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Short-Chain Fructooligosaccharide Regulates Hepatic Peroxisome Proliferator-Activated Receptor α and Farnesoid X Receptor Target Gene Expression in Rats

Tomoyuki Fukasawa,*,† Asuka Kamei,‡ Yuki Watanabe,‡ Jinichiro Koga,† and Keiko Abe§

†Food and Health R&D Laboratories, Meiji Seika Kaisha, Ltd., 5-3-1, Chiyoda, Sakado-shi, Saitama 350-0289, Japan, ‡Food Safety and Reliability Project, Kanagawa Academy of Science and Technology, KSP EAST 301, 3-2-1, Sakado, Takatsu-ku, Kawasaki-shi, Kanagawa 213-0012, Japan, and §Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Prebiotic short-chain fructooligosaccharide (scFOS) is known to have various beneficial effects in humans and animals. Using a nutrigenomic approach, we have previously identified marker genes for the intestinal immunomodulatory and lipid-lowering effects of scFOS. The present study aimed to predict novel physiological effects of scFOS through nutrigenomic analyses. DNA microarray analysis revealed that administration of scFOS changed the expression of the nuclear receptors peroxisome proliferator-activated receptor α (PPARα) and farnesoid X receptor (FXR) target genes in the rat liver. Gene expression analysis provided some new interesting hypotheses, for instance, the possible improvement of bile secretion via FXR target genes, and regulation of amino acid metabolism and the urea cycle via PPARα and/or FXR target genes. Our findings clearly indicated that nutrigenomics may make it possible to screen for novel physiological effects of dietary ingredients.

KEYWORDS: Fructooligosaccharide; nutrigenomics; DNA microarray; PPARα and FXR target genes

INTRODUCTION

Functional food components called prebiotics are defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host health" (*1*). Of the known prebiotics, oligosaccharides such as fructooligosaccharides and galactooligosaccharides, polysaccharides such as inulin, and various starch-based materials have been extensively studied (*1*−*6*).

Short-chain fructooligosaccharide (scFOS) is one of the most popular prebiotics (*1*). As scFOS is not degraded by digestive enzymes, they consequently do not increase blood glucose and insulin levels (*7*). scFOS is the mixture of 1-kestose, nystose, and 1F-β-fructofuranosylnystose and is synthesized from sucrose by fungal β-fructofuranosidase (*8*). Indigestible scFOS arrives at the human gastrointestinal tract almost intact, where bifidobacteria and lactobacilli utilize it as a carbon source (*9*, *10*). Bifidobacteria have relatively high β-fructosidase activities (*11*) and possess specific oligosaccharide transporters (*12*), and can therefore use scFOS more efficiently than other genera of human intestinal bacteria. Furthermore, some intestinal bacteria, including bifidobacteria and lactobacilli, can metabolize scFOS to produce short-chain fatty acids (SCFA) (*13*). The reported beneficial effects of scFOS administration include the improvement of gastrointestinal conditions (*9*, *10*, *14*), promotion of mineral absorption (*15*), reduction of serum lipids (*16*, *17*), down-regulation of experimental ulcerative colitis (*18*), and modulation of the intestinal immune system (*19*−*21*). These beneficial effects are thought to be due to increased bifidobacterial numbers and SCFA levels in the large intestine (*22*).

Nutrigenomics is the application of high-throughput genomic tools in nutrition research, and it attempts to study the genome-wide influences of nutrition (*23*, *24*). Although nutrigenomics is largely based on transcriptomics, there is an emerging trend toward the incorporation of proteomics, metabolomics, and other related fields. The integration of these new methodologies is critical to fully assess the overall effect of a particular food characterized by its complexity and variety of constituent components (*25*). DNA microarrays make it possible to assess the impacts of a specific diet or nutrient on the expression of a large proportion of the whole genome. Muller et al. described that, in general terms, gene expression profiling can be used for three different purposes in nutrition research: first, to provide clues about the mechanisms underlying the beneficial or adverse effects of a certain nutrient or diet; second, to identify important genes, proteins, or metabolites altered from the predisease state that might act as "molecular biomarkers"; and third, to identify and characterize the basic molecular pathways of gene regulation modified by nutrients (*23*). In recent years, nutrigenomics has provided significant insights in a number of areas, including novel functions of food factors, mechanisms of nutrient effects, and even food safety issues (*25*, *26*).

