Original paper

Excretion of 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) from Rat Liver Following Oral Administration of *Lactobacillus reuteri* and *Lactobacillus acidophilus*

Takehito Suzuki,1 Kaoru Yamazaki,2 Tadashi Shinoda,3 Mitsuyuki Shiraishi,1 Hiroshi Yoshikawa,1 Yurika Noguchi,1 Tetsuro Ito,4 Yasuo Ishii,1 Tatsuya Takizawa,1 and Hidetoshi Morita1*

1School of Veterinary Medicine, Azabu University, 1-17-71, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
2School of Contemporary Human Life Science, Tokyo Kasei Gakuin University, 2600, Aihara-machi, Machida, Tokyo 194-0292, Japan
3R & D Center, Calpis Co. Ltd., 5-11-10, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, 252-0206, Japan
4Veterinary Teaching Hospital, Azabu University, 1-17-71, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan

Received December 20, 2013 ; Accepted March 27, 2014

Oral administration of *Lactobacillus reuteri* CP3012 or *Lactobacillus acidophilus* L-92 for 60 days in rats that were previously administered 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) orally at a dose of 100 μg/kg of body weight resulted in a significant decrease in hepatic bioaccumulation of PCB126 (*p* < 0.05), with levels of 30.7 ± 3.7 ng/g and 92.6 ± 25.0 ng/g of liver tissue, respectively, compared with 133.1 ± 12.7 ng/g of liver tissue in the controls. The electron paramagnetic resonance signal level of the liver PCB126-specific *g* = 2.49 species in rats administered *L. reuteri* CP3012 decreased significantly (*p* < 0.05). Both the bile acid concentration in the feces and total stool output increased significantly following administration of lactobacilli (*p* < 0.05); however, adsorption of PCB126 onto the bacterial cells was not observed. These results suggest that these bacteria inhibit reabsorption of PCB126 with bile acid by blocking enterohepatic circulation through absorbing and/or deconjugating the bile acids in the intestinal tract and by promoting excretion of bile acids from the body, thus reducing PCB126 accumulation in the liver.

Keywords: PCB126, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, enterohepatic circulation

Introduction

Dioxins, including polychlorinated dibenzo *para*-dioxin, polychlorinated dibenzofuran, and coplanar polychlorobiphenyl, are widespread pollutants found in the atmosphere, water, soil, and food (Safe, 1998). These compounds have various adverse effects on reproduction and internal secretion of normal function as well as the nervous and immune systems (Lindstrom et al., 1995). The primary ingestion pathway of dioxins in humans is through foods (Patandin et al., 1999). Dioxins can begin to accumulate in the body upon frequent ingestion of foods containing even very small amounts of the compounds, which are neither excreted nor metabolized.

It is thought that the biological half-life of dioxins is several years to several decades in humans, as dioxins that are absorbed in the body are resistant to metabolism and excretion (Masuda, 2001). The influence of the enterohepatic circulation has been suggested as playing a role in rendering dioxins recalcitrant to excretion (Rohde et al., 1999; Kitamura et al., 2001). The enterohepatic circulation is a system for internal material recycling in which bile salts secreted in the duodenum are resorbed in the lower section of the small intestine and then reutilized in the liver. Dioxins, which are highly lipophilic and nonionic compounds, are absorbed from...
the intestinal tract by passive diffusion mediated by emulsification with bile acids in the same manner as common lipophilic materials. Absorbed dioxins are oxygenated by cytochrome P450, a drug-metabolizing enzyme in liver microsomes (Berg et al., 1994) and/or conjugated to glucuronic acid by UDP-glucuronoyl transferase in hepatocytes (Olson et al., 1983). These metabolized dioxins are secreted into bile from hepatocytes and excreted into the duodenum. Conjugated dioxins that are rendered water-soluble are excreted in the feces. Those dioxins that are deconjugated in the intestinal tract are reabsorbed from the small intestine and returned to the liver. In this case, bile salts promote internal reabsorption of the dioxins that have been rendered lipophilic again, facilitating their enterohepatic circulation.

