The inhibitory effects of yeast strains on foodborne pathogens and yoghurt starter strains were determined with the well diffusion method (Ahn & Stiles, 1990) and after simultaneous growth with the target microorganisms (Barefoot & Klaenhammer, 1983). The target microorganisms were the yoghurt starter strains *Lactobacillus casei* ATCC 334 (ATCC: American Type Culture Collection, Manassas, USA), *Lactobacillus reuteri* DSM 20016 (DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) and the foodborne pathogens *Clostridium tyrobutyricum* NCDO1754 (NCDO: National Collection of Dairy Organisms, Reading, UK), *Bacillus cereus*, *Clostridium sporogenes* C2210, *Enterococcus faecalis* EF1, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica* (collection of the Laboratory of Food Microbiology and Hygiene, Aristotle University of Thessaloniki, Greece).

For the well diffusion method sterilized supernatants from 4, 8, 16 and 24 hrs yeast cultures were studied for their inhibitory effects. A clear zone of more than 1 mm around a spot was scored as positive. Each test was performed three times. The strain *S. cerevisiae* AA4 with proven antibacterial activity against *Yersinia enterocolitica* (Nikolaou *et al*., 2006) was used as positive control.

For the simultaneous growth assay 100 μl of each target strain were plated on YEGP plates. Then, yeast strains were inoculated with a sterile microbiological loop on each plate. After 24 hrs incubation at 30˚C or 37˚C inhibition zones were read. Each test was performed three times.

Plasmid profiles

Plasmid DNA was extracted from 5 ml of two overnight cultures in YEGP broth using ChargeSwitch Plasmid Yeast kit (Invitrogen) following the proposed instructions by the manufacturer.

In vitro reduction of cholesterol

The ability of yeast strains to lower cholesterol levels *in vitro* was determined according to the method of Pereira & Gibson (2002) with some modifications. Briefly, yeast cells were cultured for 48 hrs at 37˚C in YEGP broth supplemented with 0.3% (w/v) Oxgall and 400 μg ml^{-1} of a filtered water-soluble form of cholesterol (polyoxyethanyl-cholesterol sebacate; Sigma). At 0, 24 and 48 hrs, 500 μl medium were collected and centrifuged at 18000×g for 15 min. Cholesterol concentration in the medium supernatant was measured in 100 μl freshly collected samples using the cholesterol Randox kit (Randox Laboratories Ltd.).

Adhesion assay

The culture of Caco-2 cells and adhesion assay were performed by the method described by Kumura *et al.* (2004) with the following modification. Briefly, Caco-2 cells were grown in complete Eagle Minimum Es-

sential Medium (EMEM) containing 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 × Non-Essential Amino Acids (NEAA), supplemented with 10% Foetal Bovine Serum (FBS). At postconfluence, 10^7 CFU ml^{-1} yeast cells in basal medium (EMEM without FBS and antibiotics) were added on the Caco-2 monolayers and incubated at 37°C for 90 min in 5% CO_2 atmosphere. After incubation, the monolayers were washed four times with PBS, fixed with methanol and stained with Giemsa solution. The adhesion assay was conducted in triplicate and the number of Caco-2 cells with adherent yeasts was counted in 20 random microscopic areas. Adhesion ability was expressed as the percentage of Caco-2 cells with adherent yeasts as well as the number of yeasts adhering to 100 Caco-2 cells.

Mitogenic activity of yeast extracts

The capacity of serial two fold dilutions of selected yeast strains' extracts to induce the proliferation of immune cells was performed using cultures of mouse or rat spleen cells under conditions previously described (Hadjipetrou-Kourounakis & Yiangou, 1988). Spleen cells were obtained after homogenization of spleen aseptically removed from mice or rats housed in our accredited animal facility (number EL 54 BIO 02, School of Biology, Aristotle University of Thessaloniki) in a controlled atmosphere. Mouse or rat spleen cells were suspended in RPMI-1640 complete medium containing 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 24 mM $NaHCO_3$, supplemented with 5% FBS. They were cultured in 96-well microtiter plates, at a final cell density of 1×10^6 cells ml^{-1}, in a total volume of 200 μl, at 37°C in 5% CO_2 atmosphere. In all cultures 20 μl containing 0.4 μCi tritiated thymidine (^{3}H-TdR) were added 18 hrs before cell harvesting. Proliferation of mouse or rat spleen cells was evaluated 72 hrs after culture initiation by measuring the tritiated thymidine incorporation in a liquid scintillation spectroscopy and is expressed as the mean number of Stimulation Index (SI). Samples with SI > 2 were considered positive.

