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Contents

Abbreviations

AF	Activation function
AKR1B7	Aldo-keto reductase 1B7
ANIT	α-naphthylisothiocyanate
APO	Apolipoprotein
ASBT	Apical sodium-dependent bile salt transporter
BAAT	Bile acid CoA-amino acid *N*-acetyltransferase
BACS	Ble acid CoA synthase
BDL	Bile duct ligation
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
C3	Complement component 3
CA	Cholic acid
CA12	Carbonic anhydrase 12
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
COX-1	Cycloxygenase-1
COX-2	Cycloxygenase-2
CYP7A1	Cholesterol 7α hydroxylase
CYP3A11	Cytochrome P4503A11
CYP3A4	Cytochrome P4503A4
DCA	Deoxycholic acid
DR	Direct repeat
ER	Everted repeats
FASN	Fatty acid synthase
FBP1	Fructose-1,6-bisphosphatase 1
FGF	Fibroblast growth factor
FGFR4	Fibroblast growth factor receptor 4
FRET	Fluorescence resonance energy transfer
FXR	Farnesoid X receptor
FXREs	FXR response elements

G6PC	Glucose-6-phosphatase
GC-MS	Gas chromatography-mass spectrometry
GLUT4	Glucose transporter type 4
GPR	Free fatty acid receptor
HDL	High density lipoprotein
HNF4A	Hepatocyte nuclear factor 4α
HPLC	High performance liquid chromatography
IBABP	Ileal bile acid binding protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Inverted repeat
JNK	C-Jun N-terminal kinase
LCA	Lithocholic acid
LDL	Low density lipoprotein
LDLR	low density lipoprotein receptor
LIPC	Hepatic lipase gene
LRH1	Liver receptor homologue 1
MDR	Multidrug resistance protein
MRD	Margin reflex distance
MRP	Multidrug resistance associated protein
N-CoR	Nuclear receptor co-repressor
NR	Nuclear receptor
NF-κB	Nuclear factor κB
NTCP	Sodium taurocholate cotransporting polypeptide
OST	Organic solute transporter
OATP2	Organic anion transporting polypeptide 2
PBC	Primary biliary cirrhosis
PCN	Pregnenolone 16α carbonitrile
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptorγcoactivator 1α
PLTP	Phospholipid transport protein

PPAR	Peroxisome proliferator activated receptor
PUFA	Polyunsaturated fatty acids
RXR	Retinoid X receptor
SHP	Small heterodimer partner
SMRT	Thyroid hormone receptor
SOCS3	Cytokine signaling 3
SRB1	Scavenger receptor B1
SREBP1C	Sterol-regulatory element-binding protein 1C
STAT3	Signal transducer and activator of transcription 3
SULT2A	Sulphotransferase 2A
TLR4	Toll-like receptors 4
TGR5	The G protein coupled receptor
TNFα	Tumor necrosis factors α
TTNPB	E-4-[2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid
UGT2B4	Uridine 5d-iphosphate-glucuronosyltransferase 2B4
VDR	Vitamin D receptor
VLDL	Very low-density lipoprotein
VLDLR	VLDL receptor
HER2	Human epidermal growth factor receptor 2

Chapter 1

General introduction

Chapter 1

General introduction

1.1 Background and objective

Farnesoid X receptor (FXR, NR1H4) is a ligand-activated transcription factor belonging to adopted orphan superfamily [1]. It is abundantly expressed in the liver, intestine, kidney, and adrenals [2, 3], but expressed little in fat tissue, stomach, lungs and heart [3, 4]. FXR mainly regulates intracellular levels of BAs in liver and intestine though various genes directly or intervene in other nuclear receptor signaling pathways [5, 6]. Researchers have proved FXR manages a series of genes involved in lipid and glucose homeostasis and plays a crucial role in diabetes reduction as well [7, 8]. Besides this, recent evidences have shown that FXR activation is critical in the regulation of inflammatory response [9-11].

FXR is a promiscuous receptor that can be activated by a great many of compounds not only limited to BAs [12]. The activation of FXR by its ligands has been proved effective in many diseases through FXR activation both in vitro and in vivo studies. Administration of synthetic agonist GW4064 induced a significant reduction in hepatic and serum triglycerides levels [7, 13, 14], and decrease in very low density lipoprotein (VLDL) secretion as a result of increasing small heterodimer partner (SHP) expression in mouse models of obesity and type 2 diabetes [15]. Lower incidence and extent of necrosis, reduced inflammation, and depressed bile duct proliferation were found in rats with GW4064 treatment [16]. Administration of FXR potent agonist 6α-ethyl-chenodeoxycholic acid (6ECDCA or INT747) to lithocholic acid (LCA) -induced

cholestasis increased BAs excretion and transient flee from the liver injury [17]. In addition, 6ECDCA has anti-inflammatory effect in inflammatory bowel disease (IBD) with evidence that inflammation inhibition and intestinal barrier preservation through repression of crucial pro-inflammatory cytokines, such as tumor necrosis factors α (TNFα) [10]. On the contrary, FXR-deficiency in mouse leads to increase in colon cell proliferation and carcinogenesis [18], contributes to the liver tumor formation [19] and shows impaired glucose tolerance and insulin sensitivity [20]. Due to the important role in BAs homeostasis and other metabolic diseases, FXR has a potential to be an attractive therapeutic target for common metabolic diseases treatment or prevention.

However, the clinical use of FXR ligands has not been approved yet. The most potent one is 6ECDCA which is under investigation at a phase of clinic study, and a long-term safety extension program is required [21]. Recent two studies investigating the effect of FXR activation by GW4064 on high fat diet (HFD)-induced obesity and glucose intolerance showed opposite results [22, 23]. The underlying molecular mechanisms contributing to these differences or FXR acting pathways have not been fully understood, and thus It is necessary to identify potent and selective FXR ligands or modulators which will provide us a powerful tool in analysis of unclear complex mechanism of FXR action.

A vast number of bacteria are inhabited in human body, which is a major player in maintaining human metabolism homeostasis. It is widely realized gut bacteria protect against obesity and insulin resistance [24], attenuate inflammation and restore colon homeostasis [25]. BAs levels were reduced in the gallbladder and small intestine in the presence of gut microbiota compared to germ free mouse [26, 27]. Another mouse studies indicated that there is a connection between gut microbiota and FXR function [28]. Intestine microbiome regulates BAs homeostasis by altering BAs composition resulting in FXR activation in intestine and liver [29]. In addition, a recent study has shown a functional FXR activity is necessary for the probiotic VSL#3 to exert its activity on BAs excretion and neo-synthesis in mouse [30]. Asking how intestine microbiome affects

relevant diseases via FXR activation, the work would be easier by using individual bacterial strains due to complexity of whole gut microbiome. Therefore, we aim to identify individual bacterial strains from intestine which may have important and unexpected functions via direct FXR activation.

In this study (**Figure 1.1**), at beginning a stable FXR reporter cell system was constructed for FXR-stimulating bacteria screen (chapter 2), and bacteria derived from intestine or health foods were evaluated based on their FXR activation potentials by FXR reporter system. Next, physiological effects including BAs homeostasis and anti-inflammation of two bacteria were explored in chapter 3. In the meantime, two bacteria were evaluated by using FXR containing liver hepatocellular HepG2 cells. After that, the bioactive factors that have FXR-stimulatory potentials were characterized in chapter 4. Finally, by using HFD-induced obesity mouse model, anti-obesity effects of two bacteria were investigated.

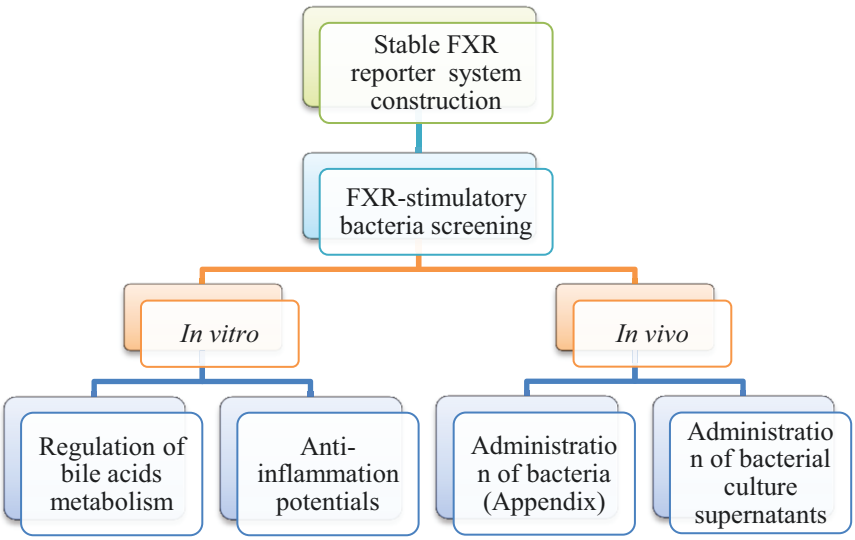

Figure 1.1 Research flow

1.2 Characterization of farnesoid X receptors (FXR)

1.2.1 Nuclear receptor (NRs)

Nuclear receptors (NRs) are great superfamily related DNA binding transcription factors

that have central roles in nearly every aspect of physiological phenomenon [31]. The research about NRs has been developed very rapidly since the first NR was recognized in 1960s. Forty-eight NRs have been identified in human genome [32] and 49 members in mouse [33, 34]. According to the ligand sensitivity, NRs can be subdivided into three subtypes [35]. The first group is steroid receptor family, which holds a high affinity for hormonal lipids. The second class is adopted orphan receptor family, which is also named "sensor" due to the sensitivity of xenobiotic or nutritional components. The third group is termed orphan receptor family which has received a high attention due to its developing understanding of function.

The structure of NRs is highly conserved. NRs can be divided into five distinct domains, named A to F (**Figure 1.2**). The N terminal region, containing A/B domain, is highly variable both in sequence and in length. Their 3D structure has not been known yet [36]. Activation function 1 (AF1) domain contains structure for gene transcription activation and repression [37]. AF1 sequence can act as an autonomous ligand-independent transcriptional activator, and can functionally synergize with ligand binding domain in AF2 [38]. Recent reports found that it can interact with co-regulators, and this co-regulator can act as bridge for AF1 domain and AF2 domain [39].

DNA binding domain (DBD) is the most conserved part in NRs. The 3D structure of DBD has been clarified in many nuclear receptors, which contains a P-box, responsible for the DNA-binding specificity; and two highly conserved zinc-fingers (C-X2-C-X13-C-X2-C and CX5-C-X9-C-X2-C) , maintaining stable domain structure [40]. DBD promotes the nuclear receptor connect to nuclear receptor response elements on the genome.

A hinge region which is a short flexible amino acid sequence, located in place between DBD and ligand binding domain (LBD). This domain can be phosphorylated, resulting increased transcriptional activation [41]. However, the full functions of the hinge domain are still unclear.

Figure 1.2 The classic structure of NRs. NRs contain an N-terminal transactivation AF1 domain, a highly conserved region DNA binding domain (DBD), a short hinge domain, a C-terminal ligand-binding domain (LBD) and a C-terminal domain.

The largest domain in NR is LBD, which is moderately conserved. The acquaintation of 3D structure which was well determined in several nuclear receptors, leads to a better understanding of the ligand binding mechanisms. LBD includes a ligand dependent activation function (AF-2) with a capability of proteins recruiting [42]. The ligand binding domain is responsible for many functions. For most ligands, a strong AF2 transactivation function and a strong dimerization interface are induced [43]. There would be an AF domain in C-terminal, which has extremely variable sequence. To date, the structure and function of this domain are unclear.

1.2.2 Farnesoid X receptor (FXR)

FXR is a ligand activated transcription factor belonging to adopted orphan receptor family. It was first cloned from rat liver cDNA library by Forman and named farnesoid X receptor based on its weak activation by farnesol [1].

1.2.2.1 Structure and distribution

There are two known FXR genes: FXRα (NR1H4) and FXRβ (NR1H5). The single FXRα gene encodes four FXRα sub-forms according to promoters and alternative splicing of the RNA, including FXRα1, FXRα2, FXRα3, FXRα4 [4]. FXRβ encodes a functional nuclear receptor in rabbits, rodents and dogs, but is a pseudogene in primate and human [44].

FXR has the common nuclear receptor superfamily modular structure as described above. In detail, the structure of FXRα four subtypes is presented in **Figure 1.3**. Compared with FXRα1 and FXRα2, FXRα3 and FXRα4 encode proteins containing an extended

N-terminus. Besides, four amino acids (MYTG) are inserted into the DBD in FXRα1 and FXRα3, while FXRα3 and FXRα4 do not contain it. The same LBD exsits in the four FXR isoforms but with different functions, e.g. DNA binding difference, varied co-regulator recruitment, or retinoid X receptor (RXR) heterodimerization properties.

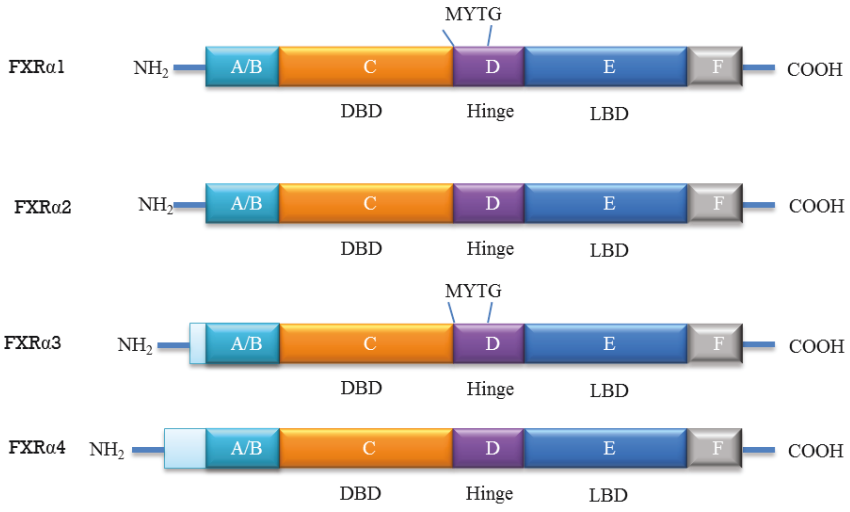

Figure 1.3 The structure of FXRα

FXR expression is limited to few tissues. As for mouse, high levels of FXR are discovered in the liver. Equal amounts of each FXR isoform are detected in mouse small intestine and liver, while adrenal glands and heart express only FXRα1 and FXRα2. Low amounts of FXR are checked in other places, such as lung and fat. For brain, spleen, or skeletal muscle, there is no FXR detectable. In adult human tissues, liver and adrenals exclusively express FXRα1/2, while FXRα3/4 is found in colon and kidney. The same expression levels of these isoforms are found in human small intestine. Immune cells, like subsets of lymphocytes and monocytes were also detected FXR expression [45]. FXR tissue distribution is summarized in table 1.1. The precise mechanisms that control FXR gene expression are not completely illuminated.

Table1.1 FXR tissue distribution

Species	Organ/tissue/cells
Mouse	Liver: gallbladder, hepatocytes, endothelial cells
	small intestine, large intestine, kidney, tongue, oesophagus, stomach, skin, eye
	White adipose tissue: adipocytes
	Lung: endothelial cells, heart
	Adrenal gland, fat
Human	Thyroid, salivary gland, prostate gland, adipose tissue
	Peripheral blood mononuclear cells (PBMC)
	CD4, CD8, CD19 and CD14 monocytes
	Adrenal gland
	Duodenum, colon, small intestine, kidney
	Liver, heart, skeletal muscle, stomach
	Atherosclerotic plaques, vasculature, metastatic cancers
	Vascular smooth muscle cells in coronary arteries and endothelial cells
	Trophoblast cells of placenta

1.2.2.2 Functional pathway

A genomic sequence analysis revealed that human FXR gene contains an assembled rough draft sequence more than 89.5 kb and contains 11 exons [46]. An interspecific backcross analysis mouse FXR gene locates in chromosome 10 region [47].

