ORIGINAL ARTICLE

Effects of *Lactobacillus casei* on the expression and the activity of cytochromes P450 and on the CYP mRNA level in the intestine and the liver of male rats

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Abstract

OBJECTIVES: The aim of the study was to find whether probiotic *Lactobacillus casei* influences the expression or the activity of cytochromes P450 (CYP) and whether it has an influence on the level of CYP mRNA in male rats.

DESIGN: Live bacterial suspension of *L. casei* was administered orally (gavage) to healthy male Wistar rats daily for 7 days. Control group of rats was treated with the saline solution. Sections of the duodenum, jejunum, ileum, caecum and colon were dissected from each experimental animal. In all individual samples, the expression of selected CYPs was determined by Western blotting. The levels of expression of CYPs were also evaluated by mRNA using the real-time PCR method.

RESULTS: There were changes observed in the expression of CYP enzymes and in the CYP mRNA levels along the intestine after application of L. casei. The expression of CYP1A1 enzyme was found to be decreased in the proximal part of the jejunum and colon, CYP1A1 mRNA level was decreased in the distal part of the jejunum, ileum and caecum. Thus, the changes in CYP1A1 protein or mRNA were observed along the intestine of male rats. Similarly, a decreased expression of the caecal CYP2E1 mRNA and of the duodenal CYP3A9 mRNA after treatment of rats with *L. casei* was found.

CONCLUSION: Probiotic L. casei might be able to contribute to prevention against colorectal cancer by decreasing levels of certain forms of xenobiotic-metabolizing enzymes; moreover, in general, there is a possibility of interactions with concomitantly taken pharmacotherapeutic agents.

Abbreviations:

cDNA	complementary deoxyribonucleic acid
CYP	cytochrome P450
EDTA	ethylenediaminetetraacetic acid
mRNA	messenger ribonucleic acid
L. casei	probiotic *Lactobacillus casei*
PAHs	polycyclic aromatic hydrocarbons

INTRODUCTION

The whole gastrointestinal tract represents an ecosystem of high complexity (Holzapfel *et al.* 2002). The intestine is important for the metabolism of xenobiotics such as dietary toxins and orally absorbed drugs due to its extremely large surface area and the presence of xenobiotic metabolism enzymes and transport systems (Ito *et al.* 2007). The complex communities of microorganisms that colonize the gastrointestinal tract play an important role in human or animal health. Probiotics are viable microbes frequently administered from food or feed supplements which favourably influence the microbial balance of the host (Fuller 1989) and are believed to temporarily colonize the intestine by adhering to intestinal surfaces. The adhesive ability of bacteria to intestinal cells has been considered as one of the selection criteria for probiotic strains (Tuomola *et al.* 1998). Specific probiotic microorganisms can modulate inflammation, produce inhibitory compounds, which down-regulate virulence factor expression by pathogens, or which directly kill or impede pathogens in the gastrointestinal tract. Probiotics are capable of inducing mucous production by enterocytes and can compete with pathogens for binding sites on the gastrointestinal enterocytes (Forssten *et al.* 2011).