Statistical Analysis

Results were expressed as mean ± SEM. Multiple comparisons were performed by one-way ANOVA followed by Tukey's test and statistical significance was accepted at values of $p < 0.05$. Each experiment was repeated three times. All statistical calculations were carried out with Microcal Origin 7.5 software (Microcal Software, Northampton, Maine).

RESULTS

Acid and bile tolerance of yeast strains

Potential probiotic strains should be able to survive within the gastrointestinal tract. Because tolerance to low pH and bile salts is seen as a prerequisite for strain survival through the gastrointestinal tract, qualitative analyses were carried out for the yeast strains. The viable counts of all yeast strains decreased after 3 hrs in PBS pH 3.0 and their inhibition ranged between 77% and 97% (Table 2). The strains *S. cerevisiae* 982, *S. boulardii* KK1 and *K. lactis* (570, 630), as well as the probiotic strain UL, exhibited significant higher capacity to survive at pH 3.0 in comparison with the control and the other yeast strains ($p < 0.05$).

Survival patterns of the yeast strains exposed to various concentrations of bile salts showed variation among the test strains regarding their ability to tolerate or grow in the presence of bile salts (Table 3). All yeasts (with the exception of the strain 952), including the control strains, could survive in the presence of bile salts. The strain 952 could not tolerate bile as the inhibition percentage was significantly higher than that of the other strains ($p < 0.05$).

Antimicrobial activity and plasmid profiles

The effects of yeast culture sterilized supernatants or yeast viable cells on the growth of selected foodborne pathogens and yoghurt starter strains were monitored. None of the yeast strains showed any antibacterial or antagonistic activity against the selected strains as no inhibition zone was observed in neither of the two assays performed (Table 4). Analysis of plasmid profiles showed that the yeast strains did not contain plasmids (data not shown).

Adhesion ability

Adherent candidate probiotic strains are considered to easily colonize the intestine which is thought to be important for their positive effects (Ouwehand & Salminen, 2003). Except the strains of the genus *Saccharomyces*, all the other strains possessed significantly higher adhesion ability to Caco-2 cells (Table 4, $p < 0.05$). Among them, the number of adherent cells of the *C. parapsilosis* KK6.5 and KK6P strains was the highest with over 300 yeasts adhering per 100 Caco-2 cells ($p < 0.05$).

TABLE 2. Ability of yeast strains to survive at pH 3.0

Strains	Survival at pH 3.0		% inhibition[1]
	Viable counts (log_{10}cfu ml^{-1})		
	0 hrs	3 hrs	
S. cerevisiae			
NCYC167	7.41 ± 0.07	5.87 ± 0.04	97.09 ± 0.16[a]
746	7.75 ± 0.04	6.56 ± 0.30	92.39 ± 4.03[a]
832	7.34 ± 0.17	6.09 ± 0.28	94.35 ± 0.15[a]
840α	7.82 ± 0.06	6.58 ± 0.18	93.59 ± 2.94[a]
952	7.66 ± 0.02	6.18 ± 0.01	96.73 ± 0.19[a]
982	7.49 ± 0.12	6.48 ± 0.02	89.80 ± 2.95[b]
S. boulardii			
KK1	7.41 ± 0.40	6.58 ± 0.45	85.07 ± 1.69[b]
UL	7.56 ± 0.43	6.56 ± 0.40	89.93 ± 0.59[b]
K. lactis			
570	7.61 ± 0.07	6.61 ± 0.04	89.63 ± 2.50[b]
630	6.95 ± 0.05	6.31 ± 0.26	77.28 ± 6.16[b]
D. hansenii			
NCYC9	7.95 ± 0.07	6.37 ± 0.02	97.39 ± 0.32[a]
414	7.50 ± 0.05	6.21 ± 0.01	94.78 ± 0.49[a]
I. orientalis			
KK5Y3	7.93 ± 0.12	6.87 ± 0.13	91.25 ± 0.16[a]
C. albicans			
KK2.1	7.67 ± 0.24	6.43 ± 0.05	93.70 ± 2.66[a]
KK2.5	7.48 ± 0.57	6.28 ± 0.36	92.90 ± 3.14[a]
KK3.1	7.70 ± 0.55	6.43 ± 0.44	94.37 ± 1.38[a]
KK4.1	7.75 ± 0.34	6.68 ± 0.35	91.38 ± 0.31[a]
C. parapsilosis			
CBS1954	7.44 ± 0.05	5.95 ± 0.02	96.71 ± 0.53[a]
KK6.5	7.26 ± 0.08	6.11 ± 0.01	92.83 ± 1.16[a]
KK6P	7.01 ± 0.50	5.85 ± 0.34	92.64 ± 2.64[a]