Considering the high toxicity of dioxins, the search for materials that will promote their excretion from the body and elucidation of the underlying mechanism of dioxin excretion are important. As alluded to above, dioxins must first be removed from the enterohepatic circulation in some way if they are to be excreted from the body. One method for removing dioxins from the enterohepatic circulation is oral administration of adsorbing materials. Dioxin excretion is reportedly promoted by ingestion of algae, such as *Chlorella*, which adsorb dioxins on the cell surface (Morita et al., 1999; Takehoshi et al., 2005). The rate of excretion of the endocrine disrupting compound bisphenol A (BPA) is reportedly accelerated by the consumption of probiotics such as *Bifidobacterium breve* and *Lactobacillus casei*, which adsorb heterocyclic amines with a chemical structure similar to BPA (Oishi et al., 2008). However, there are no reports describing bacterial promotion of dioxin excretion through adsorption.

Another method for promoting dioxin excretion is the inhibition of bile acid reabsorption. Lactobacilli can promote the excretion of bile acids through several mechanisms. One mechanism involves active uptake of bile acids by the lactobacilli (Kurdi et al., 2000, 2006), whereas another mechanism involves deconjugation of bile acids by the enzyme bile salt hydrolyase (BSH), which is secreted by lactobacilli (Gilliland and Speck, 1977; Walker and Gilliland, 1993; Taranto et al., 2000; Martoni et al., 2008). If lactobacilli containing imported bile acids are then passed out of the body in the feces, the flow of bile acids to the liver decreases, and reabsorption of dioxins will be inhibited. Bile acids that have been deconjugated by BSH resist reabsorption, thus inhibiting enterohepatic circulation and increasing excretion of the bile acids from the body.

The present study examined whether oral administration of *Lactobacillus reuteri* CP3012 or *L. acidophilus* L-92 over consecutive days reduces the hepatic bioaccumulation of the dioxins 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) in Sprague-Dawley rats. These two strains of lactobacilli were chosen because some reports indicate that *L. reuteri* and *L. acidophilus* showed high BSH activity as described above and possessed high functionality such as the effect of allergy relief and intestinal regulation as a probiotics (Torii et al., 2007; Shah et al., 2012; Goto et al., 2013), and it was predicted that these lactobacilli could promote the excretion of PCB126 by promoting the excretion of bile acid. In addition, because the future availability for ingestion as probiotics for dioxin excretion was considered, these two strains, known to have high functionality and performance as a probiotics, were chosen. PCB126 was orally administered at a dose of 100 μg/kg of body weight. Induction of hydroxylation family cytochrome P450 enzymes in the liver was determined by the electron paramagnetic resonance (EPR) method, targeting low-spin state iron in parallel as an index (Yoshikawa et al., 2002; Morita et al., 2006). In addition, because two dioxin excretion mechanisms could be involved in elimination of PCB126, the influence of lactobacillus administration on reabsorption of PCB126 through the enterohepatic circulation was addressed by measuring the ability of these bacteria to adsorb PCB126 and quantifying bile acids in the excreted feces.

Materials and Methods

**Chemicals** 3,3′,4,4′,5-Pentachlorobiphenyl (PCB IUPAC#126: PCB126, stated purity, > 99.99%) was purchased from Wellington Laboratories Inc. (Ontario, Canada). Prior to administration, PCB126 was dissolved in corn oil (Hayashi Chemical Industry Co., Ltd., Kyoto, Japan) to a final concentration of 40 μg/mL.

**Creation of a PCB exposure model for animals and conditions for rearing** Specific pathogen-free (SPF) grade, 6-week-old male Sprague-Dawley (SD) rats (Japan SLC, Shizuoka, Japan) weighing 180 – 200 g each were used. Rats were acclimatized for 7 days after being brought to the laboratory, and were maintained on a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) with tap water available ad libitum. The rats were kept at a temperature of 21 ± 2°C with a relative humidity of 55 ± 5% and a constant 12-h light:12-h dark schedule. PCB126 dissolved in corn oil was administered to the rats at a dose of 100 μg/kg of body weight using an oral gavage tube specific for use in rats (RZ-1; CLEA Japan, Tokyo, Japan). Body weight was measured daily at a fixed time.