The lactic acid bacteria are the major representatives of probiotics, both in the food and marketed food supplements (Holzapfel *et al.* 2002). Lactic acid bacteria are gram-positive organisms that ferment hexose sugars to produce primarily lactic acid (Makarova *et al.* 2006). In addition, they play an important role in the spoilage of processed and fermented foods, and beverages (the spoilage of wine, beers and fruit juices as evidenced by cloudiness and off-flavours). However, these organisms are particularly suitable as antagonistic microorganisms in foods, because they are capable of inhibiting other food-borne bacteria by a variety of means including production of organic acids, hydrogen peroxide or bacteriocins (Aguirre *et al.* 1993). Lactic acid bacteria include a variety of industrially important genera, involving *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species (Makarova *et al.* 2006). The most widely used probiotic *Lactobacillus* genus is known to colonize the human gastrointestinal tract, to protect the intestines against enteropathogenic infection (Won *et al.* 2011) and to protect against colon cancer (Liong 2008). The aim of this work was to prove whether the presence of probiotic bacteria *Lactobacillus casei* in the rat gut may influence the expression and the activity of cytochromes P450, i.e. monooxygenases metabolizing both endo- and xenobiotics (Hodek *et al.* 2009), including a plethora of widely prescribed drugs. These enzymes constitute a superfamily of heme enzymes, involved in a variety of metabolic and biosynthetic processes (Anzenbacher & Anzenbacherová 2001). In humans, approximately 80% of oxidative metabolism and almost 50% of the overall elimination of commonly used drugs can be attributed to one or more of the various CYP enzymes (Paine *et al.* 2006). Hence, the studies on possible interactions of drugs with these enzymes are of considerable importance.

MATERIAL AND METHODS

Chemicals and enzymes

All reagents and chemicals were purchased from Sigma-Aldrich CZ (Prague, Czech Republic) if not stated otherwise. Other chemicals as sodium chloride, potassium chloride, hydrochloric acid, potassium hydroxide, EDTA, sucrose and methanol, which were used for isolation of microsomal fraction and Western blotting, were obtained from Lach-Ner (Neratovice, Czech Republic). Glycerol that was used as a cryoprotectant was obtained from Merck (Prague, Czech Republic). Protease inhibitor cocktail tablets were purchased from Roche (Mannheim, Germany). The RNA stabilization reagent, RNA later®, was purchased from Quiagen (Germantown, MD, USA). Mouse anti-rat CYP3A1, 2B1/2 and 2C6 monoclonal antibodies were purchased from Abcam (Cambridge, UK). Goat anti-rat CYP1A1 polyclonal and goat anti-rat CYP2E1 monoclonal antibodies were obtained from Daiichi Pure Chemicals (Tokyo, Japan). The chemiluminiscence kit for Western blotting (Immun Star) was purchased from Bio-Rad (Hercules, CA) and the nitrocellulose membrane was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Miniprotean electrophoresis and Western blotting apparatus were purchased from Bio-Rad (Hercules, CA). The UV-Vis spectrophotometer Cary 4000 (Varian, Palo Alto, USA) was used for determination of the content of cytochromes P450. The TECAN Infinity absorbance/fluorescence/luminescence reader (Tecan, Vienna, Austria) was used for detection of the respective spectral data. HPLC-UV analyses were performed on Shimadzu LC-20A Prominence system (Kyoto, Japan).

Preparation of biological samples

Live bacterial suspension of probiotic *Lactobacillus casei* was applied intragastrically (1×10^9 CFU/dose) to male Wistar rats (body weight 300–360 g). The probiotic suspension was administered daily to six animals for 7 days. Other six rats were stressed by oral application of the saline solution daily for 7 days as well. This group was used as the control one. After 7 days the rats were sacrificed. The protocol of the experiment was

approved by the institutional Ethics Committee. The liver and all parts of the intestine (duodenum, proximal part of jejunum (A), distal part of jejunum (B), ileum, caecum and colon) were removed and weighted. The whole duodenum as well as small samples of the liver, jejunum (A), jejunum (B), caecum and colon were used for determination of CYP mRNA levels. These samples were inserted into RNA stabilization reagent. The rest of intestinal samples (without duodenum) were washed by cold saline solution; the liver was washed by cold 0.25 M sucrose in 1 mM EDTA (pH 7.4). All samples were stored at -70 °C until used. For preparation of intestinal and hepatic microsomes, the samples were rinsed in cold 0.25 M sucrose in 1 mM EDTA (pH 7.4) with 0.2 mM phenylmethanesulfonyl fluoride and a protease inhibitor cocktail. The tissue was then homogenized and subjected to differential centrifugation to obtain the microsomal fraction according to standard procedures (Lake 1990).