*To whom correspondence should be addressed. Tel: +81-49-284-7588. Fax: +81-49-284-7598. E-mail: tomoyuki_fukasawa@meiji.co.jp.

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Table 1. Diet Composition

ingredient	g/kg diet	
	control diet	scFOS diet
casein	200	200
L-cystine	3	3
vitamin mixture[a]	10	10
choline bitartrate	2.5	2.5
mineral mixture[b]	35	35
corn oil	10	10
lard	80	80
cholesterol	5	5
sodium cholate	1.25	1.25
corn starch	653.25	625.07
scFOS[c]	0	50
total[d]	1000	1021.82

[a] Vitamin mixture (Oriental Yeast, Japan) formulated according to AIN-93. [b] Mineral mixture (Oriental Yeast, Japan) formulated according to AIN-93G. [c] scFOS, fructooligosaccharides (Meioligo-P; Meiji Seika Kaisha, Japan): The concentration of oligosaccharides was greater than 95% of the total mixture. [d] Experimental diets were pair-fed to equalize the energy intake and the amount of diet components except for carbohydrate to the scFOS group and control group. Thus, control groups were administered 97.86% weight of diet of the scFOS group.

Using nutrigenomics, we identified marker genes associated with the physiological effects of scFOS administration. This included the use of DNA microarray analysis to identify marker genes for major histocompatibility complex classes I and II, interferon, and phosphatidylinositol metabolites as markers for immunomodulating effects in ileum of mice fed scFOS. In addition, subsequent experiments revealed that the recognition site in the small intestine for scFOS administration and/or consequent changes in the intestinal environment were located to the epithelium and lamina propria, rather than to the Peyer's patch (*21*). The lipid metabolism-related gene was also identified as a marker gene associated with the decrease in hepatic triglyceride concentration and epididymal adipose tissue weight following scFOS administration in the rat liver (*17*).

Various beneficial effects of scFOS administration have been identified described above. The possibility still exists that scFOS has other novel functions. To test this hypothesis, we used a nutrigenomic approach to search for novel physiological effects of scFOS ingestion. In particular, we searched for novel genes specifically expressed in the liver of rats that were altered in response to scFOS using DNA microarray analysis, and then, from these findings, we attempted to determine novel physiological effects of scFOS.

MATERIALS AND METHODS

Animals and Experimental Design. Male 4 week old Wistar rats (Charles River Japan, Tokyo, Japan) were housed in a room maintained at 22 ± 1 °C with a 12 h light−dark cycle. After 1 week of acclimatization, the rats were dichotomized by body weight-based stratification (n = 8 in each group) and given powdered experimental diets (control diet and scFOS diet) with free access to drinking water throughout the 2 week experimental period. Given that the indigestible oligosaccharide scFOS is fermented by certain intestinal bacteria and absorbed as SCFA, the energy of scFOS was calculated to be 2 kcal/g (*13*). In contrast, the energy of corn starch is 3.55 kcal/g. Therefore, experimental diets (**Table 1**) were pair-fed to equalize energy intake and amounts of dietary components except for carbohydrate between the scFOS and the control groups; that is, the control groups were administered 97.86% weight of diet of the scFOS group. The scFOS used was a mixture of 42% 1-kestose, 46% nystose, and 9% 1F-β-fructofuranosylnystose (Meioligo-P, Meiji Seika Kaisha, Tokyo, Japan). After the 2 week treatment, rats were anesthetized with nembutal and killed. Blood was collected from the carotid artery, and serum was prepared by centrifugation. The liver was excised, and a portion was stored in RNAlater (Qiagen K.K., Tokyo, Japan) at 4 °C for 15 h and then at −20 °C until use. Our study was approved by the Animal Committee of Meiji Seika Kaisha Ltd., Food & Health R&D Laboratories, with the animals receiving care under the guidelines of this committee.