The animal research carried out in this study complied with the Azabu University Animal Research Guidelines and was conducted at the Azabu University Research Institute of Biosciences. The Azabu University Animal Research Guidelines have been approved by the Office for Protection from Research Risks of Japan (Authorization No. A5393-01).

**Bacterial strains, preparation of administration samples, and administration dose** Strains of *Lactobacillus reuteri* CP3012 and *L. acidophilus* L-92, maintained as bacterial stocks in the R & D Center of Calpis Co. Ltd. (Kanagawa, Japan), were used in this study. Each strain was cultured separately in 20 mL of MRS broth (Kanto Chemical Co., Inc., Tokyo, Japan) at 37°C for 14 – 24 h and then inoculated into 2 L of MRS broth and incubated at 37°C.
PCB126 Excretion from Rat Liver Following Lactobacillus Administration

for 24 h. Bacterial cells were washed twice with saline by centrifugation and then resuspended with sterilized distilled water to a final concentration of 10⁵ colony forming units (CFU)/mL and stored at −80°C until administration. The frozen bacterial suspensions were thawed at 4°C and then administered to rats at a once-daily oral dose of 5 mL/kg of body weight, continuing for 60 consecutive days from the day after PCB126 administration. Each thawed suspension contained at least 10⁶ CFU/mL of living bacteria. Meanwhile, the control rats were administered an equivalent volume of distilled water as substitute for the lactobacilli.

Necropsy of experimental animals and preparation of liver tissues  SD rats were euthanized 60 days after PCB126 administration. After exsanguination, the liver was removed from the peritoneal cavity and immediately perfused with 1.15% KCl solution. Tissue samples were immediately placed in sample tubes, frozen in liquid nitrogen, and stored at −85°C for subsequent gas chromatography/mass spectrometry (GC/MS) analysis or EPR measurements.

Quantitative determination of PCB126 in the liver using GC/MS  The level of PCB126 in the liver was determined according to the method of Lang (1992). PCB126 was extracted with lipid from liver tissue using the Soxhlet extraction method. Extracted lipophilic substances were saponified to hydrolyzed lipids, and PCB126 was extracted from the hydrolysate and concentrated with hexane. The extracted PCB126 was analyzed using GC/MS.

EPR measurement of the liver  Liver tissues were directly transferred and packed into EPR quartz tubes, rapidly frozen in liquid nitrogen, and stored at −85°C for subsequent gas chromatography/mass spectrometry (GC/MS) analysis or EPR measurements.

Statistical analyses  Data are expressed as mean ± SD. Differences among groups of rats were assessed by analysis of Dunnett’s multiple comparison method following analysis of between-group difference of the mean values by one-way analysis of variance. *p < 0.05 were regarded as statistically significant.

Results

Observation of animals  During the entire study period, no significant difference in the rate of weight gain was detected among the experimental groups; similar increases in body weight were observed in all groups. No signs of toxicity were observed in any of the animals, and no animals died during the test period. The state and normal condition of the rats were similar to those seen in our previous studies (Yoshikawa et al., 2002; Morita et al., 2006).

Accumulation of PCB126 in the liver  Figure 1 shows accumulation of PCB126 in the liver as determined by GC/MS. The concentration of PCB126 accumulated in the liver of control rats that were not administered lactobacilli was 133.1 ± 12.7 ng/g of tissue. In contrast, the concentration in rats administered L.
CP3012 was 30.7 ± 3.7 ng/g of tissue, which was 74% lower than that of the control group. The concentration of PCB126 in the liver of rats administered \textit{L. acidophilus} L-92 was 92.6 ± 25.0 ng/g of tissue, a value that was 25% lower than that of the control group. Thus, PCB126 accumulation in these three groups differed significantly \((p < 0.05)\), with significantly less PCB126 accumulating in the liver of rats treated with lactobacilli compared with the untreated controls \((p < 0.05)\).