Determination of total protein and specific content of cytochrome P450

Total protein content was determined by bicinchoninic acid method with a standard BCA Protein Assay kit (Pierce, Rockford, IL). Determination was done in three parallel samples using a calibration curve. S.D. differences from the respective means between determinations were below 15%. Cytochrome P450 content in liver microsomes was determined by the method described by Omura and Sato (1964). Specific content of liver cytochrome P450 was obtained as a ratio of the cytochrome P450 content (in nmol) to the amount of the total protein (in mg).

Determination of activities of individual CYP forms in liver microsomes

The activities and relative amounts of selected CYP enzymes were determined by established enzymological techniques based on analogy between human and rat forms and their respective substrates: 7-ethoxyresorufin *O*-deethylation (substrate of CYP1A2) (Chang & Waxman 1998); bufuralol 1′-hydroxylation (substrate of CYP2D6) (Crespi *et al.* 1998b); testosterone 6β-hydroxylation (substrate of CYP3A4) (Guengerich et al., 1986); chlorzoxazone 6′-hydroxylation (substrate of CYP2E1) (Lucas *et al.* 1996); diclofenac 4′-hydroxylation (substrate of CYP2C9) (Crespi *et al.* 1998a). The metabolites of testosterone 6β-hydroxylation (at 245 nm), chlorzoxazone 6′-hydroxylation (at 287 nm) and diclofenac 4′-hydroxylation (at 280 nm) were measured by an HPLC with UV detection. The HPLC with fluorescence detection was used for determination of metabolites of 7-ethoxyresorufin *O*-deethylation (excitation at 535 nm, emission at 585 nm) and bufuralol 1′-hydroxylation (excitation at 252 nm, emission at 302 nm). Activities of individual CYP forms in the presence of probiotic were compared relatively to the activities of the control sample in percentage.

Western blotting. Microsomal proteins from liver (10 μg) and from all various parts of the intestine (35 μg) obtained from each experimental rat were separated on 8%-SDS (w/v) polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes according to the method of Towbin *et al.* (1979). Immunodetection of CYP was carried out using anti-rat CYP1A1, anti-rat CYP2B1/2, anti-rat CYP2C6, anti-rat CYP2E1 and anti-rat CYP3A1 as the primary antibodies. The bands were visualized with respective horseradisch peroxidase-conjugated secondary antibodies using a Luminol reagent kit (Santa Cruz, CA) and their relative intensity evaluated with Elfoman software (Semecky Inc., Prague, Czech Republic).

RNA isolation and real-time PCR procedures

Samples of intestinal and hepatic tissue (about 30 mg) of each rat was stabilized in RNA stabilization reagent and subsequently homogenized with homogenizer Diax 900 (Heidolph, Kelheim, Germany). The sample was then applied onto the QIAshredder columns to eliminate tissue microparticles. RNA was isolated with use of RNeasy® Plus Minikit (Quiagen, Germantown, MD, USA) enabling degradation of contaminating genomic DNA. One microgram of each isolated RNA was subjected to reverse-transcription using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with random hexamer primers. New synthesized cDNA was applied for real-time PCR using Light Cycler® 480 SYBR Green Master I mix in a Light Cycler 480 (Roche, Mannheim, Germany) with the following thermal cycling conditions: preincubation for 10 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, at 58 °C for 15 s and at 72 °C for 15 s for denaturation, annealing and elongation respectively. All samples for real-time PCR were prepared in triplicates. Rat primers were designed in our laboratory and synthesized by Invitrogen (Life Technologies, division Prague, Czech Republic). Absolute quantification method was applied for obtaining gene expression data. The following primer sequences were used:

