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Characterization of *Enterococcus faecium* CCDM 922 in Respect of its Technological and Probiotic Properties

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A B S T R A C T

The aim of this study was to characterize the adherence, immunomodulatory and other health properties and safety of *Enterococcus faecium* CCDM 922 from the Culture Collection of Dairy Microorganisms Laktoflora®. *E. faecium* CCDM 922 is susceptible to therapeutically important antibiotics and exhibits good adherence to Caco-2 cells (52.33 ± 10.11 %). Two different concentrations of *E. faecium* CCDM 922 were used for three and five-day stimulation of human mononuclear cells obtained from peripheral blood. Levels of IFN-γ, IL-6, IL-10, IL-17 and IL-1α were dependent on the density of *E. faecium* CCDM 922 used for stimulation and on time of stimulation. The cholesterol-lowering effect of *E. faecium* CCDM 922 was tested in Prague hereditary hypercholesterolemic rats. *E. faecium* CCDM 922 treatment of male rats reduced LDL- and VLDL-cholesterol levels significantly ($P < 0.05$). Present results indicated promising probiotic and technological potential for application of *E. faecium* CCDM 922 in functional foods or dairy products.

Introduction

Enterococci are the most controversial group of lactic acid bacteria among potential probiotic strains. Unlike most lactic acid bacteria (lactobacilli) and bifidobacteria, the genus Enterococcus has not yet obtained the status GRAS ("generally recognized as safe") (Li *et al.*, 2011). However, some Enterococcus strains act as probiotics and are used in functional food, as food supplements in pharmaceutical preparations such as Cernivet® (containing*E. faecium* SF68®, Cerbios-Pharma SA, Switzerland),

Symbioflor® 1 with *E. faecalis* (Symbio Pharm, Germany) and others (Serio *et al.*, 2010; Franz *et al.*, 2011). Probiotic enterococci can be used in the treatment and prevention of human and animal diseases such as alleviation of the symptoms of irritable bowel syndrome and diarrhoea caused by antibiotics. Additionally, anticarcinogenic and hypercholesterolemic effects, as well as immune regulation, have been described in the literature (Hlivak *et al.*, 2005; Castro *et al.*, 2007; Ooi andLiong 2010; Franz *et al.*, 2011). The immunomodulatory effect of lactic acid bacteria or probiotic bacteria is dependent on the strain used and on the dose of the strain, as demonstrated previously (Perdigon *et al.*, 2002; Castro *et al.*, 2007). The immunomodulatory properties of *E. faecium* were shown in a study by Nissen *et al.*, (2009) where *E. faecium* induced proinflammatory cytokine, interleukin-6, production by macrophages. In another study, oral administration of *E. faecium* EF1 was shown to improve microbial balance in intestines and modulate immunological homeostasis in jejunal and ileal mucosa of suckling piglets, Huang *et al.*, (2012).

In present study, we decide to extend the knowledge of the potentially probiotic strain *E. faecium* CCDM 922. We determined the adherence and immunomodulatory properties, as well as resistance of the strain *E. faecium* CCDM 922 to therapeutically important antibiotics. The cholesterol-lowering effect of *E. faecium* CCDM 922 was also investigated on hypercholesterol-emic rats.

Material and Methods

Microorganisms

Enterococcus faecium CCDM 922 was isolated from Ukrainian dairy product and deposited in Culture Collection of Dairy Microorganisms Laktoflora® (Czech Republic). Before each experiment the strain tested were cultivated overnight in M17 broth or in De Man–Rogosa–Sharpe (MRS) broth (MERCK, Germany) at 37 ˚C.

Antibiotic Resistance

Minimal inhibition concentrations (MIC) of nine antibiotics (ampicillin, streptomycin, gentamicin, tetracycline, vancomycin, kanamycin, erythromycin, clindamycin and chloramphenicol) were determined for *E. faecium* CCDM 922 using the broth microdilution method according to the approved standard of the Clinical and Laboratory Standards Institute (CLSI, 2009). Susceptibility interpretation criteria were based on The EFSA Journal (2008). Staphylococcus aureus ATCC25923 served as a reference strain for quality control purposes.