The EPR spectra of liver tissues are shown in Figure 2. We previously reported that cytochrome P450 isozymes, including CYP1A1, are induced in rats administered PCB126 (Yoshikawa et al., 2002). The active site of cytochrome P450s contains heme. In a quiescent state, the Fe component of the heme is trivalent and in a low-spin state. The low-spin ferric species \((g = 2.40, 2.24, \text{and } 1.93)\), including those of P450s, can be clearly detected using EPR analysis (Morita et al., 2006). It has been revealed that the signal of the \(g = 2.49\) species in particular changes in parallel with PCB126. Mn(II) is used as an internal index in order not to affect this measurement system, and the degree of induction of a particular cytochrome P450 is expressed by determining the ratio \((\alpha/\beta)\) of the \(g = 2.49\) species \((\alpha)\) and Mn(II) \((\beta)\) peak heights.

The EPR signal of the \(g = 2.49\) species derived from cytochrome P450 was detected in PCB126-treated animals but not in the untreated controls (Fig. 2A). Yoshikawa et al. (2002) reported that the EPR signal of the \(g = 2.49\) species can be detected in the liver of rats administered PCB126 at 100 µg/kg of body weight. Because the PCB126 dose used in this study was sufficient to detect the EPR signal of the \(g = 2.49\) species, the EPR signal observed in the liver samples in this study suggests that PCB126 was absorbed by the rats and that the compound had an effect on cytochrome P450. The EPR signal intensities of the \(g = 2.49\) species for each group are shown in Figure 2B. Signal intensities of the \(g = 2.49\) species in these three groups differed significantly \((p < 0.05)\), with significantly less signal intensity of rats treated with \textit{L. reuteri} CP3012 compared with the untreated controls \((p < 0.05)\). The signal intensity of rats treated with \textit{L. acidophilus} L-92 was lower than untreated controls, but this difference was not significant.

The molar concentration of bile acid per 0.1 g of feces is shown in Figure 3. The concentration of bile acid in the feces of PCB126-treated control rats was 15.09 ± 0.17 µM. The concentration of bile acid excreted in the feces of rats administered \textit{L. reuteri} CP3012 was 41.47 ± 2.01 µM, whereas the
concentration in the feces of rats administered *L. acidophilus* L-92 was 62.06 ± 3.00 μM. Bile acid concentration differed significantly between the three experimental groups (*p* < 0.05). Levels of bile acid in *L. reuteri* CP3012 and *L. acidophilus* L-92 administration groups were 2.7- and 4.1-fold significantly higher (respectively) than that of the PCB126-treated controls (*p* < 0.05).

**Change in feces output following administration of lactobacillus**  
Stool output per day was calculated based on the amount of all feces collected during the entire experimental period. All stool collected was freeze-dried. The cumulative fecal output for each group was normalized on a per-day, per-rat basis (Fig. 3). The daily mean feces output of the PCB126-treated control group was 7.61 ± 0.29 g. The output of rats administered *L. reuteri* CP3012 or *L. acidophilus* L-92 was 9.92 ± 0.31 g or 9.68 ± 0.33 g, respectively. Thus, these three groups differed significantly in feces output (*p* < 0.05), with significantly more feces output of rats treated with lactobacilli compared with the untreated controls (*p* < 0.05).

**Discussion**  
This study examined whether oral administration of lactobacillus reduces the accumulation of the dioxin PCB126 in the liver of exposed SD rats. Following oral administration of *L. reuteri* CP3012 or *L. acidophilus* L-92, the accumulation of PCB126 in the liver decreased (Fig. 1). A decrease in the level of g = 2.49 species EPR signals indicated attenuated induction of cytochrome P450 enzymes. We previously reported that cytochrome P450 isoforms, including CYP1A1, are induced in rats administered PCB126 (Yoshikawa *et al*., 2002). Our results thus suggested that oral administration of lactobacillus led to increased excretion of PCB126, thus reducing accumulation in the liver. Biodistribution studies with dioxins indicate that the absorption in the body is approximately 70% of the oral dose, with hepatic retention and intact excretion accounting for approximately 50% and 0.6% (respectively) of the dose (Yoshimura and Yamamoto, 1975; Yoshimura *et al*., 1984). Hepatic accumulation of PCB126 in the present study (calculated based on Fig. 1 liver concentrations and respective necropsy liver weights) was consistent with the PCB126 accumulation observed in those previous studies.