Means with different letters are significantly different at $p < 0.05$

[1] Mean [100 × (cfu 0 hrs – cfu 3 hrs at pH 3.0) / (cfu 0 hrs)] ± SEM

Cholesterol reduction

Cholesterol assimilation experiments revealed a variation among the yeast strains examined on their ability to remove cholesterol *in vitro*. All strains were able to lower cholesterol levels after 24 and 48 hrs (Fig. 1) while the strains *S. cerevisiae* 952, *S. boulardii* KK1 and *C. parapsilosis* KK6P showed maximum cholesterol removal ability after 24 hrs. The maximum percentage of cholesterol removal ranged from 4% (*S. cerevisiae* 832 strain) to 60% (*S. cerevisiae* 952 strain). The *Saccharomyces* strains exhibited the greatest variation in their ability to lower cholesterol levels (4-60%). The *S. cerevisiae* 832 strain and the *D. hansenii* strains (NCYC9, 414) exhibited low values in maximum cholesterol reducing ability (group a, $p < 0.05$). The control strains NCYC167 and CBS1954 along with the strains *S. boulardii* UL, *K. lactis* 570 and *S. cerevisiae* 746, 840α and 982 showed moderate maximum cholesterol reducing capacity (group b, $p < 0.05$). Moreover, the strains *K. lactis* 630, *S. cerevisiae* 952, *S. boulardii* KK1, *I. orientalis* KK5Y3 and the *Candida* strains exhibited the greatest ability to reduce cholesterol levels in comparison with the other strains (group c, $p < 0.05$).

TABLE 3. Ability of yeast strains to survive in the presence of various concentrations of bile salts