Autoaggregation and Hydrophobicity Properties

Cells of *E. faecium* CCDM 922 were prepared using the method of Kos *et al.*, (2003) and Vinderola *et al.*, (2004). Overnight culture of *E. faecium* CCDM 922 was centrifuged (6000 x g, 8 min.), washed twice with phosphate-buffered saline and resuspended in the same buffer to optical density 0.55-0.60 at wavelength 600 nm (A0, H0). Cell suspension for autoaggregation determination was mixed by vortexing for 10 s and incubated at 37 ˚C. Absorbance (At) was measured during 24 hours (in 2nd, 4th, 7th and 24th hour). For estimation of cell surface hydrophobicity 250 µl of hexane was added to 2.5 ml of the cell suspension and the two-phase system was mixed by vortex for one minute. After allowing the hydrocarbon rings to rise completely (10 minutes, room temperature), the decreased absorbance in the aqueous

phase (separated to cuvette) was measured at 600 nm (H1). Autoaggregation percentage was evaluated as((1-A_t)/A_0)·100. The percentage of *E. faecium* CCDM 922 adhesion to hexane was expressed as(1-H_1)/H_0 ·100.

Adherence

Human heteronuclear Caco-2 cells were selected for testing adhesion of *E. faecium* CCDM 922. The cells were cultivated in Minimum Essential Medium (MEM; Sigma-Aldrich, Austria) supplemented with fetal bovine serum (Sigma-Aldrich, Austria), non-essential amino acids and antibiotics at 37 ˚C, and in a humidified atmosphere of 5 % CO2. A suspension of *E. faecium* cells in exponential growth phase was added to eukaryote cells in vitro and the mixture was incubated at 37 ˚C for 24 hours. After cultivation, the cell layer was washed with phosphate-buffered saline and fixed in glutaraldehyde for electron microscopy and after dehydrated for scanning by electron microscopy. Adhesion of *E. faecium* CCDM 922 was expressed as the average percentage of cells adhering to Caco-2 cells.

Immunomodulation

E. faecium CCDM 922 cells in exponential growth phase were centrifuged, washed with saline buffer and resuspended in 6 ml X-vivo medium (Cambrex, USA). The final working concentration in X-vivo medium was adjusted to 3.3x105 CFU/ml (C1) and 4.5x108 CFU/ml (C2). Human peripheral blood mononuclear cells (hPBMCs) were isolated from healthy adult donors from the Blood Transfusion Centre of the General Faculty Hospital (Prague) by a Ficoll-Hypaque gradient (Sigma-Aldrich, Switzerland). After separation and purification hPBMCs were adjusted in X-vivo to a final concentration of 107 cells/ ml. Mononuclear cells (0.1 ml) were stimulated in X-vivo with 0.1 ml using two different densities (C1 and C2) of *E. faecium* CCDM 922 for 3 and 5 days at 37 ˚C. The total volume was 1 ml. Negative control was represented by unstimulated hPBMCs in X-vivo medium. Determination of cytokines was carried out using Fluorokine MAP Human Base Kit A (R&D Systems, USA) for IFN-γ, IL-6, IL-10, IL-17, IL-1α by multiplex analysis using a Luminex 200 Analyzer (Luminex Corp., USA). Results were evaluated by Luminex IS 2.3 software (Luminex Corp., USA).

Experimental Animals and Diet

A total of 24 Prague hereditary hypercholesterolemic rats (12 female and 12 male) were purchased from SEMED (Czech Republic). The rats (8 weeks old) were divided into two control groups (CM, CF) and two experimental (F, M) groups (6 rats female/male per each group). The hypercholesterolemic diet was fortified with 2.0 % (v/v) cholesterol. Rats were acclimatized to the laboratory conditions for 2 week before commencement of the experiment. They were housed at an ambient temperature of 23 ± 1 °C,12/ 12 h of light–dark cycle with ad libitum food and water. The experimental groups (F, M) received a daily dose of 400 µl fermented milk containing *E. faecium* CCDM 922 (108~109 CFU/ml) for 4 weeks. At the end of the experiment, blood samples from each rat were collected into tubes by cardiac puncture to determine serum cholesterol levels. Serum was separated from blood using centrifugation (400 x g, 10 min.). Total cholesterol and lipoprotein fractions – very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) – were analyzed by the Institute of Medical Biochemistry and Laboratory Diagnostics of the General