Like many other hormone nuclear receptors, FXR functions typically as a ligands dependent transcriptional regulator, working in with co-activators or co-repressors to activate or suppress expression of target gene (**Figure 1.4**). FXR binds to FXR response elements (FXREs) in the nucleus either as a monomer [48] or connecting with RXR as a heterodimer after ligand binding in the cytoplasm (with one known exception: uridine 5d-iphosphate-glucuronosyltransferase 2B4 gene (UGT2B4) [49]). The heterodimer form is most familiar and usually involved in gene activation, while the monomer may be related to gene repression. FXRE is typically an inverse repeat spaced in its target genes to induce FXR target genes expression. The preferred consensus FXRE for the FXR is an inverted repeat (IR)-1 element, appeared on most FXR target promoters stimulated directly by FXR. Nevertheless, FXR induces transcription by binding to DNA through multiple FXREs not

only limiting to the IR-1 consensus, like IR-0, IR-8, direct repeat (DR)-1 or everted repeats (ER)-8 [50].

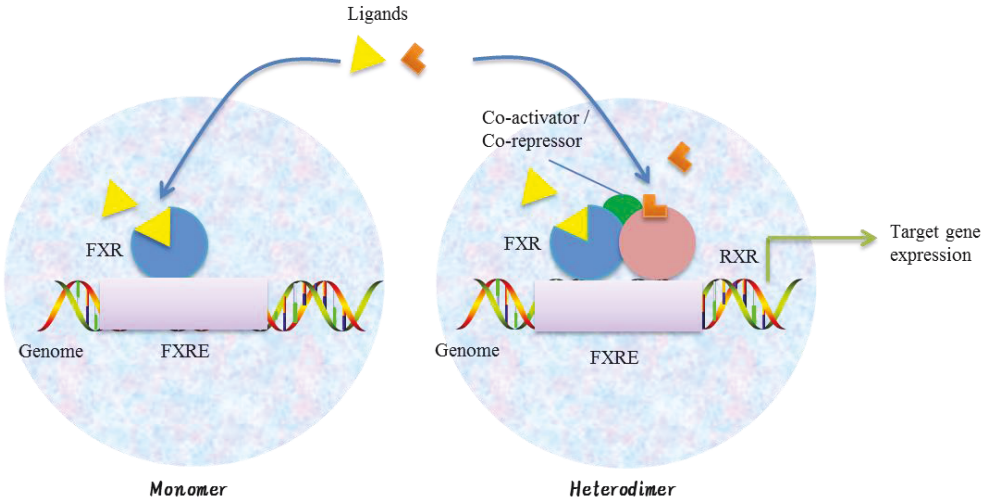

Figure 1.4 Mechanism of FXR

It is generally believed that a corepressor which inhibits the recruitment of transcriptional activation, would be involved in the FXR/RXR dimer without ligand binding [51]. Once ligand binds to FXR, it facilitates FXR conformation change, which in turn release corepressors and recruit coactivators, consequently affecting transcriptional rates of target genes [52]. However, the mechanisms that how FXR ligands regulate coactivators recruitment, and how these molecules further regulate FXR target genes expression are unknown.

1.2.3 FXR and its ligands

FXR is a receptor that can be activated by its ligands. At very beginning, FXR was identified by farnesol and farnesol metabolites activation and named farnesoid X receptor [1]. Nevertheless, these compounds only activate FXR at high concentrations that exceed physiological conditions and they cannot activate FXR directly [1]. Subsequently, some other FXR ligands have been consecutively found (Table 1.2). It is a breakthrough to discover the BAs can act as endogenous ligands of FXR at physiological concentrations [2, 53, 54].

Based on the discovery of BAs, scientists make their efforts to find effective small

molecular FXR agonists, which are modification of the parent BAs to make them more potent and stable. The approach has already resulted in some clinical success. The discovery of E-4-[2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid (TTNPB) gave the evidence to discover non-steroidal compounds for FXR. Meanwhile, some natural exacts either as FXR agonists or antagonists have been shown to act as FXR ligands. The use of these three axis of FXR ligands, including BAs, synthetic FXR ligands and natural extracts, prompts the development of the potential FXR ligands. It is expected that the new, specific, potent agonists of FXR will emerge in the future.

Table 1.2 Summary of FXR ligands

Function	Source	Compounds
Agonist	Nature	Farnesol, farnesol metabolites, CDCA, DCA, LCA, CDCA, CA,UDCA, forskolin, cafestol, xanthohumol, EGCG, ECG, EGC, coumestrol, androsterone, marchantin A, marchantin E, 22 (R)-hydroxycholesterol
	Synthesis or semi-synthesis	AGN29, TTNPB, GW4064, GW4064 derivatives, GW9047, GSK8062, fexaramate, fexarene, fexaramine, fexarine, fexarchloramide, froglitazone, rosiglitazone, pioglitazone, 6-ECDCA, CDCA derivatives, bile alcohols, GSK2324, NIHS700, WAY-362450, diphenylmethane, skeleton, methyl cholate, methyl deoxycholate, sulfated sterolspyrazolidine-3,5-dione derivatives, bile alcohols 5β-cyprinol, 1,1-Bisphosphonate esters, pyrazolidine-3,5-dione derivatives, 5β-cholanic acid, 5β-cholanic acid-7α,12α-diol, pyrrole[2,3-d] azepines, N-oxide pyridine GW4064, 6a-ethyl-24-norcholanyl-23-amine derivate
Antagonist	Nature	Guggulsterone, arachidonic acid, docosahexaenoic acid, linolenic acid, stigmasterol, oleanolic acid, tuberatolides
	Synthesis or semi-synthesis	AGN 31, bile alcohols 5β-bufol, bile alcohols 5α-cyprinol, bile alcohols 5α-bufol

1.2.3.1 BAs and BAs derivatives

Both primary BAs and secondary BAs can be FXR endogenous ligands in human. Among them, hydrophobic chenodeoxycholic acid (CDCA) is a potent BA, which was firstly described as the most potent FXR ligand with an EC50 of ~50μM. The secondary BAs

deoxycholic acid (DCA) and lithocholic acid (LCA) also showed a notable ability to activate FXR. Ursodeoxycholic acid (UDCA), a hydrophilic BAs used therapeutically in cholestatic diseases, was shown to have a weak activation ability. The order of potency of these BAs is CDCA > LCA >DCA > UDCA.

CDCA, due to its strong FXR activation, is applied to FXR studies. Oral administration of CDCA reduced cholesterol saturation of bile by decreasing hepatic cholesterol secretion and promoted cholesterol gallstone dissolution [55]. Treatment of gallstone patients with CDCA revealed decreased plasma triglyceride levels, VLDL [56] and serum triglycerides [57]. The activation of FXR in a human hepatocyte cell line by CDCA resulted in elevated low density lipoprotein receptor (LDLR) activity and the intracellular cholesterol levels [58]. CDCA is also proposed as down-regulator of lipid synthesis induced by rotavirus infection [59]. In HepG2 cells, FXR activation by CDCA has been shown to decrease sulphotransferase 2A (SULT2A, a cytosolic enzyme, critical in BAs homeostasis) transcription via the FXR response element (IR0) [60]. In wild type mouse, liver SULT2A levels were reduced after given a CDCA diet, whereas no decrease was observed in FXR-null mouse, which indicated a protective role of SULT2A against CDCA-induced hepatotoxicity.

The 6α-methyl derivate of CDCA (6α-ECDCA) was achieved by modification of the ring B of CDCA to improve the relationship of chemical properties and activity [17]. By alteration of a hydrophobic pocket in the LBD of FXR, 6α-alkyl derivates including 6α-ethyl, n-propyl and n-butyl derivatives were produced. Among them, 6α-ECDCA is the most potent FXR agonist with EC_{50}=99 nM in a fluorescence resonance energy transfer (FRET) assay which is three times higher than CDCA [17]. Many interesting derivatives for the modulation of FXR were generated, but none of them was superior to 6α-ECDCA. Therefore, 6α-ECDCA provides a powerful way for elucidation of FXR specific transcriptional signaling pathway. Administration of 6α-ECDCA promotes FXR interacts with PRMT thus enhancing FXR target genes mRNA expression [61]. When treatment of LPS-activated macrophages with 6α-ECDCA, induced SHP expression and depressed NF-κB dependent inflammatory genes,

the in vivo data also showed organ injury attenuation and immune cell activation [9]. 6α-ECDCA exhibited a protection of α-naphthylisothiocyanate and estrogen induced cholestasis, reverse ductular proliferation and necrosis by increasing bile salt export pump (BSEP), multidrug resistance associated protein 2 (MRP2) and margin reflex distance 2 (MRD2) gene expression[16, 21]. In leptin receptor mutated Zucker fa/fa rats, FXR activation by 6α-ECDCA, resulted in insulin resistance reduction both in liver and muscle cells, leading to a robust attenuation of liver steatosis [62]. 6α-ECDCA is also showed to be effective in chronic cholestasis treatment, e.g. primary biliary cirrhosis (PBC) patients [21].

1.2.3.2 Synthetic compounds

Because BAs do not strongly selective activate FXR, scientists devote themselves to develop synthetic molecules which have higher selectivity and affinity. To begin with TTNPB, a synthetic retinoid, was found to have low affinity in coactivator recruitment assays [63]. At the same time, different compounds were synthesized. These specific synthetic FXR agonists, including GW9047, GW4064, fexaramine and AGN34, provide powerful tools to explore complex FXR signaling pathways.

GW4064 (**Figure 1.5**), a selective, potent and most widely used agonist of FXR with EC50=90 nM, come from GW9047 by modification of its structure. GW4064 was considered as the first nonsteroidal FXR agonist with first high-affinity by high throughput screening and combinatorial chemistry [63]. Ever since, GW4064 has been the most widely used FXR ligand. Although GW4064 is an active FXR agonist both in vitro and in vivo, the restricted bioavailability limits its application in clinical phases.

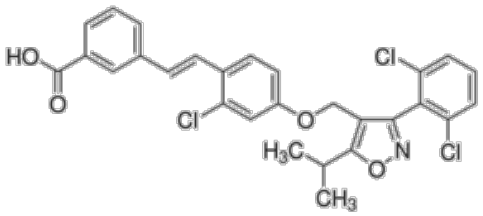

Figure 1.5 The structure of GW4064

GW4064 induced Cytochrome P450 3A4 (CYP3A4) expression in HepG2 cells and CYP3A11 expression in wild mouse, repressed hepatic and serum triglycerides levels, and increased VLDL as a result of increasing SHP expression upon FXR activation in mouse models of obesity and type 2 diabetes[15]. High triglyceride levels and low high density lipoprotein (LHDL) were appeared in FXR knock out mouse when administration of GW4064 [64]. FXR activation by GW4064 induced breast cancer cells apoptosis [65] and inhibited growth of breast cancer cells via reduction of human epidermal growth factor receptor 2 (HER2) expression [66]. Administration of GW4064 to rats led to inflammatory cell infiltration reduction and bile duct proliferation repression [16]. In bile duct proliferation, GW4064 has been proved to have hepatoprotective role, but due to the differences between rodents and humans, therapeutic benefits or risks of GW4064 in hepatoprotection still need further investigation and careful consideration [16].

1.2.3.3 Natural extracts

Many natural extracts that can act as FXR modulators have been reported. Guggulipid, the extract from the gum resin of *Commiphora mukul*, is well known for its effects in depressing of serum cholesterol, low density lipoprotein (LDL) and triglycerides. The active factor of guggulipid contributing to the metabolic property is the guggulsterone, which is a mixture of Z- and E-4, 17(20)-pregnadiene-3, 16-dione. On the contrary, guggulsterone was reported to increase bile salt export pump (BSEP) expression [67]. However, both Z- and E-guggulsterone can make theirs roles through members of endocrine NR subfamily, such as FXR and pregnane X receptor. Although guggulsterone relatively lacks selectivity, it is a gene specific antagonist of FXR activity according to its selective molecular behavior in cell free coactivator binding assays [68, 69]. This peculiar behavior could be due to a specific guggulsterone docking site in the LBD of FXR [70].

Some other natural extracts have been also indicated to be FXR modulators. Stigmasterols, the components of soy-derived lipids, were able to regulate FXR target genes expression as

FXR antagonist in cholestasis and in HepG2 cells [71]. Additionally, cafestol, a diterpene isolated from unfiltered coffee brew, displayed an agonistic effect on FXR, showing similar efficiency in HepG2 cells compared to CDCA and pregnenolone 16α carbonitrile (PCN) [72]. However, in vivo results showed FXR target genes were unaffected in the liver, but induced FGF15 gene expression in the small intestine when administration of cafestol, thus resulting in an inhibitory signaling enterohepatic loop [73]. The low bioavailability or its rapid metabolism in the liver could explain the differences in liver and gut, which indicated that ileum would be a critical FXR target organ [74]. Prenylated chalcone xanthohumol from beer hop can be a FXR activator based on the fact that it activates the BSEP in hepatoma cells and deduces plasma and hepatic triglycerides in diabetic KK-A^y mouse [75].

1.3 FXR and metabolic disorders

1.3.1 FXR and BAs metabolism

FXR makes a central role in BAs metabolism regulation. All aspects of BAs can be regulated by FXR, including BAs synthesis, conjugation, secretion, absorption and export to the gall bladder, etc. Thus, FXR is also called BAs receptor, which confirmed its special status in BAs homeostasis.

BAs may regulate their own synthesis through a negative feedback function pathway [76]. Since FXR and its target genes were discovered, this feedback pathway mechanism has been understood gradually. There are at least three pathways that could explain the negative feedback pathway. They are SHP, FGF15 in mouse (FGF19 in human) and c-Jun N-terminal kinase (JNK). Once hepatic FXR is activated, FXR target gene SHP is induced and later inactivates liver receptor homolog 1 (LRH1), which is important for cholesterol 7α hydroxylase (CYP7A1) expression [77, 78]. Hepatic CYP7A1 mRNA levels were reduced after treatment with BAs in $SHP^{-/-}$ mouse, which revealed the existing of other pathways, involved in the repression of CYP7A1 by BAs but independent of SHP [79]. The second pathway that involves gene FGF15; the expression and secretion of FGF15 in intestine was

increased when FXR was activated by GW4064 or BAs. FGF15 subsequently activate FGFR4 receptor which is localized on the hepatocytes membrane, leading to JNK pathway activation and CYP7A1 repression [73]. The critical role of FGFR4 was verified by the finding that FGFR4$^{-/-}$ mouse have larger BAs pools and increased CYP7A1 expression [80]. The third way is that BAs can rapidly down-regulate CYP7A1 gene transcription via a JNK dependent mechanism, suggesting "cross-talk" occurs between the BAs activated JNK signaling cascade and FXR in sensing BAs levels [81]. The BAs function pathway in enterohepatic cycle is shown in **Figure 1.6**.

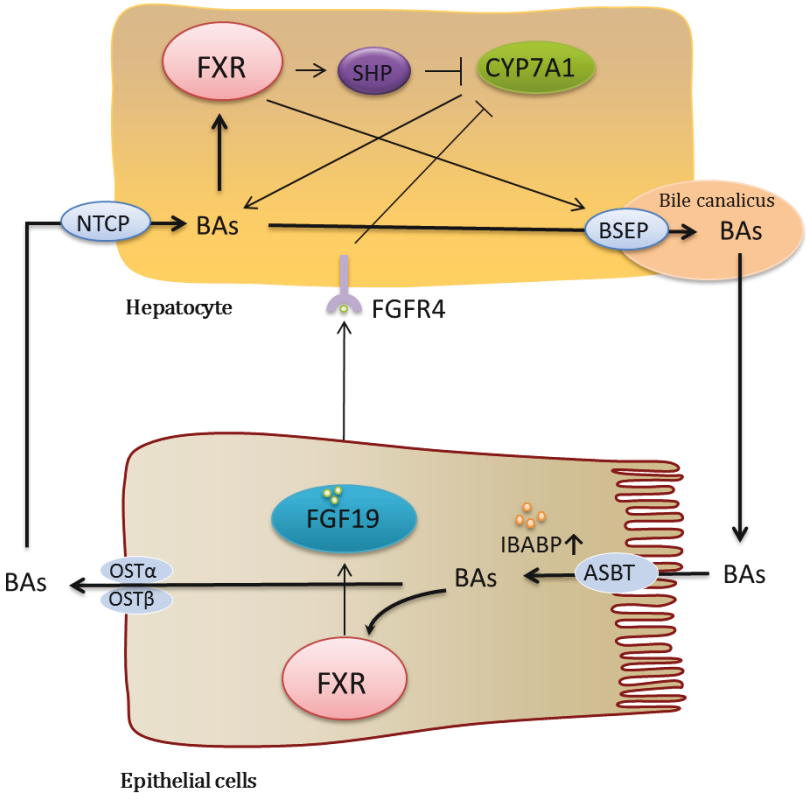

Figure 1.6 BAs pathway in enterohepatic cycle

1.3.2 FXR and hepatic disease

FXR has been shown to be effective in hepatic diseases, and thus becomes an attractive target for hepatobiliary disorders from liver regeneration, carcinogenesis, inflammation, and hepatitis C virus replication [82, 83].