RNA Isolation and DNA Microarray Analysis. Total RNA was prepared from individual liver tissue samples using Qiagen RNeasy Mini Kit (Qiagen). The appropriateness of the quality and quantity of RNA samples were confirmed by measurement of optical densities at 260 and 280 nm and by observation of electropherogram and RNA integrity number (RIN) value (RIN > 9) obtained using a BioAnalyzer (Agilent Technologies, Palo Alto, CA). Hepatic RNA samples were submitted to GeneChip assay (n = 4 in each group). For each sample, double-stranded cDNA was synthesized from 2 μg of total RNA using the Eukaryotic Poly-A RNA Control Kit (Affymetrix, Santa Clara, CA) and One-Cycle cDNA Synthesis Kit (Affymetrix). In vitro transcription from cDNA was carried out using a GeneChip IVT Labeling Kit (Affymetrix). Biotinylated cRNAs were fragmented into 50−200 base pair lengths by heating at 94 °C for 35 min, and then, 15 μg aliquots were used for hybridization to Rat Genome 230 2.0 Array (Affymetrix) according to the manufacturer's protocol. After hybridization and subsequent washing steps using the Affymetrix Fluidics station 400, bound RNAs were stained with streptavidin phycoerythrin, and signals were amplified by treatment with fluorescent-tagged streptavidin antibody. Fluorescence was measured using an Affymetrix scanner.

Data Analysis and Gene Ontology Analysis. Affymetrix GCOS software was used to reduce the array images to the intensity of each probe (CEL files). The CEL files were quantified using FARMS (Factor Analysis for Robust Microarray Summarization) (*27*), and then, comparison analysis was performed between the scFOS and the control groups by the Rank_Products program (*28*) using the statistical language R(*29*), version 2.7.1, and Bioconductor(*30*), version 2.2. Genes with annotation grade "A" and a significant level of FDR (false discovery rate) of < 0.01 were considered to have been significantly affected by scFOS administration. GeneChip data were submitted to the University of Tokyo for database inclusion. To detect over-represented Gene Ontology categories in each data set, "Functional Annotation Clustering" with DAVID (http://david.abcc.ncifcrf.gov/home.jsp) was used with the GO_BP3 threshold.

RESULTS

In our previous study of the ileum of mice fed scFOS, changes in expression of several marker genes for intestinal immunomodulation were observed at an early stage before the increase of IgA secretion (*21*). However, results from liver tissue from rats fed scFOS for 4 weeks showed only Lpl as a late-stage marker gene for the lipid-lowering effects of scFOS, even though the physiological effects of scFOS administration were observed as decreased hepatic triglyceride concentration and epididymal adipose tissue weight (*17*). These findings suggested that gene expression changes in host organs could occur at periods considerably earlier than the observation of physiological effects of scFOS administration. Therefore, we decided to investigate altered gene expression patterns at early stages after scFOS treatment to identify genes with biological functions that have not yet been recognized as being involved in physiological effects of scFOS. On the basis of such results, it might then be possible to forecast novel physiological effects of scFOS administration.

No significant changes in food intake or body weight gain were observed between the two groups (p > 0.05 each) (**Table 2**). Four samples from each group were selected at random and submitted to the DNA microarray analysis. Statistical analysis of GeneChip data revealed that the expression of 297 hepatic genes on the Rat Genome 230 2.0 Array was significantly influenced by 2 week scFOS administration (FDR < 0.01). Of the 297 genes, 160 genes were up-regulated, and 137 genes were down-regulated in response to scFOS.

To gain a comprehensive understanding of the influences of scFOS administration on the liver, the 297 genes were investigated by "Functional Annotation Clustering" using DAVID.

Table 2. Effect of scFOS Administration on the Food Intake and Body Weight[a]

	control	scFOS
food intake (g)	235.44 ± 0.53	238.60 ± 10.24
body weight (g)		
initial	150.55 ± 2.23	147.05 ± 1.24
final	248.95 ± 2.89	254.71 ± 7.30
gain	98.41 ± 1.55	107.66 ± 6.18

[a] Values are means ± SEMs.

Significantly enriched Gene Ontology categories in response to scFOS administration are shown in **Table 3**. The "primary metabolic process" category including the "lipid metabolic process" subcategory was identified as functional group 1. Functional group 2 was found to contain the "cellular metabolic process" category, including the "organic acid metabolic process" and "amino acid and derivative metabolic process" subcategories. For Functional groups 3 and 4, the genetic categories were mainly defined as "regulation of biological process". These results indicated that the major functional categories influenced by scFOS administration at the gene expression level in rat liver were lipid metabolism, organic acid metabolism, and amino acid metabolism. Therefore, we focused our attention on these metabolic-related genes for subsequent investigation.