University Hospital and of The First Faculty of Medicine of Charles University in Prague (Czech Republic). Individual lipoprotein classes VLDL, LDL and HDL were separated stepwise in solutions with the density 1.006 g/ml, 1.063 g/ml and 1.21 g/ml, respectively, in the Optima L-100XP ultracentrifuge (Beckman Coulter, USA) equipped with the rotor type 50.4 Ti. Samples were separated 20 hours in vacuum at 38000 rpm and temperature 10 ºC. Total cholesterol (TC) and triacylglycerol (TAG) concentrations were analyzed spectrophotometrically using biochemical automatic analyzator MODULAR (ROCHE, Switzerland), using spectrophotometric enzymatic test GPO-PAP for TAG and CHOD-PAP for TC. All experiment procedures were approved by ethnical committee of The Ministry of Education, Youth and Sports (jMSMT-46654/2015-8).

Statistical Analysis

All statistical analyses were performed using Statgraphics® Centurion XV (StatPoint, Inc., Warrenton, USA) with the one-way ANOVA test. Differences were considered statistically significant at the level of $P < 0.05$. Results were expressed as mean ± standard deviation (SD).

Results and Discussion

Adhesion to the epithelial cells and mucosal surfaces is one of the criteria for selecting potentially probiotic microorganisms. Intestinal cell lines (such as Caco-2, T84 and HT-29) or stainless steel have been used for in vitro determination of adhesion ability of probiotic strains (Hosseini *et al.*,2009; Marciňáková *et al.*,2010). In this study, adhesion properties of *E. faecium* CCDM 922 were evaluated using intestinal cell lines Caco-2. Additionally, autoggregation and cell surface hydrophobicity using hexane was also tested. Fortina *et al.*, (2008) tested the biotechnological potential and safety of seven strains of *E.italicus* isolated from two different Italian cheeses. Autoaggregation of these strains was moderate (between 17-30 %) in comparison to tested *E. faecium* CCDM 922 (59.98 ± 2.98 %). The cell hydrophobicity of E. italicusstrains showed a strong affinity for an apolar solvent (xylene). In our study, the hydrophobic properties of cell surface of *E. faecium* CCDM 922 to hexane (57.50 ± 10.40 %) demonstrated.

Adhesion of *E. faecium* CCDM 922 to Caco-2 cells was expressed as a percentage of the inoculum. Results showed a good adhesion of the tested strain to Caco-2 cells (53.26 ± 11.03 %). In a study of Botes *et al.*, (2008), only 6 % of E. mundtii ST4SA cells, isolated from soybeans, adhered to Caco-2 cells. The extent of adhesion is influenced by the strains used as is shown in the mentioned studies. Resistance to therapeutically important antibiotics such as chloramphenicol, ampicillin, gentamycin, erythromycin and vancomycin are undesirable for potential probiotic strains (Hosseini *et al.*, 2009; Morandi *et al.*, 2013). Vancomycin-resistant enterococci (VRE) are important initiators of nosocomial infections and other human diseases such as bacteraemia, endocarditis and urinary tract infections (Franz *et al.*, 2011). *E. faecium* CCDM 922 was found to be sensitive to all tested antibiotics (Table 1).

Immunomodulatory ability is one of the mechanisms used to demonstrate the positive influence of probiotic bacteria and lactic acid bacteria on host health (Biswas et al, 2013). Tarasova *et al.*, (2010) found that oral administration of *E. faecium*L5 resulted in increased expression of IL-10 and a decrease in IL-8 expression in rats with dysbiosis. In this study, two different

concentrations of *E. faecium* CCDM 922 were used for 3- and 5 day stimulation of hPBMCs obtained from healthy donors. As shown in Figures 1 and 2, the observed effect on cytokine production was dose dependent. A significant increase of IL-1α secretion was observed after the stimulation of hPMBCs by high density of *E. faecium* CCDM 922 ($P < 0.05$) unlike production of other tested cytokines have been stimulated by low density of *E. faecium* CCDM 922. The effect of stimulation time was also observed when levels of interleukins, after 5-days of stimulation of hPBMCs by low density of tested strain, were lower than after 3-days stimulation ($P < 0.05$). Increased production of IL-17 is related to inflammatory bowel disease and other autoimmune diseases (Donkor *et al.*, 2012).