CYP2B1/2 Fw: 5′-TCC CAG GGA GCC CCA CTG GAT CCC A-3′
CYP2B1/2 Rev: 5′-GAA CCC AGA GAA GAA CTC AAA CAC CTG G-3′
CYP2C6 Fw: 5′-GCC TTG TGG AGG AAC TGA GG-3′
CYP2C6 Rev: 5′-GCA CAG CCC AGG ATA AAC GT-3′
CYP2E1 Fw: 5′-CCA AGG GTA CAG TTG TGA TTC CAA C-3′
CYP2E1 Rev:5′-CAA CAC ACA CAC GCT TTC CTG CAG A-3′
CYP3A1 Fw: 5′-GTG CTC CTC TAC GGA TTT GGG A-3′
CYP3A1 Rev: 5′-TCC ACA TCG AAT TTC CAT AAA CCC-3′
HPRT Fw: 5′-GAAGAGCTACTGTAATGACCAGTC-3′
HPRT Rev: 5′-CGT TCT TTC CAG TTA AAG TTG AGA GA-3′

For determination of mRNA levels of the CYP1A1, CYP3A9 and CYP2E1, the universal hybridization probes with recommended primers were used (www.universalprobelibrary.com) (Roche, Germany).

RESULTS

Application of probiotic *L. casei* to rats for 7 consecutive days resulted in no significant changes in the specific content of the cytochrome P450 in the liver of experimental animals (Figure 1). Only a small increase of the specific content of cytochrome P450 in the liver was found. Figure 2 shows no significant changes in activities of liver CYP enzymes, namely of the CYP1A1/2, 2D6, 3A4, 2E1 and 2C9 prototypic activity after administration of *L. casei*. While not statistically significant, the largest change of CYP activity was with chlorzoxazone 6´-hydroxylation (substrate of rat CYP2E1 protein).

Protein expression of rat liver CYP1A2, 3A, 2E1, 2C6 and 2C11 enzymes in samples from rats without and with *L. casei* applied was detected by Western blotting (Figure 3A). Two immunoreactive bands were detected after incubation with CYP3A1 antibody due to known cross-reactivity with other isoforms of the CYP3A family. The density of both bands was taken collectively as CYP3A showing no change after administration of *L. casei*. A slight decrease of the expression of the liver CYP2E1 protein corresponded to a decrease of CYP2E1 prototypic activity (chlorzoxazone 6´-hydroxylation) (Figure 2). Expression of rat CYP2C forms, CYP2C6 and CYP2C11 (being a human CYP2C9-like rat protein according to Wang *et al.* (2009), moreover, according to BLAST (www.ncbi.nlm.nih.gov/BLAST)), exhibited a slight increase in expression in samples obtained after administration of *L. casei* in comparison to control (Figure 3A). These changes were not statistically significant again. The real-time PCR method did not reveal significant changes as well (Figure 3B).

Cytochromes P450 are localized in the liver as well as in the gastrointestinal tract; however, their localization along the intestine is variable (Mitschke *et al.* 2008). In other words, the data for each CYP from each part of the intestine could not be, in principle, obtained since not all CYP forms are present in all parts of the intestine. In addition, the jejunum is the longest part of the intestine; therefore, it was divided into two parts, A and B.

Activities of cytochrome P450 in the intestinal samples were not measured due to limited sample amount.

The expression of cytochrome P450 2B1/2 was detected by Western blotting in the small intestine; its presence in the caecum and colon was not detected. The expression of CYP2B1/2 protein among experimental animals was very variable; however, no significant changes were found after treatment with *L. casei* compared to control samples (Figure 4A). A slightly non-significant decrease of the level of CYP2B1/2 mRNA was detected in the duodenum (the sample of this smallest part of the intestine was enough only for the real-time PCR method) (Figure 4F). The level of CYP2B1/2 mRNA in the jejunum and ileum was not changed after application of probiotic *L. casei*.