Further analysis of gene expression data identified 23 genes thought to be regulated by the nuclear receptors peroxisome proliferator-activated receptor α (PPARα) and/or farnesoid X receptor (FXR). These 23 genes included 18 PPARα target genes, 11 FXR target genes, and 6 genes that were targets of both PPARα and FXR. The PPARα and/or FXR target genes were investigated for their involvement in biological pathways according to known biological functions based on Gene Ontology. This analysis yielded five genes involved in cholesterol catabolism, four genes in glutamate metabolism, and one gene in the urea cycle (**Table 4**). Of the cholesterol catabolism-related genes, nuclear receptor subfamily 0, group B, member 2 (Nr0b2, SHP), which is positively regulated by FXR, and phospholipid and bile acid transporter ATP-binding cassette, subfamily B (MDR/TAP), member 4 (Abcb4), and ATP-binding cassette, subfamily B (MDR/TAP), member 11 (Abcb11) were up-regulated. In contrast, cytochrome P450, family 7, subfamily a, polypeptide 1 (Cyp7a1), a rate-limiting enzyme in the classic bile acid synthetic pathway, and cytochrome P450, family 8, subfamily b, polypeptide 1 (Cyp8b1), which acts at a branch point in the bile acid synthetic pathway, were down-regulated. For the glutamate metabolism-related genes, glutamic pyruvic transaminase 1 (Gpt1), glutamic pyruvate transaminase 2 (Gpt2), and glutamate oxaloacetate transaminase 1 (Got1), which are key enzymes in the alanine, aspartic acid, and glutamic acid metabolism pathways, and glutaminase 2 (Gls2), which converts glutamine to glutamic acid and ammonia, were down-regulated. Down-regulation of argininosuccinate synthetase 1 (Ass1), a rate-limiting enzyme in the urea cycle, was also observed. Changes in hepatic PPARα and FXR target gene expression levels observed in our experiment were almost entirely consistent with the expected changes in target gene expression in response to activation by PPARα and FXR.

Table 3. Significantly Enriched Categories ($p < 0.005$) That Showed Significantly Altered Expression Due to scFOS Administration

GO-ID	GO term (category)	*p*-value
Functional Group 1		
	primary metabolic process	
0006629	lipid metabolic process	1.28E-07
0008610	lipid biosynthetic process	1.23E-03
0044255	cellular lipid metabolic process	3.48E-07
Functional Group 2		
	cellular metabolic process	
0006082	organic acid metabolic process	1.53E-06
0006519	amino acid and derivative metabolic process	1.74E-03
0009308	amine metabolic process	2.18 E-03
Functional Group 3		
	regulation of biological process	
0050794	regulation of cellular process	6.11E-05
0042127	regulation of cell proliferation	6.66E-06
0048523	negative regulation of cellular process	2.77E-05
0048519	negative regulation of biological process	1.14E-04
Functional Group 4		
	regulation of biological process	
0048518	positive regulation of biological process	5.16E-04
0048522	positive regulation of cellular process	2.01E-04
0050794	regulation of cellular process	6.11E-05
0043067	regulation of programmed cell death	8.98E-04
0008219	cell death	9.74E-05
0030154	cell differentiation	4.28E-04

DISCUSSION

PPARα and FXR are ligand-activated transcription factors that belong to the nuclear receptor superfamily (*31*, *32*). PPARα is highly expressed in the liver and is relatively well-expressed in the kidney, heart, intestinal mucosa, skeletal muscle, and brown fat. It is well-known that PPARα plays important roles in the regulation of various metabolic pathways, including fatty acid oxidation, lipoprotein, bile acid and amino acid metabolism, and glucose homeostasis (*33*). FXR is expressed in the liver, gall bladder, intestine, kidney, and adrenal glands (*34*, *35*) and plays crucial roles in controlling bile acid homeostasis, as well as lipoprotein and glucose metabolism (*36*, *37*). In the present study, changes in PPARα and FXR target gene expression were observed in liver tissues from scFOS-fed rats, with these changes consistent with activation of PPARα and FXR. These results indicated that scFOS administration might have activated PPARα and FXR, which in turn altered expression of the target genes for these nuclear receptors.

PPARα and FXR are ligand-activated transcription factors. It has been shown that PPARα can be activated by long-chain unsaturated fatty acids, eicosanoids, and prostaglandins as endogenous ligands (*38*), and that dietary intake of specific fatty acids can also lead to potent PPARα activation (*39*). FXR is thought to be a bile acid receptor (*36*). It remains unclear what ligands are responsible for the activation of PPARα and FXR in response to scFOS administration, and this question will be investigated in future studies.