Hepatic inflammation is involved in viral hepatitis, autoimmune hepatitis and nonalcoholic steatohepatitis. Studies have proved that FXR has a critical role in modulation of hepatic inflammatory response. Distinct inflammation and increased inflammatory genes expression were discovered in $FXR^{-/-}$ mouse liver [55]. The expression of pro-inflammatory cytokines inducible nitric oxide synthase (iNOS), Interleukin-1α (IL-1α) and IL-6 were increased by tumor necrosis factors α (TNFα) or lipopolysaccharide (LPS) in primary hepatocytes of FXR deficiency mouse, and in addition, these pro-inflammatory cytokines can be decreased by administration of GW4064 or 6α-ECDCA [56]. CDCA administration attenuates hepatocyte inflammatory damage, reduces transaminase activities, suppresses inflammation mediators, inhibits signal transducer and activator of transcription 3 (STAT3) phosphorylation and increases suppressor of cytokine signaling 3 (SOCS3) expression [84]. Another FXR synthetic ligand WAY-362450, showed protecting against inflammation and fibrosis in mouse liver, but hepatic triglyceride accumulation was not affected, whereas hepatoprotection is totally abolished in $FXR^{-/-}$ mouse [85].

Cholestasis is a pathology characterized by impairment or cessation of bile secretion and flow. Cholestatic injury is related with BAs accumulation and pro-inflammatory cytokines activation in liver, leading to biliary fibrosis and cirrhosis. FXR activation by GW4064 or 6α-ECDCA was able to protect animal models from bile duct ligation (BDL)-, α-naphthylisothiocyanate (ANIT)-, and ethinyl estradiol-induced cholestasis [16]. Activation of intestinal FXR by GW4064 protected the liver again cholestatic injury [16, 86]. FXR activation by CDCA can inhibit MRP4 expression through competition for binding to an overlapping binding site with constitutive androstane receptor (CAR), which suggesting FXR activation would be a therapy target for different cholestatic syndromes [87]. FXR agonists may be also used for cholesterol-gallstone disease treatment. While administration of lithogenic diet to $FXR^{-/-}$ mouse appeared inflammation in gall bladder wall and cholesterol gall stone formation, they can be reversed when treatment of GW4064 in wild type mouse [88]. Pharmacotherapy for cholestasis is limited. UDCA is the only drug therapy for

pharmacotherapy with evidence of efficacy that symptoms improvement, hepatic enzyme abnormalities, and death reduction and liver transplantation in patients with PBC at early stage [89].

1.3.3 FXR and gastrointestinal disease

FXR is highly expressed in the intestine especially in the ileal epithelium, which is the place of BAs absorption into intestine. FXR has been implicated that linked with many gastrointestinal diseases, such as inflammatory bowel disease and colonic cancer.

Intestinal FXR activation attenuates inflammation and preserves the intestinal epithelial barrier integrity by regulating inflammatory response, maintaining the integrity and function of the intestinal barrier, and preventing bacterial translocation. Administration of FXR ligand 6α-ECDCA, alleviated intestinal inflammation [10], protected against colitis in wild type mouse, but not in FXR knockout mouse [9]. FXR deficient mouse responded to intestinal inflammation with an uncontrolled immune reaction and inflammation-driven fibrosis in the colon, which provided the evidence that FXR is involved in IBD due to counter-regulatory effects on innate immunity [9]. Activation of FXR in the intestinal tract attenuates inflammation severity, and inhibits the production of various NF-κB target genes, such as TNF-α, IL-1, IL-6, cycloxygenase-1 (COX-1), COX-2 etc., thus contributing to a reduction in inflammation [90]. Decreasing the level of pro-inflammatory cytokines, FXR activation has indirect influence on intestinal permeability and transport. It has been demonstrated that pro-inflammatory cytokines initiate gene expression changes by regulating transcription factors as well as drug metabolizing enzymes, such as MDR-1 protein, MRP2, MRP3, BSEP, organic anion transporting polypeptide 2 (OATP2) and CYP3A [91]. FXR restores the permeability of intestinal barrier with body weight reduction, inflammatory cell infiltration decrease, goblet cell loss and inhibiting of cytokines that contributes to disruption of epithelial tight junction function [92].

Intestinal FXR activation has an important role in bacterial overgrowth inhibition, thus

protecting the intestine against bacterial damage. Bacterial overgrowth increased intestinal permeability as well as epithelium inflammation in mouse lacking FXR [93]. Expression of carbonic anhydrase 12 (CA12) was also induced by FXR activation, participating in antibacterial defense by maintaining appropriate intestinal pH and ion balance, and thus FXR activation is important for intestinal luminal contents homeostasis and the integrity of epithelial barrier [93]. These results support that a critical role of FXR for regulating intestinal bacterial growth, which has significant implications for maintaining a competent barrier and thus, contributing to the prevention of intestinal inflammation.

1.3.4 FXR and other relevant diseases

Besides its central role in BAs homeostasis, FXR is also effective in regulation of glucose and lipid homeostasis. FXR is necessary for normal glucose homeostasis, and FXR-SHP cascade provides a close relationship between lipid and glucose metabolism [7]. Plasma glucose levels were lowered by FXR activation via insulin action, which indicated that FXR is closely involved in glucose homeostasis modulation [94]. Treatment with GW4064 inhibited body weight gain and glucose intolerance in HFD feeding mouse model [23]. Also, GW4064 significantly repressed diet-induced hepatic steatosis by lower triglyceride and free fatty acid level in the liver, and attenuated hepatic inflammation while having no effect on white adipose tissue [22].

Various obese and diabetic mouse or rat models have been investigated in combination with either FXR deficiency or FXR activation by FXR ligands [22, 23, 95, 96]. However, because of experimental design differences and controversial experimental results, to date, no consensus has been achieved on FXR physiological role in metabolic disease protection or therapeutic potential as a pharmaceutical target.

1.4 FXR and bacteria

The gastrointestinal tract provides residence of a huge number of bacterial species, which contribute to not only limit to gastrointestinal health and disease, but also to biology, function, physiology, and immune response of whole body. Dysbacteriosis in intestine contributes to many diseases including irritable bowel syndrome (IBS) [97], chronic inflammatory diseases such as IBD [98], colorectal cancer [99], obesity [100] and other metabolic disorders. A great many of studies in mouse and humans have already demonstrated probiotics provide opportunities for these diseases' clinical applications.

1.4.1 Bacteria and nuclear receptors

Probiotics are closely linked with nuclear receptors to regulate metabolic homeostasis and relevant diseases. Peroxisome proliferator activated receptor (PPAR) is a nuclear receptor which plays a key role in inflammation response by inhibiting nuclear factor κB (NF-κB) pathway. Studies have shown that probiotics regulated PPAR expression in experimetal inflammatory model [101]. Probiotics can regulate NF-κB signaling and inflammatory responses by up-regulation of PPAR-γ which is highly expressed in the colon [102]. *Pediococcus pentosaceus* LP28 could act as PPAR-γ agonist with effects of triglyceride and cholesterol reduction in obese mouse [103]. Orally administration of probiotics are helpful for systemic anti-inflammatory effects and intestinal barrier function improvement through PPARγ induction [104]. *Lactobacillus casei* that increased PPARγ mRNA expression in dose-dependent manner, suppressed the expression of inflammatory mediators in intestinal epithelial cells, suggesting the anti-inflammatory action of *Lactobacillus casei* might be partially related to PPARγ activation [105].

Vitamin D receptor (VDR) also showed a critical role in regulating intestinal homeostasis by inhibiting inflammation and maintaining cells integrity. Probiotics regulate VDR expression, location, and activity, negatively regulates NF-κB [106]. Treatment with

Lactobacillus plantarum increased in VDR expression but not inhibit inflammation in mouse lacking VDR [107].

1.4.2 Bacteria and BAs metabolism

The human intestinal microbiome composition can be affected by many factors, such as age, diet, antibiotics or diseases. BAs which are reabsorbed in the gut appear to be an important impact factor of the microbiome community. It is said that the fecal BAs concentration and composition are closely connected to gut microbiota, which was confirmed by using 16S ribosomal gene quantification for analyzing microbiome community structure [108]. Mouse fed with BAs showed more complex changes in the intestinal microbiome with strike increase in *Clostridia* and *Blautia*, in the meantime, total BAs in feces increased 20-fold [109]. Actually, BAs have been proved to have strong antimicrobial activity due to the bacterial cell membranes damages [110]. High concentrations of BAs may dissolve bacterial membrane lipids very quickly which in turn dissociate with membrane proteins integrity [111]. The review concluded that low BAs levels in the intestine are more likely to affect gram negative bacteria, and some of which may produce LPS and thus have a potential to be harmful, while the high levels of BAs may favor gram positive bacteria in the intestine [29].

The normal microbiota in the gut produces secondary BAs by utilization of primary BAs [112]. The main bacteria contributing to BAs transformation are anaerobic bacteria, including *Bacteroides, Eubacterium*, and *Clostridium*. A study showed BAs synthesis changed and altered conjugated BAs signatures in the case of antibiotics-treated or germ free mouse [113]. Another study revealed the BAs pool size reduction when conventionalize germ free mouse [27]. Further studies demonstrated that the behind mechanism is probably due to activation of nuclear receptors especially FXR by the BAs [27]. BAs and their derivate by gut microbial metabolisms are major FXR ligands and strongly activate expression of FXR target genes which involved in BAs synthesis and

transport. A recent study showed when treated with antioxidant tempol, secondary BAs production was deduced by blocking bile salt hydrolase (BSH) activity, resulting in FXR activity reduction [28]. Another study exhibited that activation of FXR and the G protein coupled receptor (TGR5) receptors led to energy expenditure increase, thus protecting from diet induced obesity [114], and concluded that gut microbiota may help treatment of obesity and type 2 diabetes through lipid and glucose metabolism repression which came from FXR and TGR5 signaling activation.

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Chapter 2

Stable FXR reporter system construction

Chapter 2

Stable FXR reporter system construction

2.1 Introduction

FXR is the ligand-activated transcription factor. Upon binding to ligand, FXR will connect with FXR element (FXRE) in genome and in turn regulate FXR target genes expression (shown in **Figure 1.4**). FXR regulates a great many of target genes involved in the metabolism and homeostasis of bile acids, lipids and glucose. Many studies have indicated an important role of FXR ligands or modulators in the treatment of metabolic relevant diseases.

FXR and FXR ligands are closely linked with many diseases, which promote scientists to make a number of test systems for characterization of compounds for FXR activation. Many test systems have been developed including cell based and cell free, functional and affinity based assays and test systems to investigate the activated gene pattern. In the characterization and development of nuclear receptor, cell based test system is a standard test system, which provides a cellular background and can give information on functional activity of the candidate ligands. Recombinant protein expression and purification are not required because of the cellular background. But in the meanwhile, cell-based test systems are influenced by complex cellular factors. With cell based assays, functional activities of compounds can be easily determined but binding affinity and kinetics cannot be directly determined [1].

The most common and widely used test system for discovery and characterization of

ligands is the reporter gene assay. The first FXR reporter gene assay was used to prove that FXR is a bile acid receptor [2]. The main concept is that an expression plasmid was transfected including FXR sequence into eukaryotic cell line first, then another plasmid containing the reporter gene is transfected for DNA binding domain. Because FXR functions commonly as heterodimer with RXR, when FXR is used in a gene reporter assay, a plasmid for RXR expression is also co-transfected.

Now, many optimized selecting models have been developed for both ligands selection and genes expression exploration. Schuster et al. found a novel FXR agonist through biological evaluation in which full length of murine FXR and RXR expression plasmid and a reporter plasmid containing ECRE-*Luc* gene were transiently transfected into HEK293 cells [3]. Another group constructed a reporter gene system in HepG2 cells by transient transfection with FXR and RXR expression plasmid and the reporter vector TK-*luc* to distinguish polyhydroxylated sterols from the indonesian soft coral sinularia which acts as FXR antagonist [4].

Almost all of the FXR reporter gene assays are transiently transfected into the used cells. For the transient transfection, many protocols have been optimized in a number of cell lines and they can direct for other expect cells according to these methods. Nevertheless, since this assay is only based on the reporter gene as single readout, it needs an internal standard gene as control gene. Up to now, only a few stable cell lines for FXR reporter system are described [5, 6]. Et al. discovered a novel class of pyrrole (2, 3-d) azepines as FXR agonists by using a stable FXR reporter system which contains an expression vector for GAL4-FXR-LBD DNA binding domain and a Luc12 luciferase reporter gene in HEK293 cells [5]. Because of stable transfection, this system is both time-saving and cost-economic, and more importantly, desirable. In transfection, a β-galactosidase or a fluorescent gene is always transfected, and thus they can be detected simultaneously without affecting the reporter gene. Second, there is no need to find an internal gene as control gene due to reason that the internal control gene is located on an additional plasmid and under control of a constitutive promoter [1]. The stable cell line

can be incubated with test compounds directly, and after the co-incubation period usually 24 h, luciferase activity can be measured.

In this part, in order to screen FXR modulators, I aimed to construct a stable reporter system as FXR modulator screening system. Although reporter gene system is a good method for FXR ligand screening, it lacks the information on FXR target genes expression regulated by its ligands [1]. Thus, we combined these two methods together for FXR modulators screening and physiological evaluation.

2.2 Material and methods

2.2.1 Cell culture

Human colon adenocarcinoma cell line SW480 (ECACC, EC87092801) was grown at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle's medium (Wako, Osaka, Japan), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, New York, USA) and 1% Penicillin-Streptomycin Solution (×100) (Wako, Osaka, Japan).

2.2.2 FXRE-driven firefly luciferase reporter vector

A DNA fragment containing four copies of the FXRE (5'-aaactgaGGGTCAgTGACCCaaggtgaa-3') from the phospholipid transfer protein promoter [7, 8] and *Xho* Ⅰ and *Bgl* Ⅱ restriction enzyme sites was synthesized and cloned in the vector pUC19 (Greiner Bio-One, Frickenhausen, Germany). The *Xho* Ⅰ / *Bgl* Ⅱ fragment of pUC19-4×FXRE was ligated into a pGL4.27 [luc2p/minP/Hygro] vector (Promega, Madison, USA) digested with *Xho*/*Bgl* Ⅱ (Promega) to generate a FXRE-driven firefly luciferase reporter vector (pGL4-4×FXRE-*luc*).

2.2.3 Establishment of a stable FXR reporter cell line

SW480 cells do not respond to a FXR agonist (e.g. GW4064), because SW480 cells do not endogenously express FXR [9]. In order to generate a stable FXR expressing cell,

SW480 cells were transfected with a FXR expression vector EX-T0601-M02 (Genecopeia, MD, USA) using FuGENE reagent (Promega, WI, USA). For the selection of stable FXR expressing cells, the cells were cultured in DMEM medium which containing 800 μg/ml G418 (Wako, Osaka, Japan).