Similarly, the expression of cytochrome P450 2E1 was detected by Western blotting mainly in the small intestine. No significant changes in the expression of CYP2E1 protein were found; only slight decrease at the beginning and at the end of the small intestine after administration of *L. casei* in comparison to control samples was observed (Figure 4B). A significant decrease of

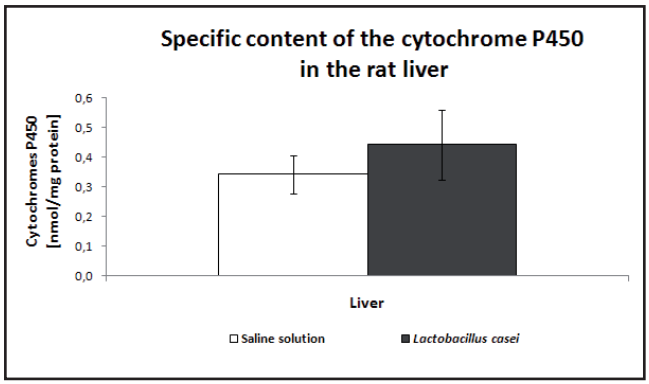

Fig. 1. The specific content of the cytochrome P450 in the hepatic microsomes in rats after treatment with probiotic *L. casei*. Control rats were treated with the saline solution. The specific content of the CYP is expressed as nmol CYP/mg protein. Results are presented as means ± S.D.; N = 6.

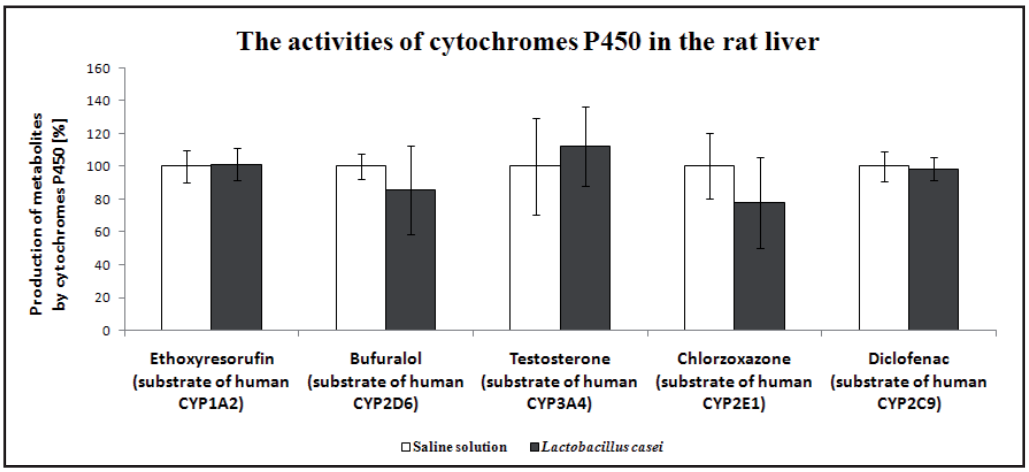

Fig. 2. Relative activities of CYP enzymes in rat liver microsomes after treatment with probiotic *L. casei* daily for 7 days. Control samples were obtained by treatment with the saline solution for 7 days as well. Results presented as means ± S.D; N = 6.