Nr0b2 (small heterodimer partner, SHP) is a nuclear receptor positively regulated by FXR and represses the transcription of Cyp7a1 and Cyp8b1 in a direct or indirect manner (*37*). The Abcb4, Abcb11, Cyp7a1, and Cyp8b1 are all key molecules in the bile acid synthetic pathway. Both Abcb4 and Abcb11 belong to the ATP-binding cassette transporter family and export phospholipids and bile acid, respectively, from the inner to the outer leaflet of the canalicular membrane (*40*, *41*). Cyp7a1 is a rate-limiting enzyme in the bile acid synthetic pathway. The series of gene expression changes related to bile acid synthesis suggested that the accumulation of bile acid in the liver was repressed by scFOS administration. The observation of up-regulated Abcb4 and Abcb11 expression and down-regulated Cyp7a1 and Cyp8b1

Table 4. Hepatic PPARα and/or FXR Target Genes Related to Cholesterol Catabolism, Glutamate Metabolism, and the Urea Cycle That Showed Altered Expression Due to scFOS Administration

gene title	gene symbol	representative public ID	up/down	rank	targeted by PPARα/FXR
cholesterol catabolism					
ATP-binding cassette, subfamily B (MDR/TAP), member 4	Abcb4	NM_012690	up	50	PPARα(*44*)/FXR (*45*)
ATP-binding cassette, subfamily B (MDR/TAP), member 11	Abcb11	NM_031760	up	168	PPARα(*46*)/FXR (*47*)
nuclear receptor subfamily 0, group B, member 2	Nr0b2	NM_057133	up	177	FXR (*37*)
cytochrome P450, family 8, subfamily b, polypeptide 1	Cyp8b1	NM_031241	down	8	PPARα(*48*)/FXR (*37*)
cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	NM_012942	down	48	PPARα(*49*)/FXR (*37*)
glutamate metabolism					
glutamic pyruvate transaminase (alanine aminotransferase) 2 (predicted)	Gpt2_predicted	AI535168	down	37	FXR(*50*)
glutaminase 2 (liver, mitochondrial)	Gls2	J05499	down	54	PPARα(*51*)
glutamic pyruvic transaminase 1, soluble	Gpt1	NM_031039	down	110	FXR(*50*)
glutamate oxaloacetate transaminase 1, soluble	Got1	D00252	down	123	PPARα(*51*)/FXR (*50*)
urea cycle					
argininosuccinate synthetase 1	Ass1	BF283456	down	172	PPARα(*51*)

expression in response to scFOS was consistent with the transcription pattern obtained after administration of an FXR agonist (*42*). Moschetta et al. reported that in the presence of the lithogenic diet when the FXR pathway is compromised, the biliary lipid profile is unbalanced, and bile becomes supersaturated with cholesterol. In contrast, stimulation of FXR activity by a synthetic agonist rebalances the physical/chemical interactions of lipids in bile. The experimental diet that we used in this study included cholesterol, sodium cholate, and lard as animal fats and could therefore be classed as a "lithogenic diet". The set of genes related to bile acid synthesis and export that were altered by scFOS administration suggested that the lithogenic state with a high-fat and high-cholesterol diet could be improved by scFOS administration via the transcriptional regulation of FXR target genes Abcb4, Abcb11, Cyp7a1, and Cyp8b1 (**Figure 1a**). To our knowledge, there have been no previous reports of improved bile secretion in response to scFOS administration. Thus, the present study is the first to show the possibility of enhanced bile secretion via transcription control of cholesterol catabolism.

Down-regulation of amino acid metabolism-related genes Gpt1, Gpt2, Got1, and Gls2, all known to be PPARα and/or FXR target genes, was observed. In addition, Ass1, a rate-limiting enzyme of the urea cycle, was also down-regulated, which suggested that the urea cycle was suppressed by scFOS administration. Gls2 encodes a glutaminase that converts glutamine into glutaminic acid and ammonia. Down-regulation of hepatic Gls2 suggested that scFOS administration suppressed ammonia production in the liver and, as a result of this phenomenon, inhibited the urea cycle. On the other hand, Gls2 down-regulation also suppressed the production of glutamic acid, which is another product of glutamine. In addition, the conversion of alanine to glutamic acid was suppressed by the down-regulation of Gpt1 and Gpt2, which is also known as alanine aminotransferase (ALT). Given this pathway of suppressed glutaminic acid production, it was interesting that Got1, also known as aspartate aminotransferase (AST), which interconverts glutamic acid and aspartic acid, was also down-regulated by scFOS administration. This is because as the result of these changes, provision of aspartic acid to urea cycle could be suppressed. Thus, scFOS administration appeared to facilitate two pathways of urea cycle suppression, that is, suppression of ammonia supply via down-regulation of Gls2 and suppression of aspartic acid supply via down-regulation of Gls2, Gpt1, Gpt2, and Got1. Furthermore, if ammonia and