G418-resistant SW480 cells were further transfected with a reporter pGL4-4×FXRE -*luc* with FuGENE transfect reagent. For a selection of a stable FXR reporter cell, the cells were cultured in DMEM medium containing 800 μg/ml G418 and 300 μg/ml hygromycin B (Invitrogen, CA, USA). G418 and hygromycin B-resistant cells were collected with a cloning cylinder, and subcultured into fresh medium containing G418 and hygromycin B.

2.2.4 Reporter assay

FXR reporter cells were seeded at a density of 5×10^4 cells/well in 96-well plates. Twenty-four hours after seeding, cells were incubated with DMSO (0.1% v/v) or GW4064 (10 μM) for 24 hours. After that, cell supernatants were removed and cells were washed twice by phosphate-buffered saline (PBS) and lysed by adding 20 μl of passive lysis 5×buffer (Promega) with gentle rocking for 20min. Luciferase activities were measured with administration of luciferase assay reagent (Promega, Madison, USA) by using GloMax® 96 Microplate Luminometer (Promega). The ratio of treatment over control was served as -fold activation.

2.3 Results and discussions

FXR activation has been proved to be beneficial to the intestinal health. Reduced FXR expression in the intestine would increase colorectal cancer susceptibility in mouse [10]. When FXR is activated in colon cancer cells, it induces cells' apoptosis and removes genetically altered cells, suggesting that promotion of FXR expression in colon tumors could be an useful tool in treatment of colon cancer [11].

SW480 cell line is a kind of human colorectal carcinoma cells which is commonly applied to *in vitro* research. In the research of FXR, the hepatocyte cell lines have been

widely used but not intestinal epithelial cell line SW480. My target is to screen intestinal bacteria that can activate FXR activity. In the meantime, intestine is the place that bacteria inhabit. Thus intestinal bacteria may have cross-talk with intestinal FXR. In addition, the report stated that intestinal microbiota mainly affect FXR targets in the ileum but not in the liver [12]. Thus, the intestinal cells are more suitable for FXR-stimulatory bacteria screening.

Compared with other intestinal cell line such as CaCO2 and HT29, which are widely used, SW480 cell does not express FXR [9]. However, the levels of FXR expression of CaCO2 and HT29 cells changed with their degree of differentiation level [9], which might affect the repeatability and reproducibility of the assay system. And in the meantime, FXR target gene IBABP was not detected in SW480 cell when given FXR agonist CDCD or GW4064, which revealed the expression of IBABP is strictly dependent on FXR in SW480 cells. Based on this recognition, SW480 cell line was selected for stable cell line construction.

Stable expression of target genes can be influenced by two factors: The transfection method used and the vector containing the gene of interest. First, we chose FuGENE® HD Transfection Reagent, which is suitable for stable transfection. Because the level and time of expression of target gene (FXR) depend on the promoter cloned upstream on the expression vector and on the particular integration site. An EX-T0601-M02 vector, which containing CMV promoter, can ensure FXR constitutive expression.

To be sure that SW480 cells have successfully integrated FXR, the cells were under antibiotics G418 selection, because a neomycin is co-expressed on EX-T0601-M02 vector. Antibiotics G418 enriched and enabled the growth of a subpopulation where FXR has been incorporated into the genome.

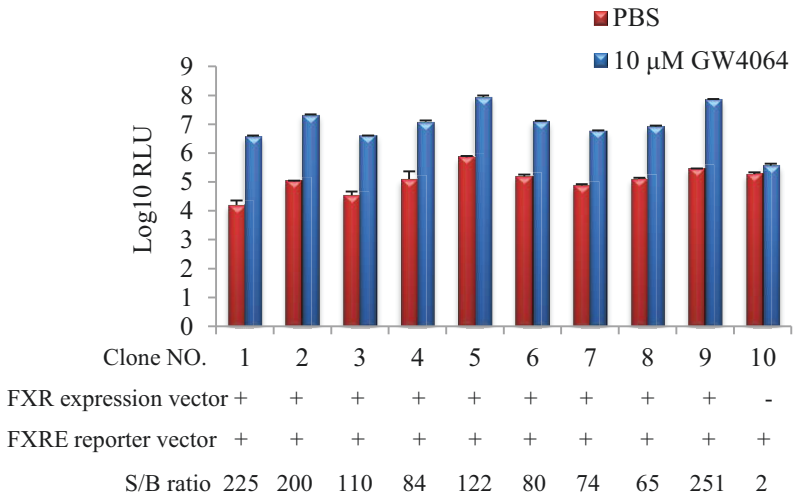

Figure 2.1 Luciferase activity of each clone. Cells were seeded to white 96-well plates with density of 5×10^4/well 24 h before administration of GW4064 or PBS. After 24 h cultivation, cells were lysed and chemiluminescense was evaluated by administration of luciferase assay reagent. RLU: Relative chemiluminescent units = GW4064/DMSO, S/B: Signal-background. Experiments were performed in triplicate with the mean ± SD shown.

In order to monitor FXR transcriptional activity in intestinal epithelial cell line, a stably FXR reporter cell line was obtained by co-transfecting a reporter vector pGL4-4xFXRE-luc and human FXR expression vector into SW480 cells. The site of integration can have an effect on the transcription rate of target gene. Usually a regular expression plasmid is integrated into the genome of the target cell randomly. Integration into inactive heterochromatin results in little or no transgene expression, whereas integration into active euchromatin frequently allows transgene expression. In order to keep homogeneity and uniformity, single cell culture instead of transfected cells in bulk was obtained by cloning cylinder. Nine of single cell clones isolated from the transfected cell populations were applied for determining the responsiveness to synthetic FXR agonist (GW4064). Each FXR reporter cell was treated with 10 μM GW4064 or DMSO control. As a result, clones 1, 2 and 9 were selected owing to its high chemiluminescence value (RLU) and signal-to-background (S/B) ratio (**Figure 2.1**). On the other hand, responsiveness of the SW480 cells transfected with only a reporter vector pGL4-4xFXRE-luc to 10 μM GW4064 was very low (S/B ratio = 2), indicating that endogenous FXR expression level in SW480 cells is very low. Thus, agonist-induced

chemilumirescence induction in FXR reporter cells constructed in this study was due to the exogenous FXR expression.

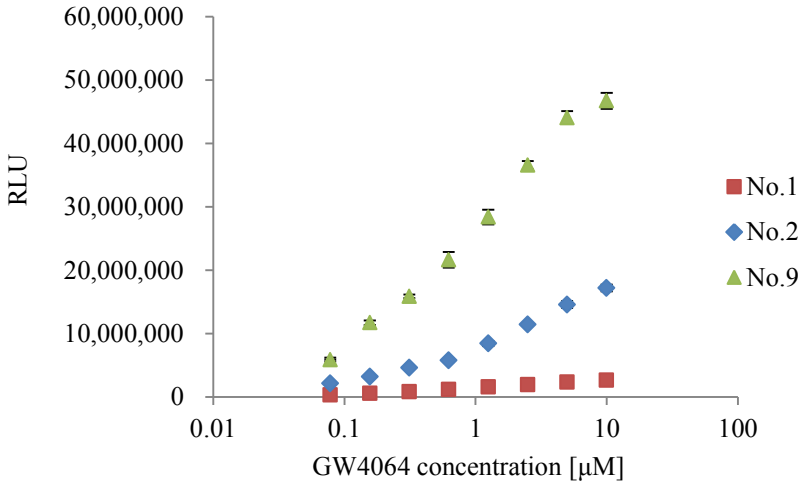

Figure 2.2 Dose response of FXR agonist (GW4064). Cells were seeded to white 96-well plates with density of 5×10^4/well 24 h before administration of GW4064 or DMSO. After 24 h cultivation, cells were lysed and chemiluminescense was evaluated by administration of luciferase assay reagent. Experiments were performed in triplicate with the mean ± SD shown.

Next, FXR reporter cell lines were exposed to different concentrations of FXR agonist GW4064 for characterizing agonist dose-response. As shown in **Figure 2.2**, only clone 9 showed sharp rising when GW4064 concentration was increased, indicating clone 9 is sensitive to GW4064 concentration changes. However, clone 2 presented consistent growing, and the activity changes in clone 1 were very slight. As a result, the level of FXR reporter activity increased in a dose-dependent manner. Undoubtedly, clone 9 is the best choice for my later research.

FXR has been shown to activate various genes by FXR ligands. It has also been demonstrated that FXR ligands including GW4064 may induce FXR target genes expression in gene-specific manner. Because GW4064 was used as positive control in this study, thus it's necessary to find out GW4064 dependent expression of FXR target genes. FXR activation by treatment with GW4064 induced gene IBABP [9], OSTα and FGF19 expressions [13]. Upon this understanding, I determined whether stimulation by GW4064 is able to transactivate FXR target genes by real-time PCR in the FXR reporter

cells. Results demonstrated that GW4064 induced the mRNA expression of IBABP, OSTα and FGF19 in a dose-dependent manner (**Figure 2.3**). The mRNA levels of IBABP and OSTα reached their maximum expression level when given 500 nM GW4064, while 100 nM GW4064 induced most expression of FGF19. However, GW4064 did not induce the similar level of FGF19 mRNA as genes IBABP and OSTα in the FXR stable cell line.

To discover new FXR ligands [2], elucidate FXR target genes expression [14, 15], and study the relationship between FXR and other receptors [14, 16], a FXR reporter system is often selected. Usually, an expression vector containing FXR, a reporter plasmid containing FXR response element with a fluorescent gene, and an internal control plasmid are co-transfected into cells. Because FXR binds with RXR as heterodimer, a RXR expression vector is often transfected together. However, I did not co-transfect RXR in this study. This is because some compounds have been shown to interact with not only one receptor. One paper reported that the RXR agonist LG100268 repressed FXR activity and FXR target BSEP expression [14]. Therefore, in order to exclude the effects from other receptor, only FXR expression plasmid was transfected.

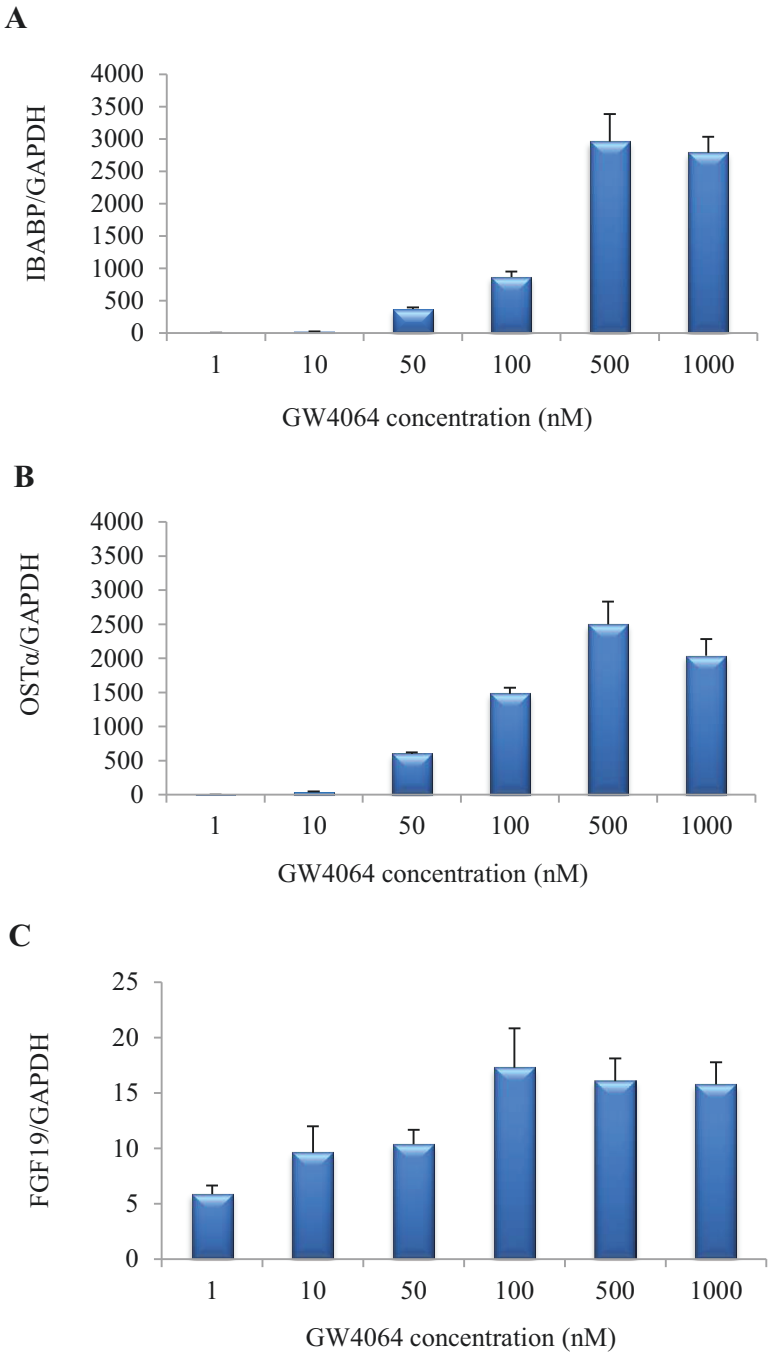

Figure 2.3 Dose response of GW4064 in induction of FXR target genes (IBABP, OSTα, FGF19). SW480-FXR-FXRE cells were incubated with various concentration of FXR agonist GW4064 for 24h. Total RNA was extracted from cells and mRNA levels were evaluated by real-time PCR as described in 2.2.4. The results are normalized to GAPDH mRNA level. Experiments were performed in triplicate (n = 2) with the mean ± SD shown.

Besides SW480 cells, many other cells are selected for reporter system construction. To test whether bile acids are the physiologic ligands of FXR, HepG2 cells or African

green monkey kidney (CV-1) cells murine were co-transfected with a rat FXR expression plasmid and a luciferase reporter gene containing multiple copies of an inverted repeat response element (IR-1) [2]. Another study discovered compound T0901317 can be FXR agonist, by using HEK293 cells which was transfected with a Gal4 DBD-FXR-LBD receptor and a Gal4-responsive luciferase reporter [17]. Molecular cross-talk between FXR and PPARα was confirmed by co-transfection of FXR and RXR receptor in HepG2 cells, with induction of PPARα activity in the presence of CDCA [16].

2.4 Conclusion

In this chapter, a stable FXR reporter system was obtained for FXR modulators screening by transfection of a FXR expression plasmid and a FXRE-*luc* reporter vector into colon cancer cell line SW480. In addition, FXR agonist GW4064 induced target genes IBABP, OSTα and FGF19 mRNA expression in this stable cell line.

2.5 Reference

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Chapter 3

FXR-stimulatory bacteria screening and physiological effects exploration

Chapter 3

FXR-stimulatory bacteria screening and physiological effects exploration

3.1 Introduction

FXR can be activated by various compounds (**Table 1.3**) from bile acids to synthetic compounds. Some natural extracts also have been found as FXR modulators. Guggulsterone, a steroid found in the guggul plant, as FXR antagonist, decreased hepatic cholesterol in wild type mouse that received a high cholesterol diet but had no effect in FXR deficient mice, demonstrating that guggulsterone can inhibit cholesterol through inhibition of FXR transcriptional activity [1]. Xanthohumol from beer hops attenuated diabetes in KK-*Ay* mice by stimulation of FXR and regulation of genes involved in lipid and glucose metabolism [2]. Although not so many natural extracts have been discovered as FXR modulators up to now, the undiscovered world provides us a big chance to find out more modulators that possess unexpected functions.

Intestinal bacteria, as a whole, have important functions in metabolism, intestinal epithelial cell functions and health, and inflammation response. However, bacteria species and strains affect host metabolism a lot, which makes it difficult to elucidate the mechanism of host-microbiome interactions. What's more, different strains may have different and even opposite actions. Thus, with the purpose of understanding of molecular mechanisms of micro-host interactions, it is necessary to find out specific bacteria individuals.