Strains	Survival in the presence of bile salts				% inhibition[1]		
	Viable counts (log_{10}cfu ml^{-1})						
	0%	0.15%	0.30 %	0.50%	0.15%	0.30%	0.50%
S. cerevisiae							
NCYC167	6.79 ± 0.15	6.73 ± 0.16	6.72 ± 0.19	6.69 ± 0.20	15.34 ± 0.57[a]	14.78 ± 7.96[a]	20.46 ± 9.10[a]
746	6.75 ± 0.04	6.69 ± 0.03	6.83 ± 0.02	6.72 ± 0.02	13.10 ± 3.30[a]	–21.10 ± 6.35[a]	5.59 ± 4.22[a]
832	7.32 ± 0.23	7.29 ± 0.24	7.27 ± 0.26	7.27 ± 0.27	6.27 ± 3.41[a]	11.73 ± 6.02[a]	11.51 ± 8.65[a]
840α	7.67 ± 0.14	7.70 ± 0.24	7.65 ± 0.12	7.65 ± 0.14	–5.81 ± 3.02[a]	6.16 ± 1.66[a]	4.50 ± 1.38[a]
952	6.98 ± 0.26	6.69 ± 0.28	4.36 ± 0.06	3.89 ± 0.04	48.73 ± 2.22[b]	99.74 ± 0.12[b]	99.90 ± 0.06[b]
982	7.30 ± 0.27	7.24 ± 0.21	7.24 ± 0.20	7.21 ± 0.18	12.16 ±11.14[a]	12.59 ± 14.44[a]	18.50 ± 16.65[a]
S. boulardii							
KK1	7.68 ± 0.10	7.67 ± 0.10	7.65 ± 0.08	7.64 ± 0.08	1.32 ± 1.12[a]	7.25 ± 4.62[a]	6.93 ± 3.24[a]
UL	7.67 ± 0.15	7.64 ± 0.13	7.63 ± 0.17	7.61 ± 0.15	6.21 ± 3.18[a]	8.41 ± 3.72[a]	12.31 ± 0.19[a]
K. lactis							
570	6.99 ± 0.01	6.97 ± 0.01	6.96 ± 0.01	6.92 ± 0.01	2.60 ± 0.56[a]	6.73 ± 0.41[a]	13.49 ± 1.25[a]
630	7.62 ± 0.03	7.60 ± 0.02	7.58 ± 0.04	7.57 ± 0.01	4.69 ± 2.13[a]	8.54 ± 1.72[a]	11.80 ± 4.11[a]
D. hansenii							
NCYC9	7.81 ± 0.05	7.89 ± 0.01	7.86 ± 0.03	7.85 ± 0.02	–21.78 ± 16.15[a]	–13.4 ± 17.63[a]	–9.26 ± 14.89[a]
414	7.65 ± 0.02	7.67 ± 0.05	7.72 ± 0.04	7.72 ± 0.01	-4.16 ± 6.49[a]	–18.69 ± 16.20[a]	–16.41 ± 2.20[a]
I. orientalis							
KK5Y3	7.85 ± 0.05	7.87 ± 0.04	7.83 ± 0.06	7.82 ± 0.06	–5.74 ± 0.61[a]	3.18 ± 2.90[a]	7.33 ± 2.20[a]
C. albicans							
KK2.1	7.91 ± 0.15	7.89 ± 0.14	7.98 ± 0.09	7.93 ± 0.13	4.41 ± 0.96[a]	–18.27 ± 14.50[a]	–5.73 ± 2.89[a]
KK2.5	7.73 ± 0.15	7.72 ± 0.15	7.72 ± 0.14	7.71 ± 0.12	2.20 ± 0.44[a]	3.95 ± 1.32[a]	1.98 ± 4.61[a]
KK3.1	7.73 ± 0.10	7.68 ± 0.09	7.73 ± 0.09	7.72 ± 0.14	11.27 ± 1.97[a]	1.78 ± 4.11[a]	3.61 ± 8.02[a]
KK4.1	7.87 ± 0.19	7.82 ± 0.19	7.83 ± 0.22	7.84 ± 0.19	11.68 ± 1.26[a]	9.02 ± 5.57[a]	7.01 ± 0.76[a]
C. parapsilosis							
CBS1954	7.40 ± 0.36	7.34 ± 0.35	7.41 ± 0.32	7.39 ± 0.30	12.60 ± 1.69[a]	–2.80 ± 9.94[a]	0.80 ± 11.71[a]
KK6.5	7.33 ± 0.10	7.28 ± 0.10	7.41 ± 0.03	7.41 ± 0.02	11.23 ± 0.54[a]	–22.94 ± 18.24[a]	–22.55 ± 18.64[a]
KK6P	7.83 ± 0.02	7.82 ± 0.03	7.72 ± 0.13	7.73 ± 0.09	3.75 ± 0.48[a]	21.39 ± 18.57[a]	20.33 ± 11.88[a]

Means with different letters are significantly different at $p < 0.05$

[1] Mean [100 × (cfu w/o Oxgall – cfu in the presence of Oxgall) / (cfu w/o Oxgall)] ± SEM

TABLE 4. Determination of yeast antibacterial activity and their adhesion ability on Caco-2 cells. Means with different letters are significantly different at $p < 0.05$; ND: not determined