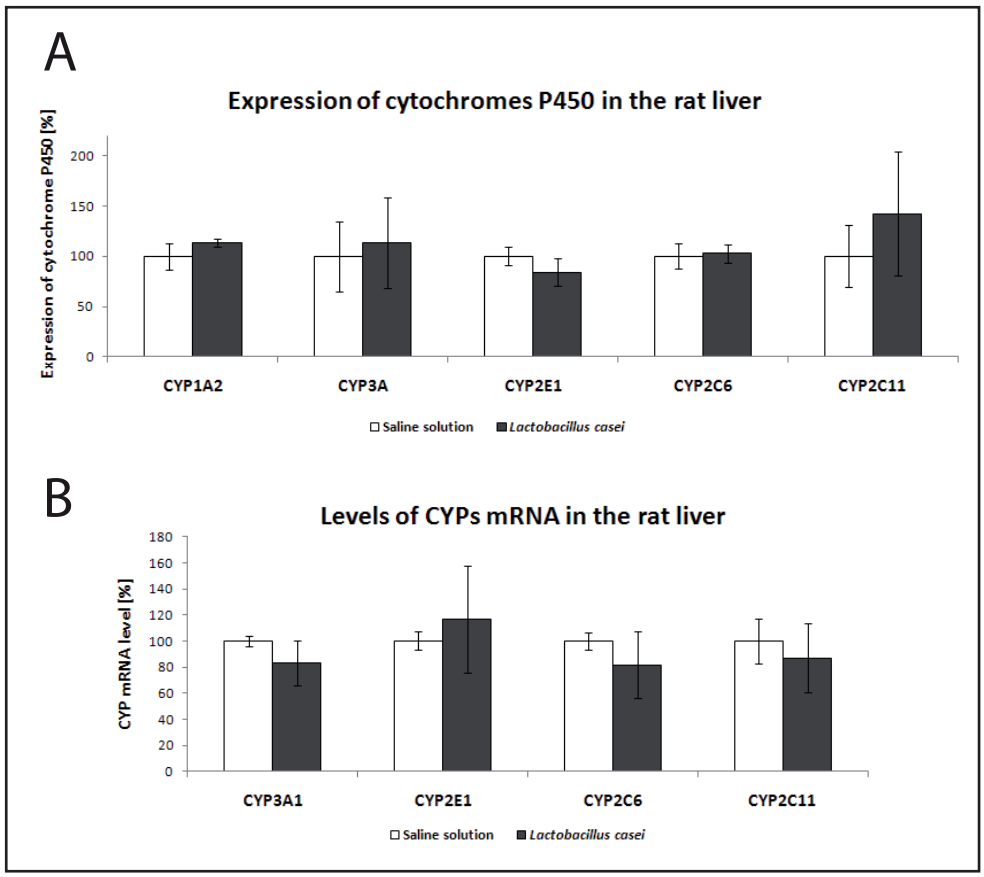

Fig. 3. Expression of cytochrome P450 (A) and the level of the CYP mRNA (B) in the rat liver after treatment by probiotic *Lactobacillus casei* daily for 7 days. Control rats were treated with the saline solution for 7 days as well. Results are presented as means ± S.D.; N = 6.

the CYP2E1 mRNA level was found in the caecum after application of *L. casei* by real-time PCR method (Figure 4G). In the other parts of the rat intestine (duodenum, whole jejunum, ileum, colon), no changes of CYP2E1 mRNA level were detected.

The expression of rat CYP3A form was found by Western blotting all over the intestine of all experimental animals. A slight decrease in the expression of CYP3A was detected at the beginning of the jejunum (sample "jejunum A") after treatment of *L. casei* (Figure 4C). This change was confirmed by real-time PCR method (Figure 4H). A significant decrease of the level of CYP3A9 mRNA after application of *L. casei* was found in the duodenum (the smallest part of the intestine, enough only for real-time PCR method).

The CYP2C6 protein has been shown to be present mainly in the caecum and the colon (Matuskova *et al.* 2010). A small increase in the expression of CYP2C6 protein was observed in these parts of the intestine (after treatment with probiotic *L. casei*) (Figure 4D). These changes were not significant and real-time PCR method confirmed this observation (Figure 4I).

Expression of cytochrome P450 1A1 was also studied by Western blotting in all parts of the intestine. The CYP1A1 protein was detected mainly in the small intestine; it was also localized in a smaller extent in the caecum and colon (as described also in Matuskova *et al.* 2010). A decrease in the expression of CYP1A1 enzyme was observed in the jejunum (part A) as well as in the colon (after application of probiotic *L. casei*) (Figure 4E). The decrease of the CYP1A1 mRNA level in the jejunum (part A) and colon was found also by real-time PCR method (Figure 4J); however, this change was not significant. On the other hand, a significant decrease of CYP1A1 mRNA was observed in the jejunum B, ileum as well as in the caecum.