Mice lacking microbiota are unable to induce expression of IBABP or FGF 15, which revealed the FXR activation require colonic bacteria for the initiation [3]. Probiotics induced alterations in bile acids metabolism were abolished once FXR or FGF15 knockout, indicating that FXR-FGF15 axis is necessary for bile acids regulation by probiotics [4]. These studies emerged that bacteria or their metabolites have a potential to activate FXR. In this chapter, I intended to access bacteria in different forms for FXR stimulatory potentials by using a reporter system constructed in the last chapter.

Activation of FXR by its ligands plays an important role in the treatment of wide range of disorders. Besides its especial role in bile acids metabolism, increasing evidences have revealed that FXR regulates inflammatory processes as a key modulator. FXR agonist GW4064 repressed the expression of NF-κB regulated genes both in HepG2 cells and mouse primary hepatocytes through decreasing the binding activity between NF-κB and DNA sequence, which suggested FXR can act as a potential regulator for hepatic inflammation and application of its ligands in the treatment of liver inflammatory diseases [5]. FXR activation in intestinal epithelial cells reduces expression of genes regulated by toll-like receptors 4 (TLR4) receptor through repression of pro-inflammatory cytokines and chemokines [6]. Upon anti-inflammation function of FXR activation by its ligands, I would like to know whether FXR stimulatory bacteria hold anti-inflammatory effect through FXR activation. Thus, the anti-inflammatory effect of candidate bacteria was also assessed in this chapter.

3.2 Material and methods

3.2.1 Cell culture

FXR stable reporter cells SW480 cells , human hepatocellular liver carcinoma cell line HepG2 (ECACC, No. 85011430) and epithelial colorectal adenocarcinoma cell line CaCO-2 (CACC, No. 86010202) were grown at 37°C with 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (Wako, Osaka, Japan), supplemented with 10%

heat-inactivated fetal bovine serum (Invitrogen, New York, USA), 1% Penicillin-Streptomycin Solution (×100) (Wako, Osaka, Japan). As for FXR stable reporter cells, 800 μg/ml of G418 and 300 μg/ml of hygromycin B were supplemented.

3.2.2 Preparation of bacterial suspensions and bacterial culture supernatant

Thirty-eight bacterial isolates were used in this study (**Table 3.1**). Each bacterial isolate was identified based on a nearly full length of 16S rRNA sequence, which was deposited in DNA Data Bank of Japan (DDBJ). Bacterial strains were seeded into GAM medium (Nissui, Tokyo, Japan) for 24-40 hours in anaerobic condition to obtain enough bacteria, and culture supernatants were achieved at 40 hours. The culture broth was centrifuged at 9,000 rpm for 10 min to separate bacteria and culture supernatants. After washed by 10 ml PBS twice, wet weight of bacteria pellets was measured. Then, bacteria pellets were suspended with PBS at final concentration of 100 mg /ml as follows: PBS (ml) =W×9, [W (g) =wet weight of bacteria pellets]. Bacterial suspensions (0.5 ml) added with 0.2 g of 0.1 mm silica/zirconium beads (BioSpec, OK, USA) were beaten at 5,500 rpm for 2 min to make bacteria disrupt. Bacterial suspensions were kept at 100°C for 10 min to be heat-killed. Intact bacterial suspensions were directly preserved. Bacterial culture supernatant was filtered through cellulose acetate filters with a pore size of 0.2 μm (ADVANTEC Toyo, Tokyo, Japan) to get rid of bacteria from supernatant. All samples were stored at -80°C until use.

Table 3.1 Bacteria information

Strain	Accession number	Isolation source	Taxonomic assignment	16S rRNA sequence similarity (%)
W1		Culture collections	*Lactobacillus casei* [NBRC 15883]	-
W2		Culture collections	*Lactobacillus fermentum* [NBRC 15885]	-
W3		Culture collections	*Lactobacillus plantarum* [NBRC 15891]	-
W4		Culture collections	*Lactococcus lactis* [NBRC 100933]	-
W5	LC061609	Dairy foods	*Lactobacillus gasseri* [FJ557004]	99
W6	LC061610	Dairy foods	*Lactobacillus delbrueckii* [CP000156]	99
W7	LC061611	Dairy foods	*Streptococcus thermophilus* [FR875178]	99
W8	LC061612	Dairy foods	*Lactobacillus helveticus* [CP011386]	99
W9	LC061613	Dairy foods	*Lactobacillus gasseri* [FJ557004]	99
W10	LC061614	Dairy foods	*Streptococcus thermophilus* [FR875178]	99
W11	LC061615	Dairy foods	*Lactobacillus reuteri* [EU722746]	99
W12	LC033789	Dairy foods	*Lactobacillus helveticus* [HM218413]	99
W13	AB932539	Human feces	*Bifidobacterium bifidum* [AP012323]	100
W14	AB932540	Human fces	*Bifidobacterium longum* [FP929034]	100
W15	AB932542	Human feces	*Bifidobacterium adolescentis* [CP010437]	99
W16	AB932544	Human feces	*Bifidobacterium bifidum* [KJ160509]	99
W18	LC033790	Human feces	*Bacteroides dorei* [EU722737]	99
W19	LC033791	Human feces	*Eubacterium limosum* [AB638446]	99
W20	LC033792	Human feces	*Bacteroides* sp.W20 [EU728710]	99
W21	LC033793	Human feces	*Bacteroides fragilis* [AB618792]	98
W22	LC033794	Human feces	*Ruminococcus* sp.W22 [FJ611794]	99
W23	LC033795	Human feces	*Clostridiales bacterium* W23 [HQ452859]	98
W24	LC033796	Human feces	*Bacteroides uniformis* [AB247142]	99
W25	LC033797	Mouse feces	*Parabacteroides distasonis* [AB238924]	98
W26	LC033798	Mouse feces	*Bacteroides acidifaciens* [AB510696]	97
W27	LC033799	Mouse feces	*Bacteroides thetaiotaomicron* [AE015928]	97
W28	LC033800	Mouse feces	*Lactobacillus johnsonii* [FN298497]	99
W29	LC033801	Mouse feces	*Lactobacillus reuteri* [KR492886]	97
W30	LC033802	Mouse feces	*Lactobacillus animalis* [AB911535]	98
W31	LC033803	Mouse feces	*Bacteroides sartorii* [AB572597]	98
W32	LC033804	Mouse feces	*Bacteroides* sp.W32 [AB599946]	99
W33	LC033805	Mouse feces	*Parabacteroides goldsteinii* [AB547650]	99
W34	LC033806	Mouse feces	*Enterococcus faecalis* [FJ378702]	99
W35	LC033807	Human feces	*Enterococcus durans* [AJ276354]	99
W36	AB932524	Human feces	*Enterococcus raffinosus* [AF061003]	99
W37	AB932534	Human feces	*Enterococcus cecorum* [AF061009]	99
W38	AB932546	Human feces	*Enterococcus avium* [DQ779961]	100
W39	LC033808	Human feces	*Enterococcus faecium* [FJ378690]	99

3.2.3 Bacteria screening by luciferase assay

FXR reporter SW480 cells or HepG2 cells were seeded at a density of 5×10^4 cells/well (96-well plates). Twenty-four hours after seeding, cells were incubated with bacterial suspensions, culture supernatants, DMSO (0.1% v/v) or GW4064 (10 nM) respectively for 24 hours. After that, cell supernatants were removed and cells were washed twice by phosphate-buffered saline (PBS) and lysed by adding 20 µl of passive lysis 5×buffer (Promega, WI, USA) with gentle rocking for 20 min. Luciferase activities were measured with administration of luciferase assay reagent (Promega, Madison, USA) by using GloMax® 96 Microplate Luminometer (Promega). The ratio of treatment over control was served as -fold activation.

Table 3.2 Primers used in real-time PCR

Gene	Forward	Reverse	Reference
GAPDH	GAAGGTGAAGGTCGGAGT	CATGGGTGGAATCATATTGGAA	[7]
IBABP	TCACTTGGTCCCAGCACTA	CTTGTCACCCACGATCTCT	
OSTα	CTACACCTGGGTGAGCAGAA	AGAGGAATAGGGAGGCGAAC	[8]
IL8	AGAGTGATTGAGAGTGGACC	ACTTCTCCACAACCCTCTG	[9]
FGF19	CACGGGCTCTCCAGCTGCTTCCTGCG	TCCTCCTCGAAAGCACAGTCTTCCTCCG	[10]
FXR	CCGTGAATGAAGACAGTGAAGGTCG	ACCCTTTCAGCAAAGCAATCTGGTC	
SHP	GGCTGGCAGTGCTGATTCAG	TGGGGTGTGGCTGAGTGAAG	[11]
BSEP	AGTTGCTCATCGCTTGTCTACG	GCTTGATTTCCCTGGCTTTG	

GAPDH, IBABP, OSTα and hIL8 were analyzed using the following conditions: 95°C 2 min, 40 cycles of 95°C for 10 sec, and 60°C for 30 sec. FGF19 was analyzed using the following conditions: 95°C 15 min, 40 cycles of 95°C for 15 sec, and 62°C for 30 sec. FXR, SHP and BSEP were analyzed using the following conditions: 95°C 2 min, 40 cycles of 95°C for 10 sec, and 56°C for 10 sec.

3.2.4 RNA isolation and Real-time PCR

Stably FXR-expressing SW480 cells (3×10^5 cells/well) were seeded in 24-well plate 24 hours before administration of bacterial supernatant samples. After 24 hours of incubation with bacterial supernatant samples, total RNA was extracted from the cells by using Qiagen RNeasy mini kit (Qiagen, Tokyo, Japan). RNA concentration and purity was determined by a NanoDrop™ spectrophotometer (Thermo, MA, USA). For reverse

transcription reaction, 1 μg of total RNA was used in a final volume of 20 μl with PrimeScript RT reagent Kit (Takara, Tokyo, Japan) at 37°C for 15 min and 85°C for 5 sec. The real-time PCR reactions were performed using iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, CA, USA). For each reaction, the final volume of 20 μl was composed of 10 μl of SYBR Green PCR Mix (Bio-Rad), 1 μl of each primer (10 μM) (**Table 3.2**), and 2 μl of RT product diluted 10 times. After PCR, melting curve analysis was performed to ensure the specificity of the assay. Each analysis was performed in triplicate and GAPDH was used as endogenous gene. Relative gene expression was calculated via the Relative standard curve method [12].

3.2.5 Cell viability analysis

HepG2 cells were seeded at a density of 5×10^4 cells/well (96-well plates). Twenty-four hours after seeding, the cells were incubated with bacterial culture supernatants, DMSO (0.1% v/v) or GW4064 (10 nM) for 24 hours. The cells were equilibrated at room temperature for 30 minutes before administration of a volume of Cell Titer-Glo Reagent (Promega) equal to the volume of cell culture medium present in each well. After mixing for 2 min on an orbital shaker to induce cell lysis, the plate was kept at room temperature for 20 min to stabilize luminescent signal. Finally the luminescence was recorded by using GloMax® 96 Microplate Luminometer.

3.3 Results and discussion

3.3.1 Screening of bacteria for FXR activation by luciferase assay

By using a well-constructed FXR reporter cells line (clone 9), a total of 38 bacterial strains which affiliated with the genera *Bacteroides*, *Bifidobacterium, Enterococcus*, *Eubacterium*, *Lactobacillus*, *Parabacteroides*, *Ruminococcus* and *Streptococcus*, were assessed whether they can be modulators for FXR activation. FXR-stimulatory potentials of intact bacterial cells, mechanical disrupted bacterial cells, heat-killed bacterial cells, or

bacterial culture supernatants of each isolate were evaluated by using FXR reporter cells. The intact bacteria kept integral part of the bacterial outer membrane, while fractionated subcellular components were exposed when bacteria was disrupted by beads beating. Heat treatment of bacterial cells leads to the conformational change and degradation of bacterial cell wall components. As a result, FXR-stimulatory activities in most of bacterial cell samples were less than 2-fold change (**Figure 3.1**). Interestingly, culture supernatants derived from *B. dorei* and *E. limosum* intensely activated FXR, which indicated that these two bacteria would function as FXR modulators (**Figure 3.1D**).

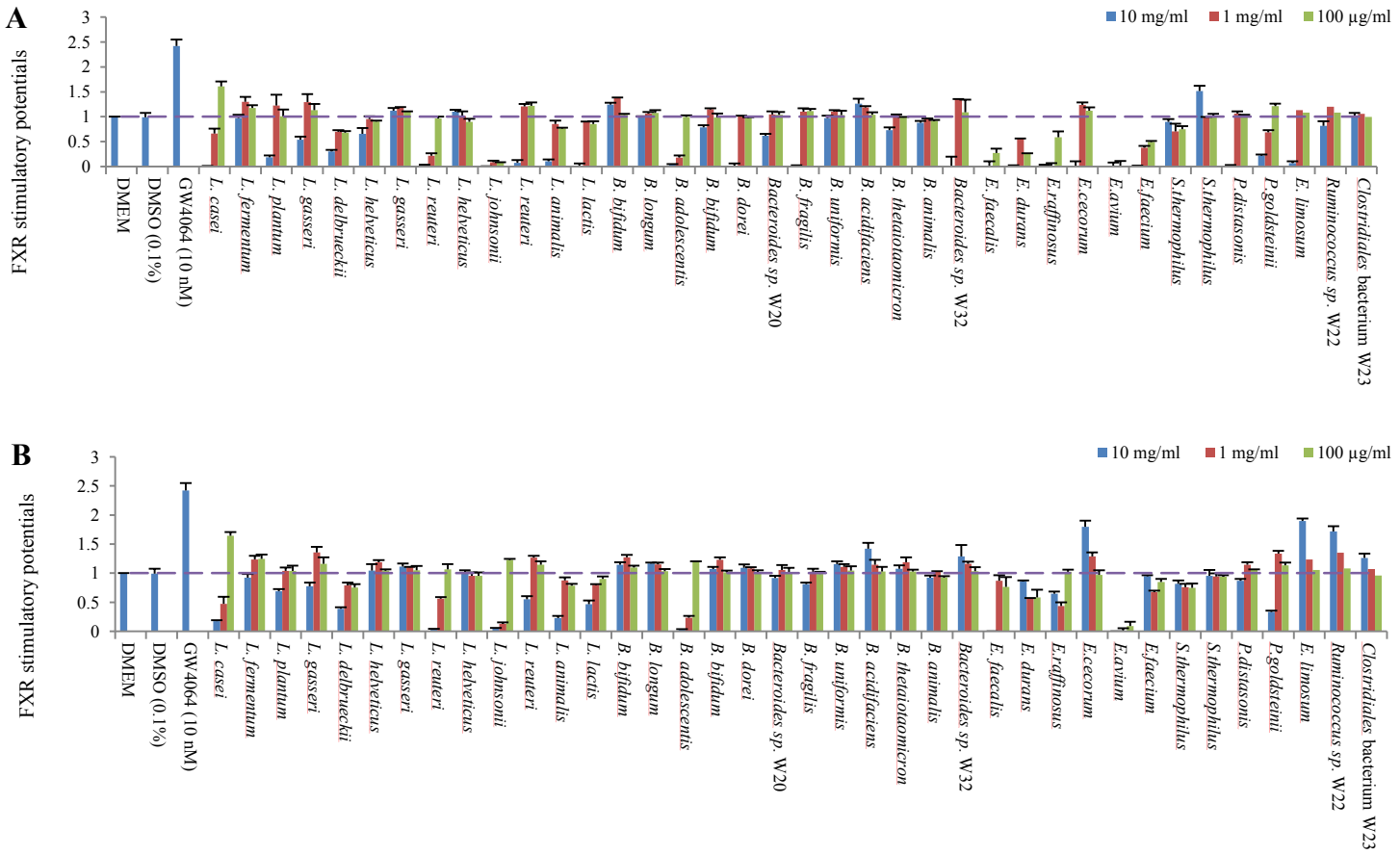

Figure 3.1 After cells were cultured in white 96-well plates with density of 5×10^4/well for 24 h, bacterial suspensions or culture supernatants were introduced for 24 h incubation before measurement of chemiluminescense by administration of luciferase assay reagent. (A) Intact bacteria. (B) Mechanical disrupted bacteria. (C) Heat-killed bacteria. (D) Culture supernatants. Experiments were performed in triplicate. Values are the mean ± SD. 3.3.2 Evaluation of two FXR-stimulatory bacteria.