Strain	Antimicrobial activity		Percentage of Caco-2 cells with adherent yeasts[1]	Adherent yeasts per 100 Caco-2 cells[2]
	Well diffusion	Antagonistic activity		
S. cerevisiae				
A64	+*	ND	ND	ND
NCYC167	–	–	3.4 ± 1.21[a]	3.4 ± 0.51[a]
746	–	–	3.3 ± 0.51[a]	3.2 ± 0.58[a]
832	–	–	2.5 ± 0.43[a]	2.6 ± 0.68[a]
840α	–	–	3.2 ± 0.74[a]	2.8 ± 0.37[a]
952	–	–	1.8 ± 0.31[a]	2.2 ± 0.49[a]
982	–	–	3.1 ± 1.16[a]	3.4 ± 0.51[a]
S. boulardii				
KK1	–	–	4.3 ± 0.91[a]	4.6 ± 0.52[a]
UL	–	–	4.1 ± 1.29[a]	4.2 ± 0.59[a]
K. lactis				
570	–	–	98.2 ± 1.52[b]	112.4 ± 7.03[b]
630	–	–	96.4 ± 2.12[b]	97.8 ± 5.64[b]
D. hansenii				
NCYC9	–	–	84.3 ± 3.94[b]	75.4 ± 8.62[b]
414	–	–	80.1 ± 3.55[b]	88.1 ± 3.42[b]
C. albicans				
KK2.1	–	–	93.3 ± 2.56[b]	100.1 ± 5.45[b]
KK2.5	–	–	92.1 ± 1.92[b]	157.2 ± 3.63[b]
KK3.1	–	–	91.2 ± 2.62[b]	98.6 ± 2.13[b]
KK4.1	–	–	93.3 ± 2.48[b]	103.6 ± 6.58[b]
C. parapsilosis				
CBS1954	–	–	94.5 ± 2.92[b]	109.1 ± 8.74[b]
KK6.5	–	–	98.7 ± 0.62[b]	314.4 ± 20.71[c]
KK6P	–	–	98.9 ± 0.53[b]	332.6 ± 28.61[c]
I. orientalis				
KK5Y3	–	–	97.4 ± 2.56[b]	100.2 ± 7.75[b]

(–) negative activity against *L. casei* ATCC 334, *L. reuteri* DSM 20016, *C. tyrobutyricum* NCDO1754, *B. cereus*, *C. sporogenes* C2210, *E. faecalis* EF1, *E. coli*, *L. monocytogenes*, *S. aureus* and *Y. enterocolitica*

[1] Mean percentage of Caco-2 cells with adherent yeasts ± SEM

[2] Mean number of adherent yeasts per 100 Caco-2 cells ± SEM

* antibacterial activity observed only against *Y. enterocolitica*

Effect of yeast extracts on the mitogenic activity of mouse or rat spleen cells

Probiotics exert their beneficial effects through the modulation of the immune responses. The *in vitro* probiotic characterization of yeast strains showed that the strains 982, KK1, 570 and 630 were the most promising to exert probiotic effects. Thus, these strains were selected to determine their ability to induce the *ex vivo* activation of rat and mouse leukocytes. The strain 832 – exhibiting no probiotic characteristics and low *in vivo* immunomodulatory activity (unpublished data) – was used as control strain. None of the inactivated cell extracts of the selected yeast strains induced the proliferation of mouse splenocytes (Fig. 2). However, all strains induced the activation of rat splenocytes at only a basal level (Fig. 2).