DISCUSSION

The experiments discussed here were performed *in vivo* with rat experimental model; for this reason, the results show, in principle, a great variability. Despite the variability among the biological samples within one group, the tendencies and statistical significance of differences between the data for samples with or without *L. casei* administered can be seen.

In this study, a marked decrease of CYP1A1 mRNA level was found in the distal jejunum (part B), ileum and caecum; a decrease in the CYP1A1 protein expression was found in the jejunum (part A) and colon; as for the CYP2E1, a decrease of CYP2E1 mRNA level was found in the caecum. Cytochrome P450 1A1 is one of the well-characterized xenobiotic-metabolizing enzymes regulated by the arylhydrocarbon receptor. The CYP1A1

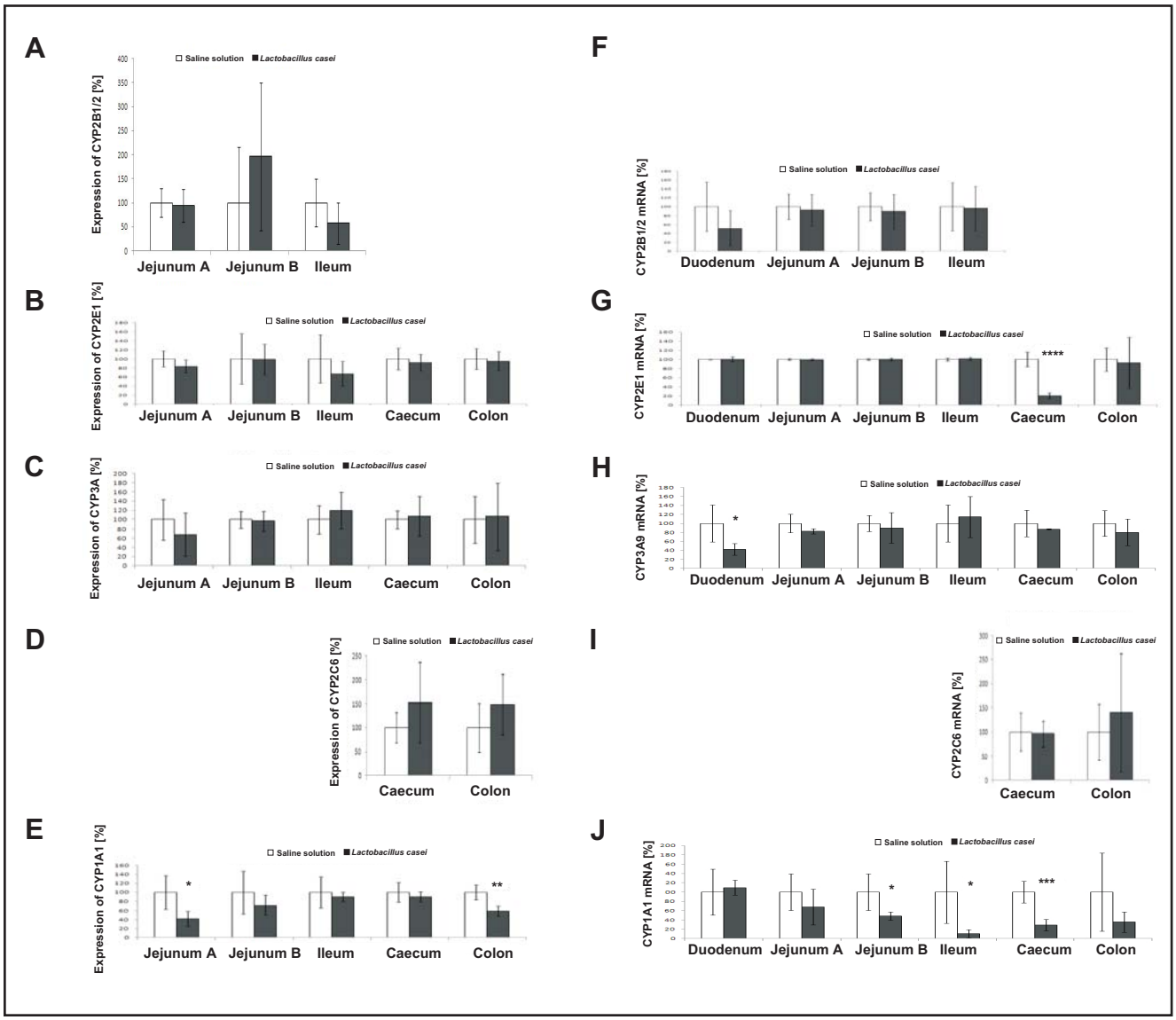

Fig. 4. Expression of rat CYP2B1/2 (A), CYP2E1 (B), CYP3A (C), CYP2C6 (D), CYP1A1 (E) protein and the level of the CYP2B1/2 (F), CYP2E1 (G), CYP3A9 (H), CYP2C6 (I), CYP1A1 (J) mRNA along the intestine after treatment by probiotic *Lactobacillus casei* daily for 7 days. Control rats were treated with the saline solution for 7 days as well. Un-reported values in different parts of the intestine were under detection limit of the used method. Results are presented as means ± S.D.; N = 6; *p<0.05, **p=0.0011, ***p=0.000125, ****p=0.000049.

enzyme has an important role in metabolic activation of polycyclic aromatic hydrocarbons (PAHs), a group of stable and ubiquitous organic contaminants released in the environment (Khan *et al.* 2008). These PAHs are converted to highly reactive electrophilic metabolites that can form DNA adducts and lead to gene mutations and cellular transformation. The CYP2E1 enzyme metabolizes a large number of low-molecular-weight compounds including compounds of toxicological and carcinogenic significance (Guengerich 2005). The inhibition of these two carcinogen-activating CYP enzymes is considered to be one of the major health promoting effects of chemopreventive compounds (Krizkova *et al.