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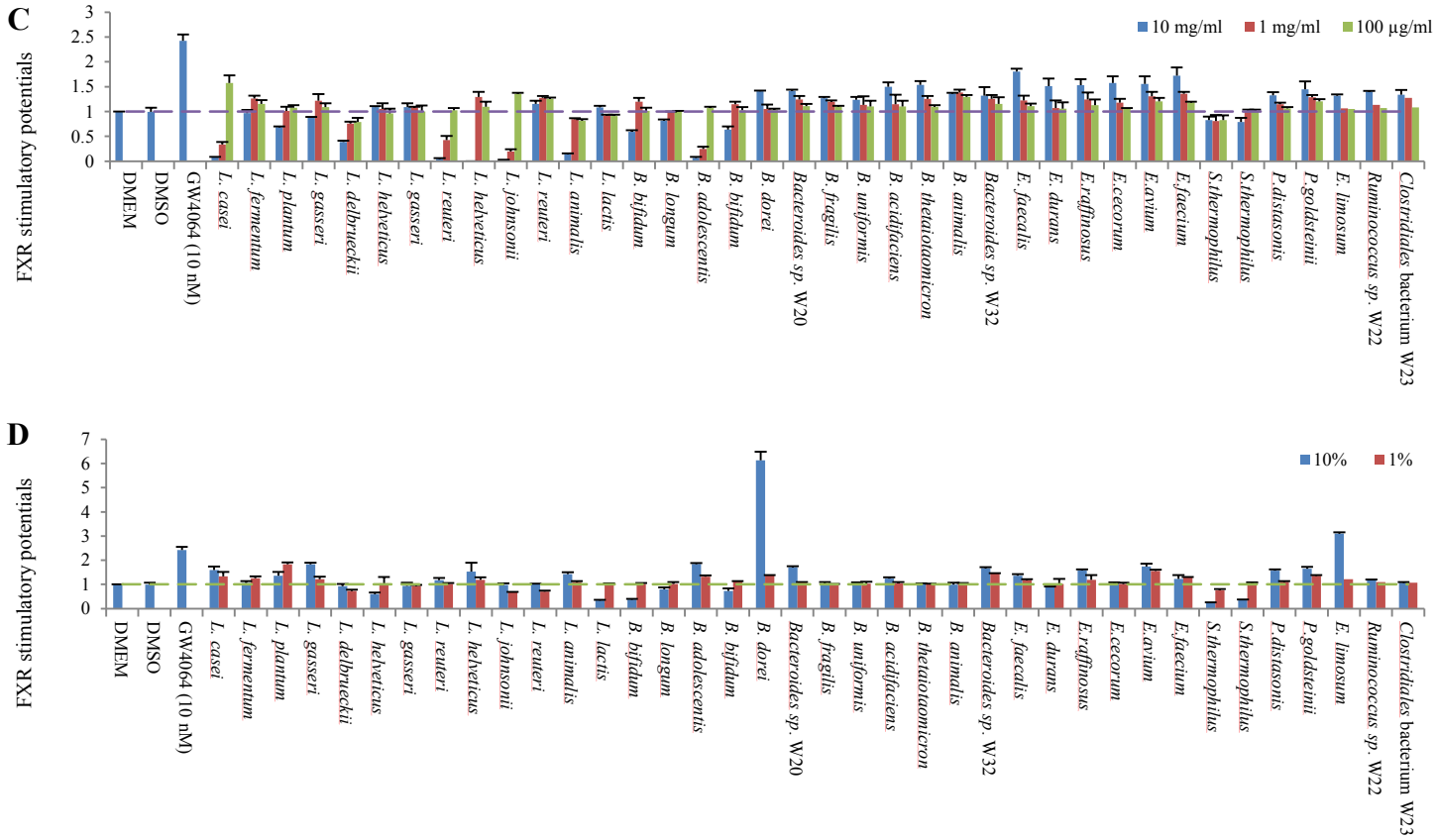

Figure 3.1 After cells were cultured in white 96-well plates with density of 5×10^4/well for 24 h, bacterial suspensions or culture supernatants were introduced for 24 h incubation before measurement of chemiluminescense by administration of luciferase assay reagent. (A) Intact bacteria. (B) Mechanical disrupted bacteria. (C) Heat-killed bacteria. (D) Culture supernatants. Experiments were performed in triplicate. Values are the mean ± SD. 3.3.2 Evaluation of two FXR-stimulatory bacteria.

By repeating three independent experiments in triplicate, I confirmed the reproducibility of FXR-stimulatory activity of culture supernatants derived from *B. dorei* and *E. limosum* (**Figure 3.2A**). As a result, 10% of culture supernatant of *B.dorei* strongly induced FXR activation, while FXR-stimulatory potential of *E.limosum* was the same as that of 10 nM GW4064. Palmitic acid, which is the PPAR ligand, did not activate FXR in SW480 reporter system even at concentration of 1 μM (Appendix Figure 13).

Next, FXR-stimulatory activities of bacterial culture supernatants sampled every 12 hours and bacterial cell growth during cultivation were determined (**Figure 3.2B, C**). For *B. dorei*, FXR stimulatory potentials in the culture supernatant increased continuously with the growth of bacterial cells. On the other hand, FXR-stimulatory potentials in the culture supernatants of *E. limosum* began to increase after 12 hours and reached a plateau at 36 hours. Taken together, I decided to get culture supernatants about 40 hours since bacteria inoculation for my later experiment.

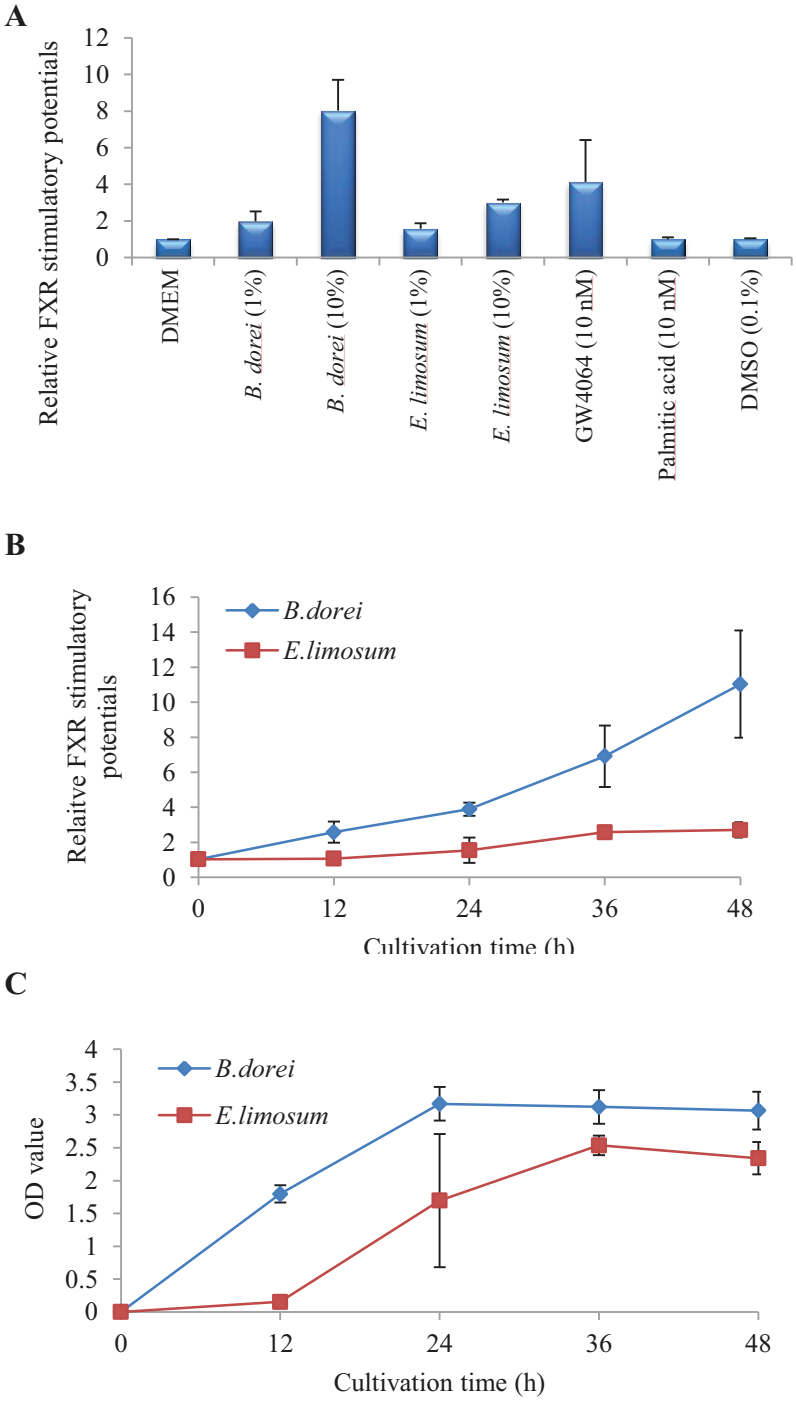

Figure 3.2 After cells were cultured in white 96-well plates with density of 5×10^4/well for 24 h, bacterial culture supernatants were introduced for 24 h incubation before measurement of chemiluminescense by administration of luciferase assay reagent: (A) FXR activation by culture supernatants of *B. dorei* and *E. limosum*, (B) FXR activation by culture supernatants sampled at various times, (C) The quantity of two bacterial cells at different times. Values are the mean ± SD, n = 3 (Palmitic acid: n=1).

3.3.3 Regulation of FXR targets involved in bile acids metabolism by bacterial culture supernatant

FXR activation by bile acids or other ligands like GW4064, would facilitate bile acids transport into portal vein by stimulating the up-regulation of IBABP, promote bile acids recycle to the liver by expression of OSTα and OSTβ, and facilitate the release of FGF19 travel to the liver to repress bile acid synthesis through JNK dependent pathway. By using the SW480 reporter cell line, I would like to know whether these two bacterial culture supernatants regulate genes involved in bile acid metabolism in FXR reporter cells. Same with genes in the last chapter, IBABP, FGF19 and transporter OSTα were picked up for real-time PCR analysis.

As shown in **Figure 3.3**, the culture supernatants derived from both *B. dorei* and *E. limosum* appreciably transactivated the FXR target genes IBABP and OSTα. *B.dorei* induced FXR target gene IBABP and OSTα mRNA expression with 947.34 and 177.57 respectively, while *E.limosum* also strongly increased their expression with 112.62 in IBABP and 57.48 in OSTα. Interestingly, treatment with *B. dorei*-derived metabolites exhibited the strong induction of IBABP mRNA (8.4-fold) and OSTα (3.1-fold) compared with *E.limosum*-derived metabolites. Nevertheless, neither of these two culture supernatant induced gene FGF19 mRNA expression in a FXR reporter cells. These results indicated that FXR activation induced by treatment with these bacterial metabolites did not lead to the transactivation of all FXR target genes.

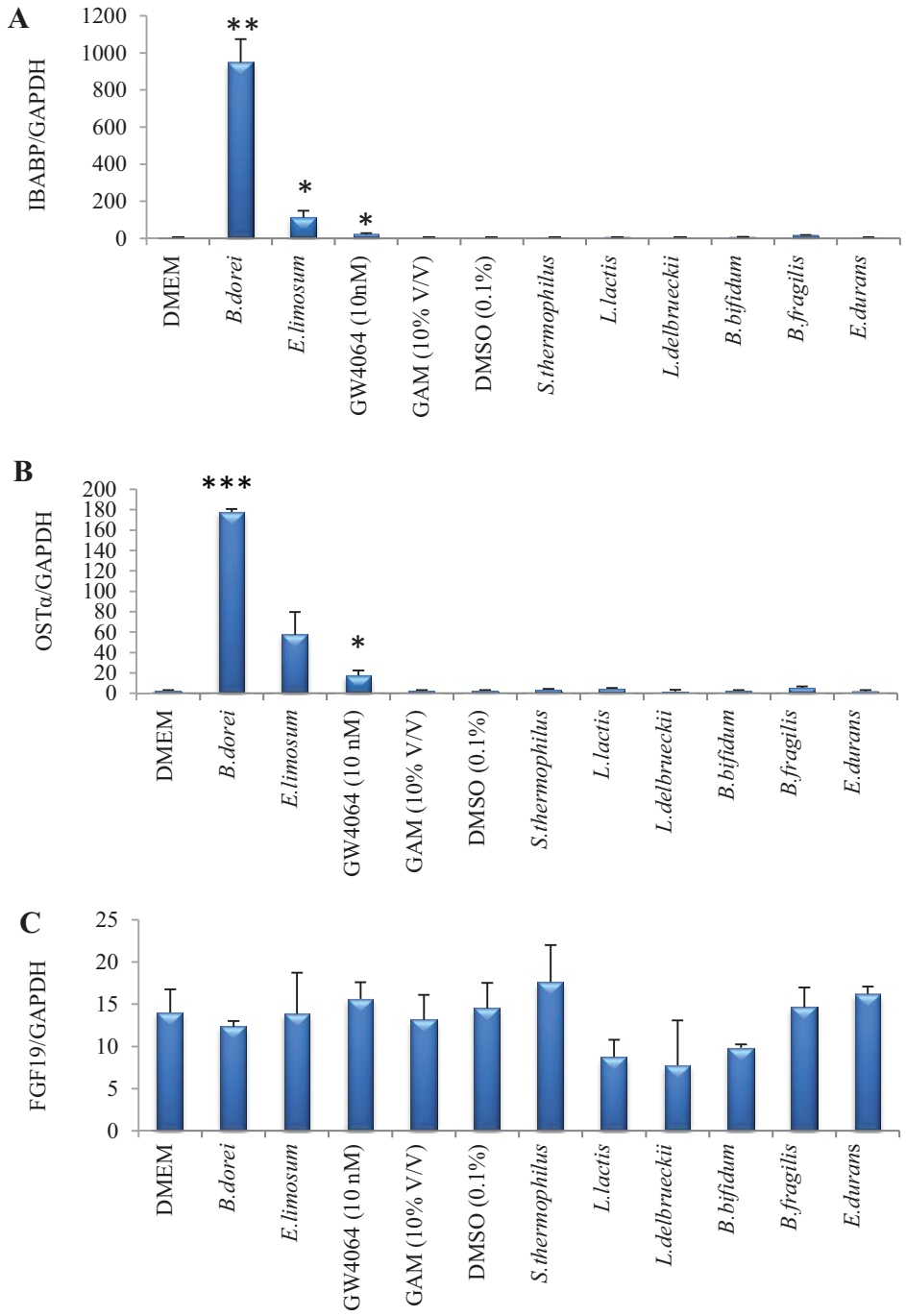

Figure 3.3 mRNA expression of FXR target genes induced by *B. dorei* or *E. limosum*. Cells were cultured in 24-well plates with density of 3×10^5/well for 24 h, bacterial culture supernatants were introduced for 24 h incubation before total RNA isolation. mRNA levels were normalized to GAPDH mRNA levels via the relative standard curve method: (A) IBABP expression levels, (B) OSTα expression levels, (C) FGF19 expression levels. Values are the mean ± SD (n = 3). Differences compared with the GAM culture treatment group were calculated using Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001).

The results showed that the culture supernatants of *B.dorei* and *E.limosum* significantly induced the expression of FXR target gene IBABP and OSTα, suggesting two bacterial culture supernatants function as FXR 'ligands'. In bile acids metabolism, FXR activation would facilitate bile acids transport into portal vein by stimulating the upregulation of IBABP, promote bile acids recycle to the liver by expression of OSTα and OSTβ [13-15]. Using a stable HEK293 reporter cell line, WAY-362450 was proved to be FXR potent agonist; later it was confirmed its role in repression of serum cholesterol, TG and VLDL levels dependent on FXR activation [16]. Therefore, within functions of bacterial culture supernatants in vitro, I may predict that these two bacteria may intervene in bile acids metabolism through regulation of FXR target genes expression.