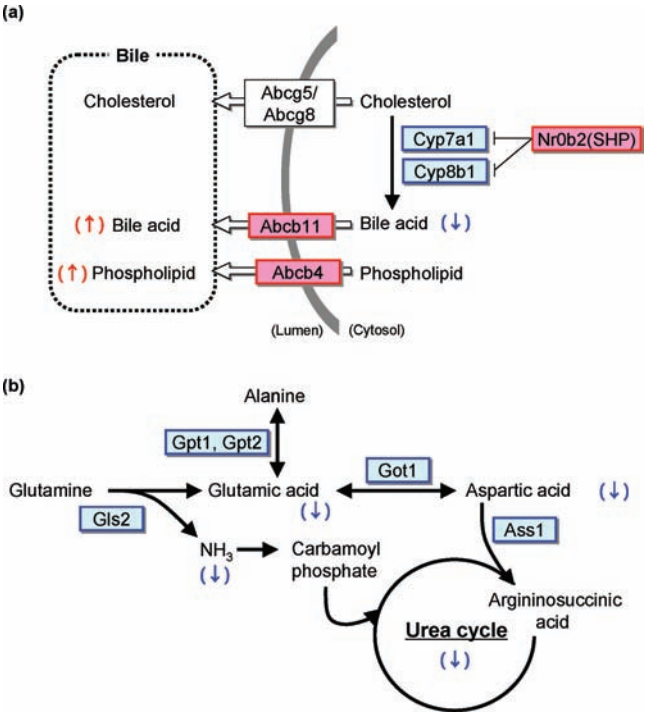

Figure 1. (**a**) FXR target genes involved in the bile acid synthetic pathway and biliary secretion and their possible responses to scFOS administration and (**b**) PPARα and FXR target genes involved in amino acid metabolic pathways and the urea cycle and their possible responses to scFOS administration. Indicated genes show the regulation following scFOS administration, with red boxes showing up-regulation and blue boxes showing down-regulation. The up and down arrows indicate the speculated effects of scFOS administration on bile acid synthesis and secretion, or amino acid metabolic pathways and the urea cycle.

aspartic acid, starting substances of the urea cycle, are suppressed via scFOS-mediated Gls2, Gpt1, Gpt2, and Got1 down-regulation, then the down-regulation of Ass1, the rate-limiting enzyme that incorporates aspartic acid into the urea cycle, is consistent. Thus, our results indicated the possibility that scFOS administration might suppress ammonia production and the urea cycle in the liver via down-regulation of Gls2, Gpt1, Gpt2, Got1, and Ass1, target genes of PPARα and/or FXR (**Figure 1b**).

It has been reported that scFOS administration reduces the production of intestinal toxic metabolites such as indole and skatol (*43*). The possibility that ammonia production and the urea cycle are suppressed in the liver by scFOS administration may reflect the phenomenon that the detoxification, more specifically activation of urea cycle, was not necessarily due to decreases in intestinal toxic metabolites.

Our present study revealed that scFOS administration changed the expression of PPARα and FXR target genes in the liver. DNA microarray analysis provided some interesting new insights, including the possibility of improved bile secretion via FXR target genes and regulation of amino acid metabolism as well as the urea cycle via PPARα and/or FXR target genes. Our previous study showed that the physiological effects associated with these gene expression changes occurred at a late stage after scFOS administration (*17*,*21*). In further studies, we intend to investigate whether the novel physiological effects associated with the observed gene expression changes actually occur by examination of biochemical markers at late stages after scFOS administration. Employing PPARα and FXR target genes identified in this study as "marker" genes should help clarify the mechanisms that underlie any new physiological effects of scFOS administration via PPARα and FXR target genes.

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