Table.1 Antibiotic resistance and Adhesion properties of *E. faecium* CCDM 922

Antibiotics	**MIC (µg/ml)**	
Ampicillin	0.125	S
Streptomycin	32	S
Gentamycin	2	S
Tetracycline	0.5	S
Vancomycin	0.125	S
Kanamycin	8	S
Erythromycin	0.125	S
Clindamycin	0.125	S
Chloramphenicol	4	S
Adhesion properties (%)		
adherence to Caco-2	52.33 ± 10.11	
Autoaggregation	59.98 ± 2.98	
hydrophobicity (hexane adhesion)	57.50 ± 10.40	

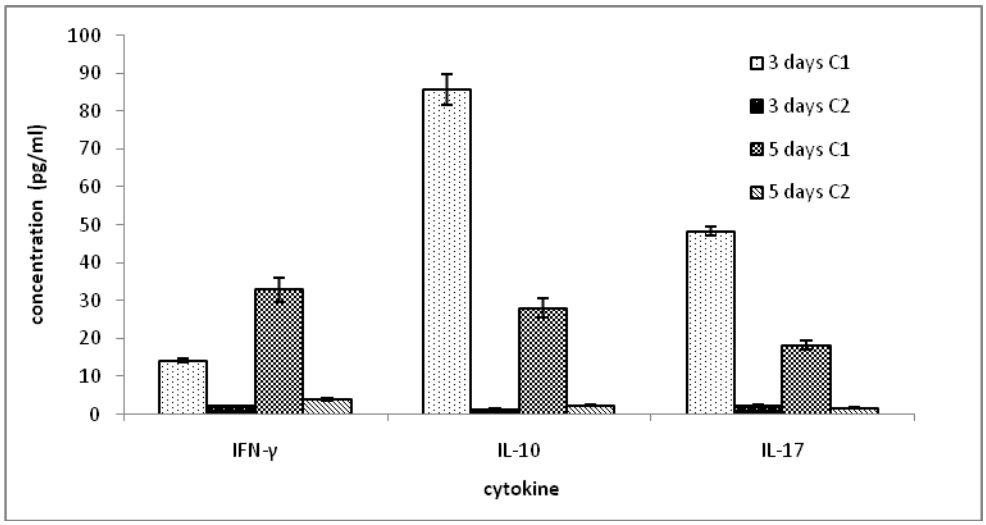
R-resistant; S-sensitive; Valuemean ± SD, n = 3;MIC-minimalinhibitoryconcentration

Table.2 Effect of *E. faecium* CCDM 922 Administration on Levels of Lipoproteins in Blood of Prague hereditary Hypercholesterolemic Rats

gender	**treatment**	**lipoproteins**		
		VLDL (mmol/ml)	**LDL (mmol/ml)**	**HDL (mmol/l)**
male	control	2.4 ± 0.8*	1.4 ± 0.5*	0.5 ± 0.1*
	E. faecium CCDM 922	0.7 ± 0.2*	0.6 ± 0.3*	0.2 ± 0.1*
female	control	3.0 ± 2.2	1.6 ± 0.4	1.8 ± 1.5
	E. faecium CCDM 922	0.7 ± 0.1	0.7 ± 0.3	0.5 ± 0.1