* 2008). Hence, the results presented here indicate that the probiotic strain *L. casei* might help in the defence of the organism against formation of reactive metabolites in the gastrointestinal tract by CYP2E1 and CYP1A1.

In rats, the CYP3A subfamily consists of five related genes, CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23 (Anakk *et al.* 2003). In fact, the rat CYP3A forms (3A1, 3A2, 3A9, 3A18 and 3A23) are similar to human CYP3A4 (the most important CYP form in human liver) and CYP3A5 (Matsubara *et al.* 2004). While CYP3A1 and CYP3A2 mRNA were detected by real-time PCR only in the liver, CYP3A9 mRNA was detected in both, in the liver and in the intestinal tract (Matsubara *et al.* 2004) and this was why the CYP3A9 mRNA probe for the real-time PCR method was used in this study. It was found here that the CYP3A9 mRNA level was reduced in duodenum by approximately 50%

after application of probiotic *L. casei*. In other words, a potential decrease in the CYP3A level in duodenum could lead to a less effective metabolism of drugs by CYP3A in this first part of the intestine.

In conclusion, in this work, the influence of probiotic *Lactobacillus casei* on the level of intestinal CYP1A1, 2E1 and 3A9 mRNA was found. This *in vivo* study revealed that treatment of rats with *L. casei* could exhibit, in defined parts of the intestine, an influence on the level of CYP enzymes. Probiotic *L. casei* can possibly contribute to prevention against colorectal cancer by decreasing levels of certain forms of xenobiotic-metabolizing enzymes. The results obtained in this study, however, cannot be easily extrapolated to the man; on the other hand, they document the complexity of effects associated with the intestinal microbiome.

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