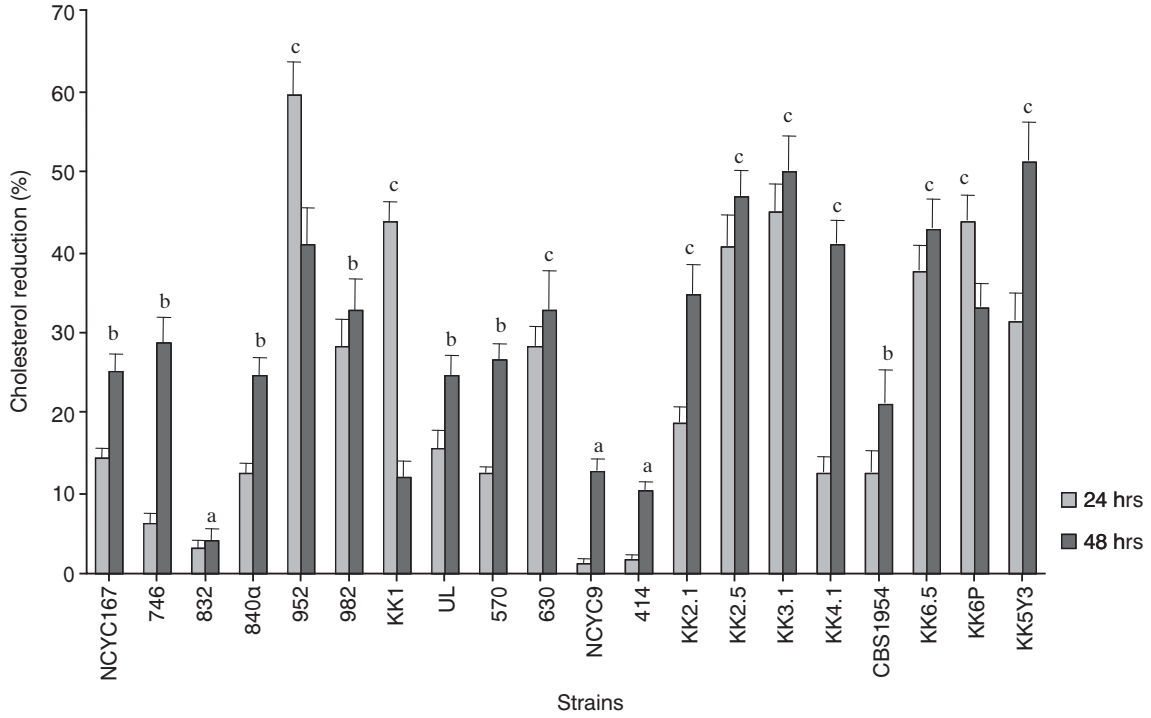

FIG. 1. *In vitro* reduction of cholesterol levels in the culture medium of several yeast strains. Means with different letters are significantly different at $p < 0.05$.

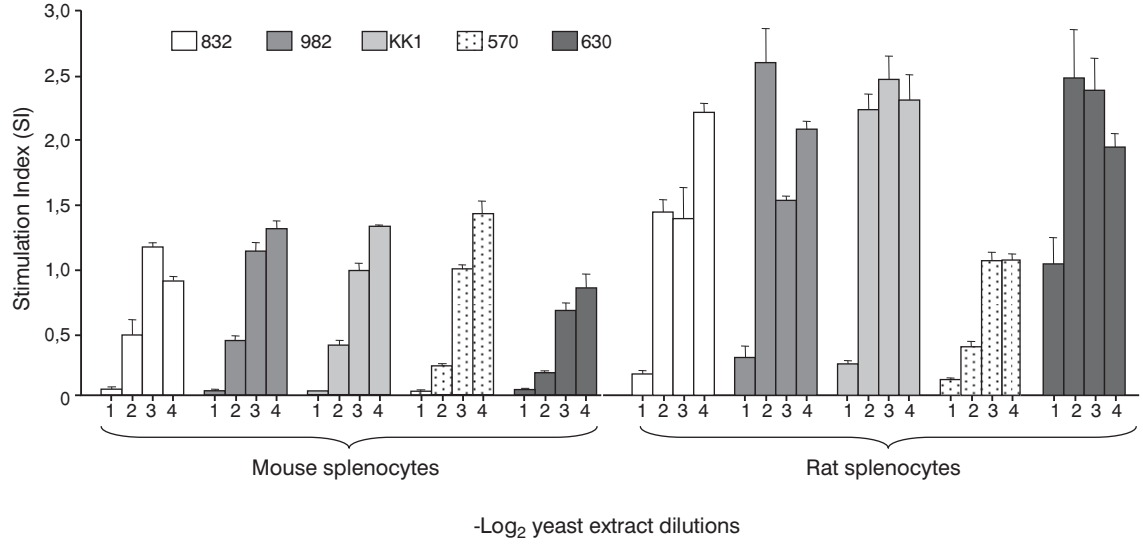

FIG. 2. Effect of yeast extracts on the *ex vivo* proliferative responses of mouse and rat splenocytes.