Because PCB126 is an extraneous substance, the compound is excreted from the body by biological defense mechanisms following ingestion. Chemical substances that are highly lipophilic, such as PCB126, are metabolized and converted in the liver into a water-soluble metabolic product, which is then excreted into the urine or bile. The accumulation of lipophilic PCB126 *in vivo* occurs by reabsorption through the renal tubules of the low amounts of PCB126 excreted into the urine. In addition, it has been reported that even if PCB126 is conjugated in the liver and then excreted to the bile, accumulation of PCB126 in the liver does not change over the long term (Olson *et al*., 1983; Berg *et al*., 1994). This mechanism includes enterohepatic cycling, through which PCB126 is reabsorbed following deconjugation and then combined with bile acid in the intestinal tract. In fact, lipophilic PCB126 is reabsorbed via the small intestine in the same manner as bile. It is likely that PCB126 is only minimally metabolized and excreted by the body, given that no significant decrease in the liver concentration of PCB126 was detected in a previous study in which rats were administered a single dose of PCB126 and maintained for 60 days after administration under the same conditions used in this study (Yoshikawa *et al*., 2002).

Some materials, such as *Chlorella*, may adsorb dioxins following ingestion; however, they may not promote excretion of dioxins from the body. The adsorption of BPA by *B. breve* and *L. casei* and of aflatoxin B1 by *L. rhamnosus* involves hydrophobic interactions (Haskard, *et al*., 2000). There are considerable data indicating that the excretion of toxic hydrophobic compounds can be facilitated by probiotic bacteria. Although this previous work suggests that lactobacillus should adsorb dioxins, our assay did not
permit detection of adsorption of PCB126 to the surface of *L. reuteri* CP3012 or *L. acidophilus* L-92 cells in the present study. In addition, there are no other reports describing bacterial adsorption of dioxins. Thus, we concluded that administration of probiotics does not contribute to PCB126 excretion through adsorption to the bacterial cells in the intestinal tract.

A notable observation in this study is the general increase in the quantity of bile acids excreted from the body through probiotic-induced increases in the bile acid content in the feces and total fecal output. Administration of *L. reuteri* CP3012 or *L. acidophilus* L-92 resulted in a 4.1- to 2.7-fold increase, respectively, in the bile acid concentration in the feces, most likely representing bile acids derived from the enterohepatic circulation (Fig. 3).