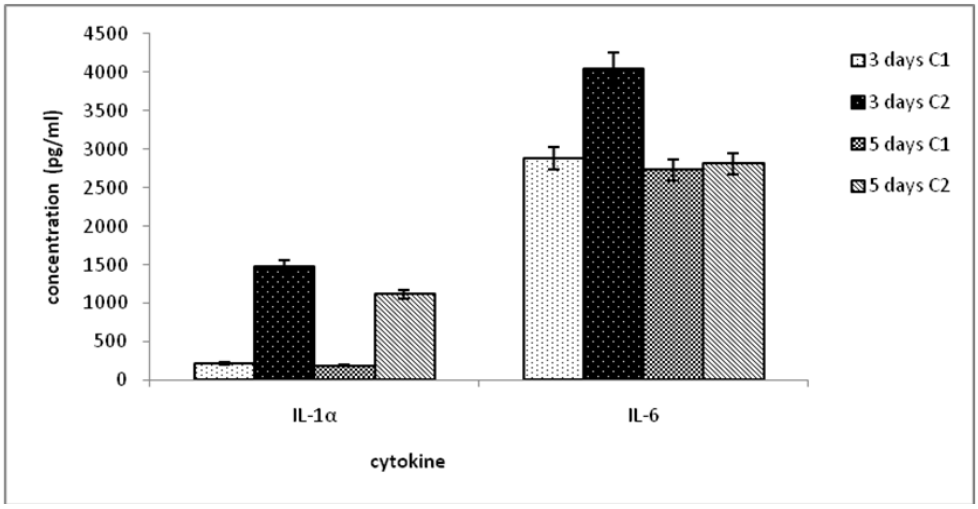
VLDL –very low density lipoprotein, LDL-low density lipoprotein, HDL – high density lipoprotein; Value mean ± SD, n = 6; Concentration of lipoproteins were compared with control by one-way ANOVA.*values with superscripts differ ($P < 0.05$).

Fig.1 Comparison of IFN-γ, IL-10 and IL-17 levels (pg/ml) produced by hPBMCs following 3- and 5-days stimulation of two different densities of *E. faecium* CDDM 922 (C1 and C2)



IL: interleukin; IFN: interferon; Value mean ± SD, n = 6; Two concentrations of *E. faecium* CCDM 922 (C1=5.52 log CFU/ml and C2= 8.65 log CFU/ml) and 10^6hPBMC/ ml from two healthy donors were used. Levels of produced cytokines were compared by the one-way ANOVA.

Fig.2 Comparison of IL-1α and IL-6 levels (pg/ml) produced by hPBMCs following 3- and 5-days stimulation of two different densities of *E. faecium* CDDM 922 (C1 and C2)



IL: interleukin; Value mean ± SD, n = 6; Two concentrations of *E. faecium* CCDM 922 (C1=5.52 log CFU/ml and C2= 8.65 log CFU/ml) and 10^6hPBMC/ ml from two healthy donors were used. Levels of produced cytokines were compared by one-way ANOVA.

Hypercholesterolemia is related to coronary heart disease and arteriosclerosis (Lee *et al.*, 2009; Ooi and Liong, 2010). Decreasing LDL cholesterol, which accumulates cholesterol in blood vessels, is one of possible treatment. Consuming fermented products containing probiotic microorganism can reduce cholesterol, as supported in many studies using animal and human models (Fukushima and Nakano 1996; Xiao *et al.*, 2003; Hlivak *et al.*, 2005; Nguyen *et al.*, 2007). In this study, the

cholesterol-lowering effect of *E. faecium* CCDM 922 was tested in female and male Prague hereditary hypercholesterolemic rats. *E. faecium* CCDM 922 treatment in male rats reduced LDL- and VLDL-cholesterol levels significantly ($P < 0.05$), as is shown Table 2. Decrease of the levels of LDL- and VLDL- cholesterol in female rats was not significant ($P > 0.05$) due to the wide variability in the control groups (Table 2) and hormonal cycle hormonal cycle which could have the influence on fractions of cholesterol of female rats because rats become sexually mature at 6 weeks.The strain CCDM 922 is also characterized by production of extracellular polysaccharide substances (EPS) and occurrence of bsh (bile salt hydrolase) genes. Both EPS and bsh genes may have a positive effect in reducing cholesterol (Tok and Aslim 2010; Pavlovis *et al.*, 2012). Hlivak *et al.*,(2005) tested the probiotic strain *E. faecium* M-74 (single daily capsule at 2 x 109 CFU/capsule) in 43 human volunteers. The administration of *E. faecium* M-74 reduced the concentration of serum cholesterol by 12 % after 56 weeks compared to the control.

In conclusion, this study demonstrated that *E. faecium* CCDM 922 is susceptible to therapeutically important antibiotics and exhibits good adhesion and immuno-modulatory abilities. Oral administration of *E. faecium* CCDM 922 to rats was associated with reduction of LDL and VLDL cholesterol that can help prevent or treat hypercholesterolemia. Tested *E. faecium* CCDM 922 seems to be promising for use as a probiotic strain in the functional food and dairy industry.

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