DISCUSSION

Yeasts are used in fermentation and food since the ancient times and several yeast strains are present in dairy related products or occur as a part of gastrointestinal tract. However, their use as probiotic agents is limited only to strains of the genus *Saccharomyces*. In this study we have investigated the *in vitro* probiotic properties of several yeast strains isolated from feta cheese or human gastrointestinal tract. We have demonstrated that, besides the strains of the *Saccharomyces* genus, other yeast species (such as *K. lactis*) may also be considered as presumptive probiotic candidates. Four yeast strains, the *S. cerevisiae* 982, *S. boulardii* KK1 and *K. lactis* 570, 630 strains were the most promising to be characterized as presumptive probiotic candidates, since they exhibited acid and bile tolerance and cholesterol reduction capacity. This study also shows that adhesion of yeasts to Caco-2 cells is restricted only to *K. lactis*, *D. hansenii*, *I. orientalis* and *Candida* strains.

Among yeast strains included in this study the *S. cerevisiae* 982, *S. boulardii* KK1 and *K. lactis* 570, 630 strains exhibited significantly higher resistance to pH 3.0 and bile salts than the rest of the strains. The above data indicate that these strains could potentially remain viable and metabolically active within the gastrointestinal tract which is considered as a prerequisite for probiotics to exert their beneficial effects.

Probiotics should be able to colonize the intestine, at least temporarily, by adhering to the intestinal epithelium (Ouwehand *et al.*, 1999; Tuomola *et al.*, 2001). Several *in vitro* models, such as the Caco-2 cell line have been developed for the determination of the adhesion ability (Ouwehand & Salminen, 2003). *Kluyveromyces lactis*, *D. hansenii*, *I. orientalis*, *C. albicans* and *C. parapsilosis* strains showed strong adhesive properties, which suggests that these strains could also colonize the intestine. Their adhesive ability is probably due to their capacity to rapidly adapt to new habitats and form biofilms (Verstrepen & Klis, 2006). Only few of the foodborne yeast strains exhibited adhesive capacity. On the other hand, the *Saccharomyces* strains exhibited low capacity to adhere to Caco-2 cells suggesting potential inability of these strains to colonize the intestine. These findings are also in agreement with those of Kumura *et al.* (2004), van der Aa Kuhle *et al*. (2005) and Edwards-Ingram *et al*. (2007) who have shown the adhesive ability of *K. lactis* and *D. hansenii* strains and the inability of *S. cerevisiae* and *S. boulardii* strains to adhere *in vitro*. However, feeding studies have clearly shown that in general probiotics, with *in vitro* adherence capacity, do not colonize the gastrointestinal tract permanently (Ouwehand & Salminen, 2003). For this reason, adhesion ability should not be considered as a prerequisite for probiotic functionality. Furthermore, pathogenicity of *C. albicans* strains is directly linked to its capacity to colonize the intestine. The complexity of these interactions is further seen by the fact that the virulence of *C. albicans in vivo* also depends on the formation of biofilms with certain bacteria (Wargo & Hogan, 2006). Several *Candida* strains are common constituents of the human intestine microflora and some of them are opportunistic pathogens. The *Candida* strains used in this study, exhibit *in vitro* probiotic properties and no pathogenic characteristics or *in vivo* immunostimulatory activity (unpublished data) and thus, further experimentation is required to determine their safety. Non-pathogenic and non-colonizing microorganisms are used as vectors for *in vivo* delivery of genes or for vaccination (Medina & Guzman, 2001; Fernandez-Arenas *et al.*, 2004). In addition, baker's yeast was recently administered as a live recombinant vaccine in mice (Upadhyaya & Manjunath, 2009). Thus, all the strains of the *Saccharomyces* genus included in our study may also be considered as presumptive candidate microorganisms for gene delivery or vaccination.