However, FGF19 did not show any differences with treatment of two bacterial culture supernatants, which indicated a selective modulatory role of two bacterial culture supernatants. Many FXR ligands have shown a gene selective regulation role. Oleanolic acid (OCA), a composition of some traditional Chinese medicine, induced BSEP expression by partially blocking the ability of CDCA, modestly enhanced SHP expression, but had no effect on OSTβ expression [17]. A grape seed procyanidin extract which decreased serum TG level selectively modulated FXR-target gene expression [18]. As for positive control GW4064, it is a selective agonist for FXR in itself [19].

Besides, FXR function is so complicated that it can intervene in other receptors. FXR is one of receptors that regulate FGF19 expression in SW480 cells. For example, FGF19 can be regulated by a series of FGFR which is the main receptor for FGF19 [20]. Moreover, the culture supernatant is a hotchpotch containing not only one compound. It is possible that FXR is differentially-activated, and thus gene expressions are multiple active results of culture mixture. Therefore, it is necessary to find out which components in bacterial culture supernatant can stimulate FXR.

3.3.4 Specificity of FXR activation by two bacterial culture supernatants

To screen FXR-stimulatory bacteria, SW480 cells co-transfected FXR and a reporter plasmid containing FXRE were used. However, FXR modulators have a potential to activate FXRE, which may in turn induce luciferase activity as well. To pursue this possibility further, the specificity of FXR activation by two bacterial culture supernatants was validated in cells with or without FXR transfection in SW480 cells. As shown in **Figure 3.4A**, two bacterial culture supernatants did not induce luciferase activity in FXR null SW480 cells, indicating that the luciferase activity stimulated by two bacterial metabolites was dependent on FXR. In addition, the levels of FXR target gene IBABP and OSTα were very low compared with those in FXR containing cells (**Figure 3.4B, C**), revealing that FXR target genes expression was dependent on FXR in SW480 cells by two bacterial culture supernatants.

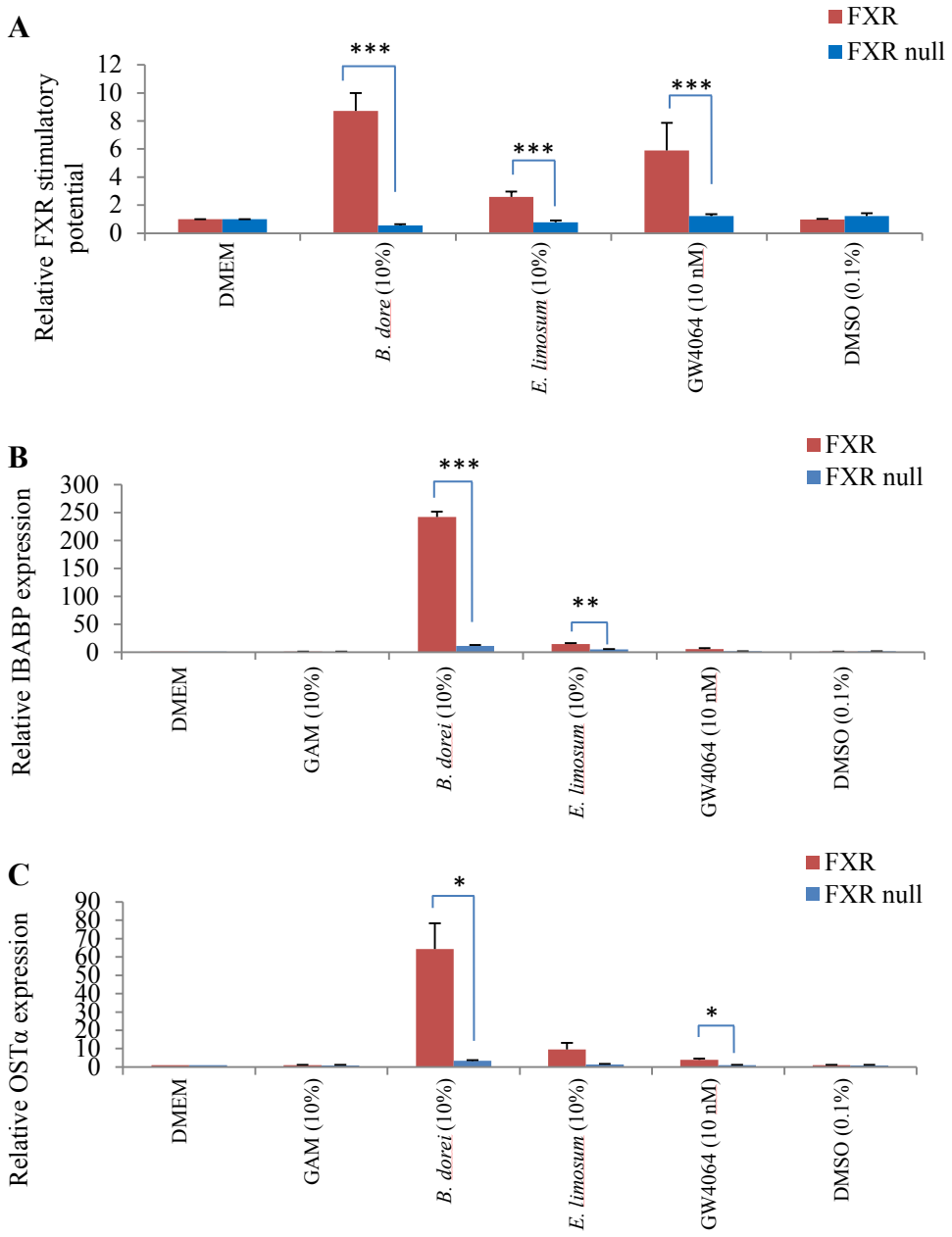

Figure 3.4 The specificity of FXR activation by two bacterial culture supernatants. FXR-expressed or FXR-null SW480 cells were treated with bacterial culture supernatants (10% v/v) for 24 h incubation before measurement by administration of luciferase assay reagent. (A) The activation of FXR in each cell was measured by luciferase reporter construct FXRE-luc (n=2). The induction of FXR target gene by bacterial culture supernatants was determined with quantitative real-time reverse transcription-PCR analysis (n=1): (B) *Ibabp* gene, (C) *Ostα* gene. The mRNA levels were normalized to GAPDH mRNA level via the relative standard curve method. Relative mRNA expression: Compared to DMEM medium group. Experiments were performed in triplicate. Values are the mean ± SD. Differences were calculated using Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.3.5 Cell type specific activation by two bacterial culture supernatants

In this study, colon cell SW480 was picked up for FXR reporter system construction to screen FXR-stimulatory bacteria. Although this system has its incomparable merits to select ligands for FXR, considering the fact that FXR is not detected in SW480 cells, it is necessary to use some other cell lines to evaluate FXR expression with these two bacterial culture supernatants.

To investigate whether two bacterial metabolites activate FXR target genes in other cell lines, I used two different cell lines (i.e. CaCO-2 and HepG2), which endogenously express FXR [21, 22]. For human colon epithelial cancer cell line CaCO-2, cells were cultured for 7 days or 21 days before the treatment with bacterial supernatants, because FXR expression level depends on cell differentiation degree [23]. The induction of FXR target gene (IBABP) by bacterial culture supernatant was determined in undifferentiated (7 days) or fully differentiated CaCO-2 cells (21 days). The results showed that two bacterial culture supernatants induced IBABP expression (**Figure 3.5**). The *B. dorei*-derived metabolites induced much higher levels of IBABP expression than the *E. limosum*-derived metabolites in CaCO-2 cells, which is a similar trend with that in SW480 cells. In addition, the levels of IBABP were a little higher in differentiated CaCO-2 cells, suggesting that the levels of IBABP exhibited positive correlation with differentiation degree of CaCO-2 cells. However, two bacterial metabolites did not induce IBABP expression in FXR-null SW480 cells. In FXR containing SW480 cells, two bacterial metabolites induced IBABP expression but the levels did not show significant differences between undifferentiated and differentiated cells.

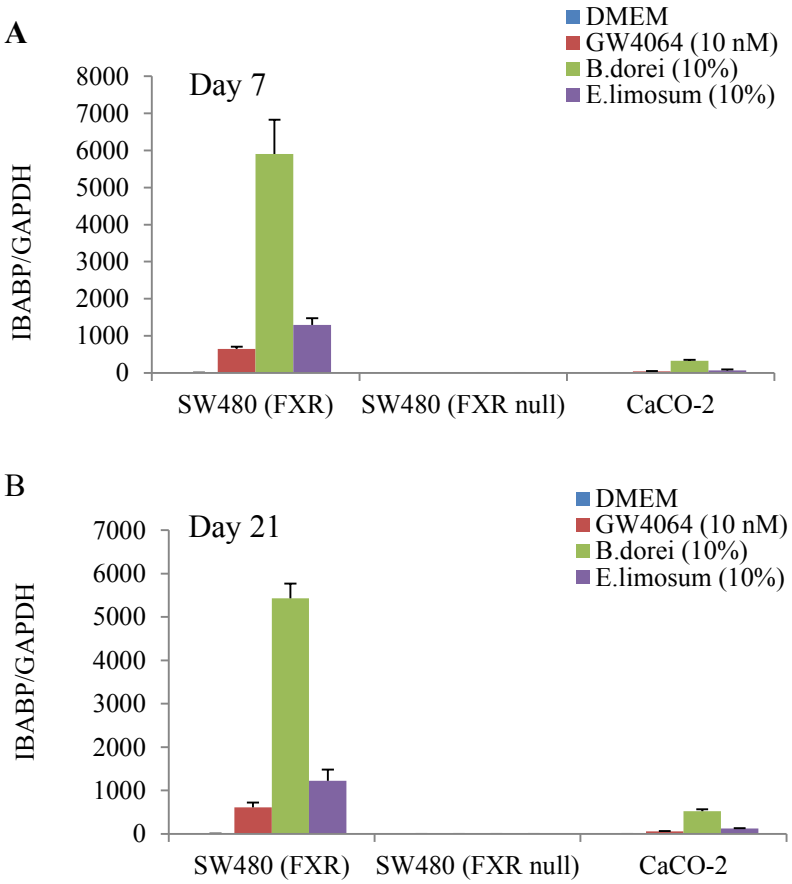

Figure 3.5 Gene expression levels in intestinal epithelial cells. Before the treatment with bacterial supernatants, cells were cultured for 7 days or 21 days. The induction of FXR target gene (IBABP) by bacterial culture supernatant was determined in undifferentiated (A) or fully differentiated Caco-2 cells (B). The mRNA levels were normalized to GAPDH mRNA levels via the relative standard curve method. Experiments were performed in triplicate. Values are the mean ± SD.

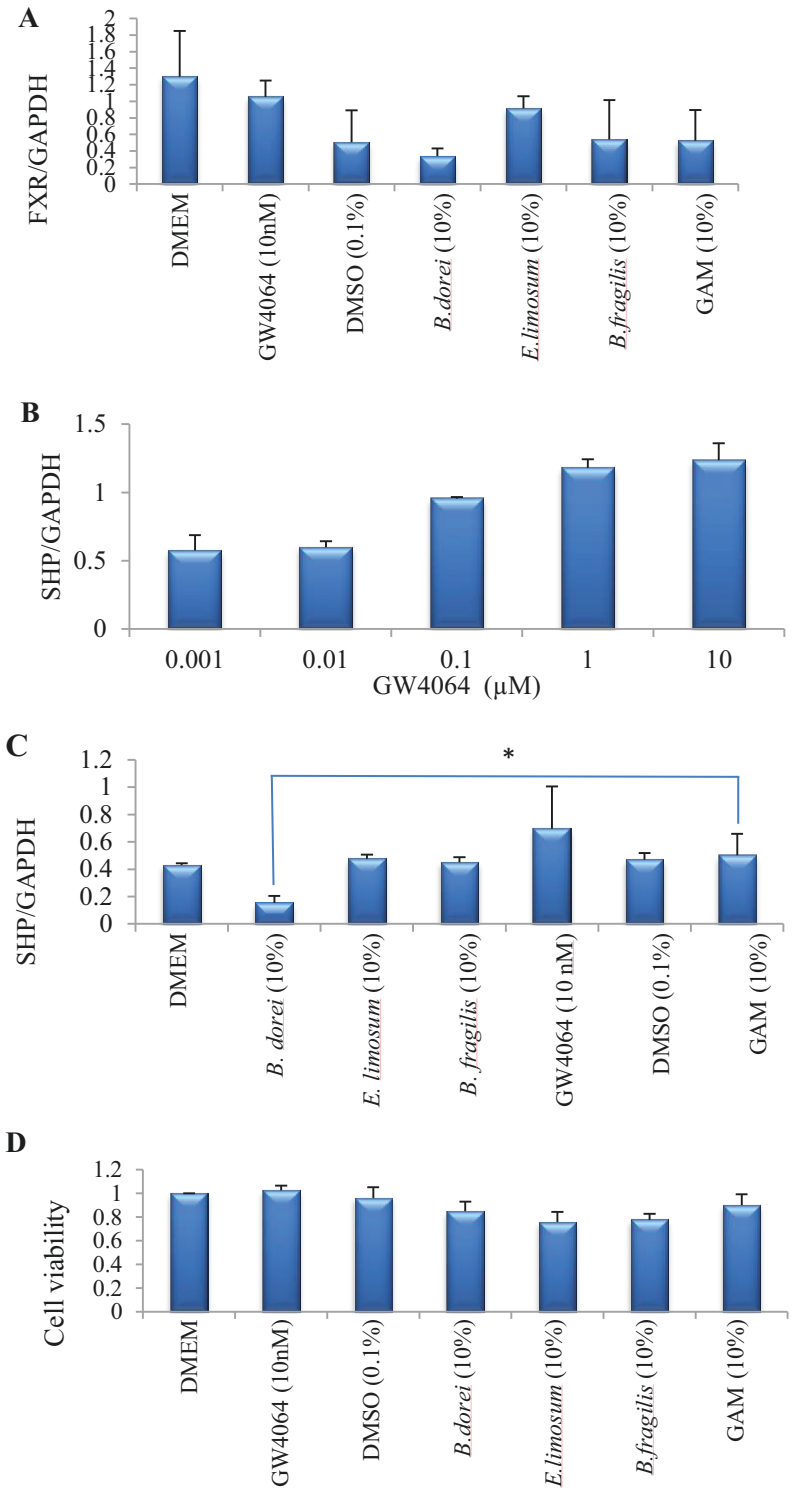

Figure 3.6 Evaluation of mRNA expression with two culture supernatants in HepG2 cell. Cells were cultured in 24-well plates with density of 3×10^5/well for 24 h, bacterial culture supernatants were introduced for 24 h before total RNA extration. mRNA levels were normalized to GAPDH mRNA level. Values are means ± SD, n=3 (n=2 in B). Differences were calculated using Student's t test (*$p < 0.05$).

Furthermore, the culture supernatant derived from *B. dorei* did not stimulate FXR expression in human hepatocellular liver carcinoma cell line HepG2 cells (**Figure 3.6A**). Considering that FXR expression level did not show difference when given 10 nM GW4064, GW4064 was insufficient to be a positive control when evaluation of two bacteria for FXR stimulation in HepG2 cells. Thus, FXR target gene SHP was further investigated. According to **Figure 3.6B**, FXR target gene SHP showed dose-dependent manner in HepG2 cells. GW4064 of 10 nM can induce SHP expression compared to control DMEM group (**Figure 3.6C**). However, the culture supernatant derived from *B. dorei* did not stimulate FXR of a hepatocyte-derived cell line, HepG2 cells, by measuring the *Shp* gene that induced directly by FXR activation.

So, why two bacterial culture supernatants did not regulate SHP expression in HepG2 cells? I assumed that the bacterial culture supernatants may be harmful to HepG2 cells. Based on this hypothesis, the cells viability was checked. As shown in **Figure 3.6D**, two bacterial culture supernatants did not reduce the viability of HepG2 cells, indicating the growth of HepG2 cell was under good condition.