The main bile acid in bile is cholic acid, which is conjugated with either glycine or taurine and thus contains a carboxyl group derived from the amino acid. Due to the negative charge imparted by ionization of its hydrogen molecule at intestinal pH, the carboxyl group binds easily with positively charged materials in the intestinal tract. Ingestion of insoluble dietary fiber derived from material such as chitosan or seaweed reportedly shortens the biological half-life of dioxins, thus limiting bioaccumulation by suppressing dioxin reabsorption in the gastrointestinal tract and promoting excretion in the feces (Kimura et al., 2004; Morita, 2002). Chitosan promotes excretion of dioxins with bile acids in the feces through the formation of a covalent bond between the positively charged free amino group of the chitosan molecule and the carboxyl group of the conjugated bile acid, eliminating dioxins without digestion and absorption (Kimura et al., 2004). Enterobacteriaceae such as *Escherichia coli* and *Salmonella enterica* are resistant to bile acids, including cholic acid (Thanassi et al., 1997; Nikaido et al., 2008). These bacteria excrete bile acids that have entered the cell using the proton motive force in order to avoid toxicity. *Lactococcus lactis* reportedly excretes bile acids from the cell through an ATP-dependent transporter (Yokota et al., 2000; Zaidi et al., 2008). Interestingly, some lactobacilli actively import bile acids into the cell, where the bile acids are neither metabolized nor excreted (Kurdi et al., 2000, 2006). In addition, high BSH activity has been reported for both *L. reuteri* (Taranto et al., 2000; Martoni et al., 2008) and *L. acidophilus* (Gilliland and Speck, 1977; Walker and Gilliland, 1993). Bile acids deconjugated by BSH resist absorption and inhibit enterohepatic circulation, and *ex vivo* excretion of the bile acids increases. The stimulatory effect of *Lactobacillus casei* TMC0409 and *L. acidophilus* ATCC 43121 ingestion on bile acid excretion is similar to the effect of chitosan ingestion on cholesterol and blood lipids (Kawase et al., 2000; Rodas et al., 1996), and there is a possibility that bile acid excretion could be generally promoted by this mechanism using lactobacilli. Our results suggest that administration of *L. reuteri* CP3012 or *L. acidophilus* L-92 inhibit the reabsorption of PCB126 in the intestinal tract, thus decreasing PCB126 accumulation in the liver by promoting an increase in bile acid excretion. Dioxin absorbed in the body is distributed to adipose tissue and to the skin, but is distributed predominantly to the liver (Yoshimura, 1984). Following accumulation in target organs, dioxin can persist long term in the body without redistribution; subsequent spontaneous excretion of accumulated dioxin is rare (Masuda, 2001). In the present study, PCB126 content was measured in the liver, the primary site of bioaccumulation; repeat-dose administration of lactobacillus resulted in decreased PCB126 accumulation. We did not measure PCB126 content in the feces directly, but the decreased liver concentration of PCB126 indicates PCB126 fecal excretion without reabsorption.

The effect of probiotics on the *ex vivo* excretion of cholesterol has been actively studied in recent years due to potential benefits associated with prevention or improvement of hyperlipidemia (Ejtahed et al., 2011; Awaishheh et al., 2013; Oner et al., 2013). The promotion of cholesterol excretion through probiotic-associated enhancement of bile acid excretion has been reported with many species and strains of probiotic bacteria, including *L. reuteri* and *L. acidophilus*. High BSH activity and promotion of high levels of bile acid excretion in the feces have been reported for many species of lactobacilli that exhibit hypocholesterolemic activity in the blood (Ejtahed et al., 2011; Awaishheh et al., 2013; Oner et al., 2013). Increased cholesterol-7a-hydroxylase activity in the liver promotes biosynthesis of bile acids from cholesterol to replenish bile acids lost through excretion (Kawase, 2000). Therefore, hypocholesterolemic activity may be important as an index when screening for bacteria that promote PCB126 excretion through inhibition of enterohepatic circulation. In addition, we found that administration of *L. reuteri* CP3012 or *L. acidophilus* L-92 led to significantly increased stool output. It is not only the increase in the bile acid content in the feces but also the increased stool output that contributes to the increase in the total amount of bile acid excreted from the body. Increasing stool output thus may become an effective mechanism to promote the discharge of toxic substances. Therefore, lactobacilli (e.g., administered as fermented milk (yogurt) starters) might be used to promote the continuous excretion of lipophilic toxic substances from the body. In addition, we previously (Yamazaki et al., 2008) have observed that fecal levels of *Lactobacillus* and *Bifidobacterium* were significantly decreased in rats administered PCB126 under the same conditions as those of the present study. Thus, ingestion of lactobacillus is very important to ensure recovery of *Lactobacillus* numbers adversely affected by PCB126 exposure.

This study demonstrated that oral administration of *L. reuteri* CP3012 or *L. acidophilus* L-92 reduces the accumulation of PCB126 in rat liver. Our data indicate that a possible mechanism for this effect is the promotion of bile acid excretion in the intestinal tract. Ingestion of lactobacilli promotes increased excretion of bile acids into the feces through absorption or deconjugation. Our results suggest that the increased excretion of PCB126 from the body results from inhibition of bile acid
PCB126 Excretion from Rat Liver Following Lactobacillus Administration
reabsorption.

References


Taranto, MP, Medici, M, Perigon, G, Ruiz Holgado, AP, and Valdez, GF.