One of the probiotic mechanisms of action is the antibacterial activity against pathogens that penetrate the various mucosa sites (Saxelin *et al.*, 2005; Boirivant & Strober, 2007). Although several yeast strains have been shown to have antibacterial activity (Magliani *et al.*, 1997; Nikolaou *et al.*, 2006), none of the yeast strains in our study could produce antibacterial metabolites or had antagonistic activity *in vitro* against the selected foodborne pathogens and yoghurt starter strains.

In addition, several studies have shown the ability of probiotic microorganisms to lower cholesterol levels *in vitro* (Xanthopoulos *et al.*, 1998; Pereira & Gibson, 2002; Psomas *et al.*, 2003; Dilmi-Bouras, 2006) and *in vivo* (Fukushima & Nakano, 1995; Endo *et al.*, 1999; Sindhu & Khaterpaul, 2003). All yeast strains used in this study could remove cholesterol with the strains *K. lactis* 570, 630, *S. cerevisiae* 746, 840α, 952, 982, *S. boulardii* KK1, *I. orientalis* KK5Y3 and the *Candida* strains showing the greatest potential to reduce blood cholesterol. These results could be considered promising, as even 1% reduction in cholesterol can reduce the risk of cardiovascular diseases for 2-3% (Manson *et al.*, 1992). The cholesterol-low-

ering action of probiotics may be achieved through cholesterol assimilation (Xanthopoulos *et al.*, 1998; Pereira & Gibson, 2002; Psomas *et al.*, 2003; Dilmi-Bouras, 2006). Moreover, the different cholesterol concentrations or the various forms of soluble cholesterol as well as the cholesterol measurement assays used for *in vitro* studies may also affect the cholesterol removal ability of yeast strains. The strain 832 previously shown to possess high removal ability (Psomas *et al.*, 2003) showed only low removal ability in our study and this may be due to the higher concentration of cholesterol used in this study indicating its potential sensitivity in high cholesterol concentrations. In addition, the lower reduction of cholesterol levels by the strains 952, KK1 and KK6P, determined by 48 hrs compared to the respective one at 24 hours, could be due to higher sensitivity of these strains to the assimilated cholesterol. However, clear evidence about the degree of cholesterol removal ability of potential probiotic yeast strains must await *in vivo* experimentation.

The yeast strains were also tested for the presence of plasmids to exclude the possibility that they may carry potentially transmissible plasmid-encoded genes. Since potential transfer of plasmid-encoded genes to pathogens or other microorganisms of the intestinal flora could lead to disturbance of the microbial balance, strains harboring mobile elements should not be used as probiotics (Marteau & Shanahan, 2003). However, from the yeasts tested, none of the strains may carry plasmid-encoded genes as no plasmid DNA was isolated.

Most microorganisms encountered *via* the enteric route will be disrupted, their cell walls will be broken, and their cell contents will be released (Conway *et al.*, 1987). Bacterial cell extracts have been reported to show *in vitro* stimulatory (Nagafuchi *et al.*, 1999) or suppressive effects (Pessi *et al.*, 1999; Kankaanpää *et al.*, 2003). Diluted homogenates from the strains 570, 630, 982 and KK1 exhibited *in vitro* immunostimulatory activity by inducing the proliferation of rat lymphocytes. This response was not observed with mouse splenocytes indicating potential species specific immunoactivation by yeast strains. Moreover, the above data suggest that the immunomodulatory activity of yeast strains could be attributed to heat-sensitive cytoplasmic factors or indirectly through the induction of specific agents by viable yeast cells.

In summary, yeast strains isolated from feta cheese and infants' gastrointestinal tract could be used as gene delivery and expression systems for therapeutic or vaccination purposes as well as probiotics, since they do not contain plasmids and possess *in vitro* probiotic properties such as acid and bile resistance, adherence ability and cholesterol removal ability (Psomas *et al.*, 2001, 2003). In conclusion, our data show that the yeast strains *K. lactis* 570, 630, *S. cerevisiae* 982 isolated from feta cheese and *S. boulardii* KK1 isolated from infants' gastrointestinal tract (faeces) exhibit *in vitro* probiotic properties and are promising to be used as probiotics with the ability to decrease cholesterol levels. However, additional experimentation is required to examine the effects, and especially the immune effects, of these yeast strains in the gastrointestinal tract after their oral administration.

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