The pharmacological activations of FXR result in complicated responses including both conductive actions and underlying side effects. The exploration of FXR modulators is important for the development of compounds for metabolic syndrome treatment. Some FXR modulators have been demonstrated to regulate FXR target genes in a cell and with a gene specific fashion. Suzukibile et al. found an acid derivative MeDCA as FXR ligand by a reporter system, which appeared cell type selective property [24]. This compound induced BSEP expression in HuH-7 cells, but it did not affect FXR target gene SHP, CYP7A1 and BSEP expression in HepG2 cells. Guggulsterone, the widely studied natural extract for FXR activation, was initially described as an FXR antagonist, but later it was demonstrated to be a partial FXR agonist due to the fact that inducement of BSEP expression via FXR [25]. Considering that only one compound can be FXR selective modulator with cell or gene selective manner, my selected bacterial culture supernatants

are multiply compounds mixture that will make the situation much more complicated. The compounds can be metabolized by different cells; therefore, their metabolites would bind to FXR as ligands. Under the circumstances, the varied compounds may keep silent by metabolism in some cells, thus resulting in differential activation of FXR. Furthermore, the truth that I cannot ignore is the compounds permeability is different depending on cell types, which would be the clearest and simplest explanation for the cell selective activation. The mechanism of selective FXR modulation action remains unclear. However, it was suggested that co-regulator recruitment differences would contribute to the cell type and promoter specific regulation [26].

3.3.6 Anti-inflammatory effects evaluation of bacterial culture supernatants

In order to explore anti-inflammatory effect of two bacterial culture supernatants, I first applied FXR agonist GW4064 to check its anti-inflammatory effect through evaluation of TNFα induced inflammatory cytokine IL8 mRNA expression in FXR reporter cells. At beginning, IL8 mRNA expression levels were measured after administration of TNFα with different times. One ng/ml TNFα induced IL8 mRNA expression, got maximum expression in 1 hour, and followed by a continuous decline (**Figure 3.7A**).

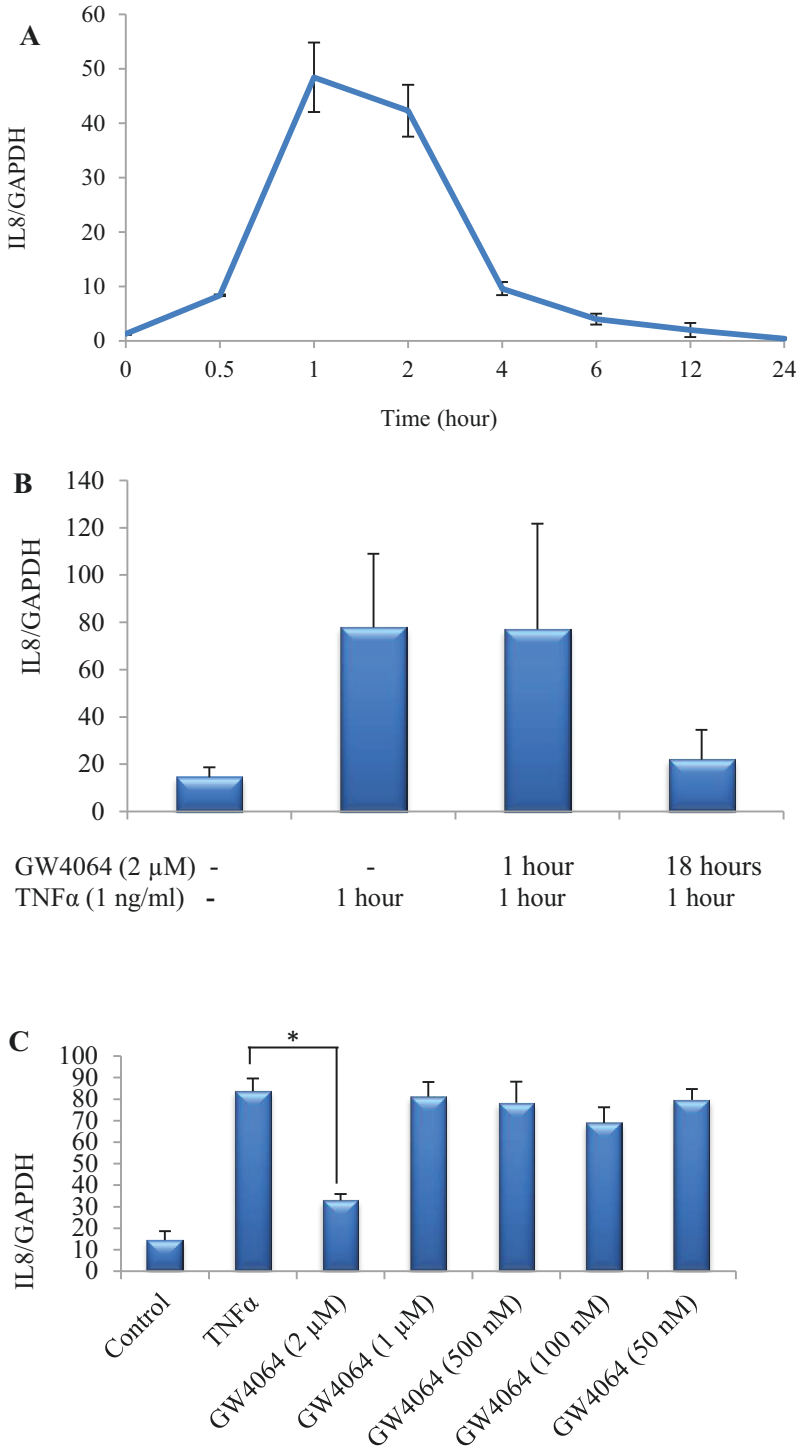

Figure 3.7 IL8 mRNA expression level at different times. Cells were cultured in 24-well plates with density of 3×10^5/well for 24 h, bacterial culture supernatants were introduced before TNFα administration. mRNA levels were normalized to GAPDH mRNA level. Values are means ± SD. Differences from the TNFα only treatment group were calculated using Student's t test (*$p < 0.05$).

Next, the cells were cultured in 24-well plate with density of 3×10^5 cells/well. The following day, the medium was changed with new DMEM medium containing GW4064 (2 μM) and treated with TNFα (1 ng/ml) for 1 hour or pretreated with various concentration of GW4064 for 18 hours before given TNFα (1 ng/ml) for another 1 hour. Total RNA was isolated for IL8 mRNA expression analysis. As shown in **Figure 3.7B**, GW4064 did not decrease IL8 expression when introduced GW4064 and TNFα simultaneous for 1 hour. Although IL8 got its maximum expression in 1 hour, the time may not enough for GW4064 to make its role to deduce inflammatory cytokine. IL8 mRNA expression was depressed when pretreatment GW4064 at concentration of 2 μM for 18 hours (**Figure 3.7C**). However, whether this anti-inflammatory effect is due to GW4064 or cells death caused by cytotoxicity is to be questioned. Next, whether GW4064 can suppress IL8 expression at lower concentrations was checked. Beyond our expectation, IL8 mRNA expression were not affected when treatment with lower concentration of GW4064 compared with group that given TNFα only. Thus, the toxicity from GW4064 in FXR reporter cells should be confirmed. From **Figure 2.2**, it is known that GW4064 increased FXR activity at a range of 0.1 μM to 10 μM. Moreover, 2 μM is a concentration that is commonly used by other scholars, and they even use 10 μM cells transiently transfected with FXR [27]. Therefore, I conclude that 2 μM GW4064 is proper in FXR reporter cells for my study, and pretreatment of 2 μM GW4064 depressed TNFα induced IL 8mRNA expression.

To investigate whether two FXR-activating bacterial supernatants possess anti-inflammatory effect, cells were pretreatment with culture supernatant for 18 hours before TNFα administration. As shown in **Figure 3.8A**, IL8 expression decreased when treated with lower concentration of both bacterial culture supernatants. However, IL8 expression levels were much higher than that induced by 1 ng/ml TNFα, which implied that there may exist endotoxin in supernatants that induce inflammatory cytokine IL8

expression in FXR reporter cells, or the density of 10% culture supernatant may be harmful to cells growth.

To exclude the harm from bacteria culture supernatants, FXR activity was measured at various concentrations of culture supernatants. According to **Figure 3.8B**, the culture supernatant of *B.dorei* increased FXR activity and 25% of culture supernatant induced maximum FXR activity; while FXR activation got peak when given 50% of *E.limosum* culture supernatant. FXR activation declined considerably after it got peak when administration of increased culture supernatant concentration. Cells growth was affected when introduction of high concentration of culture supernatant was introduced. So, I convinced that cells were in good growth condition before FXR activation peaked.

Based on these results, I got a conclusion that the stimulation of TNFα-induced IL8 expression by culture supernatants was due to the endotoxins in culture supernatants. Endotoxins were chemically characterized and identified as lipopolysaccharide (LPS), which is the most abundant component within the cell wall in gram-negative bacteria. It has been widely recognized that LPS can promote the release of inflammatory cytokines, including IL8 in various cell types, leading to an acute inflammatory response [28]. FXR-activation metabolites in culture supernatant may have anti-inflammatory effect, but it was covered by the pro-inflammatory effect from endotoxins. Thus, it is necessary to find out the exact factors in the culture supernatants that can increase FXR activity in the later study.

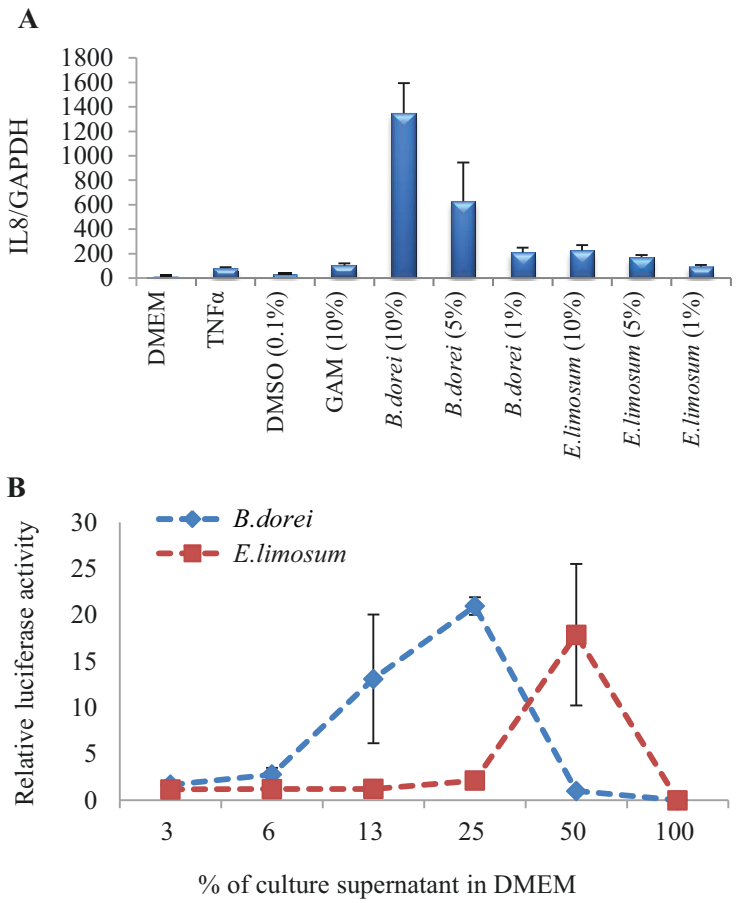

Figure 3.8 Exploration of anti-inflammatory effect of culture supernatants. (A) Cells were cultured in 24-well plates with density of 3×10^5/well for 24 h, bacterial culture supernatants were introduced for 24 h incubation before administration of TNFα for 1 h. mRNA levels were normalized to GAPDH mRNA level. (B) After cells were cultured in white 96-well plates with density of 5×10^4/well for 24 h, bacterial culture supernatants were introduced for 24 h incubation before measurement of chemiluminescense by administration of luciferase assay reagent. Values are means ± SD in triplicate (n=2).

3.4 Conclusion

In this chapter, it was demonstrated that two bacteria *B.dorei* and *E.limosum,* especially their culture supernatants, can directly induce FXR activity based on FXR reporter system and selectively induced FXR target genes expression in reporter system. In addition, two bacterial metabolites stimulated FXR target gene IBABP expression in the intestinal epithelial cell CaCO-2 cells. However, they did not affect FXR target gene SHP expression in HepG2 cells. Moreover, neither of two bacteria showed anti-inflammatory

property. The results revealed that two bacterial culture supernatants would be both gene- and cell-selective FXR modulators, and IEC-based FXR reporter cell is useful for screening intestinal FXR modulators including bacteria.

3.5 Reference

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Chapter 4

Evaluation of two FXR-stimulatory bacteria *in vivo*

Chapter 4

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4.1 Introduction

FXR, a key regulator of bile acids metabolism, regulates the bile acids enterohepatic circulation by inhibiting hepatic bile acids synthesis, promoting bile acids efflux from the liver and intestinal absorption. In addition to well-established roles in bile acids metabolism (Chapter 1.4.1), FXR activation also regulates lipid metabolism, insulin sensitivity, and energy homeostasis [1].

FXR is closely involved in many diseases, including hypertriglyceridemia, non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and obesity mainly through the way of decreasing triglyceride levels both in liver and serum [2]. The accumulation of hepatic lipids and circulating cholesterol and triglycerides were detected in $FXR^{-/-}$ mice [3]. FXR deficiency facilitated NASH pathophysiology with the evidence of macrosteatosis, hepatocyte ballooning and inflammation when mouse was fed with a high fat diet (HFD) [4].

Many of studies reported that the activation of FXR by its ligands reduces both lipogenesis in the liver and the levels of plasma cholesterol and triglyceride. Activation of FXR in obese and diabetic db/db mice by bile acids or GW4064 induced fasting plasma glucose decreasing and insulin sensitivity improvement [3, 5].

6ECDCA protected Zucker fa/fa rats from insulin resistance and NAFLD with repression of body weight gain and fat deposition in muscle and liver, which can be explained by down-regulated genes expression participated in gluconeogenesis, fatty acids synthesis, lipogenesis [6]. Treatment of humans or animals with bile acids (CDCA or CA) lead to plasma triglyceride deduction, low density lipoprotein (LDL) induction (reviewed in [7]). Another bile acid, ursodeoxycholic acids (UDCA), improved hepatic insulin resistance and steatosis in HFD-fed KK-A(y) mice with liver disease [8].

Administration of GW4064 or CA led to a significant decrease in plasma glucose levels and improvement of insulin sensitivity in mouse [9-11]. Activation of FXR by GW4064 suppressed weight gain through significantly repression of diet-induced hepatic steatosis, triglyceride and free fatty acids level in the liver when C57BL/6 mice were fed with either HFD or high fat and high cholesterol diet [12].

It was proved that the culture supernatants of *B. dorei* and *E. limosum* can induce FXR activity in FXR stable reporter system in chapter 3. However, it's incomplete to characterize their roles only *in vitro*. HFD can induce obesity and metabolic disorders in rodents whose pathophysiology is very similar to the human metabolic syndrome. HFD-induced obese mice model contributes immensely for understanding of diet induced obesity, insulin resistance or other metabolic diseases. Thus, in this chapter, a HFD induced obese mice model was used for two bacteria assessment.

4.2 Material and methods

4.2.1 Animals

Four-week old C57BL/6J male mice were obtained from CLEA Japan (Tokyo, Japan). Mice were housed in a temperature controlled room (23°C) under a 12 hours light-dark cycle. After stabilized in specific pathogen free (SPF) room for two weeks,

the mice were first given standard diet (STD, CLEA Japan) for one week before high fat diet (HFD, CLEA Japan) administration for another 10 weeks. During the whole feeding period, the mice received bacterial culture supernatants (0.1 ml/mouse) or PBS every day by intragastric administration (**Figure 4.1**). Body weight was recorded weekly.

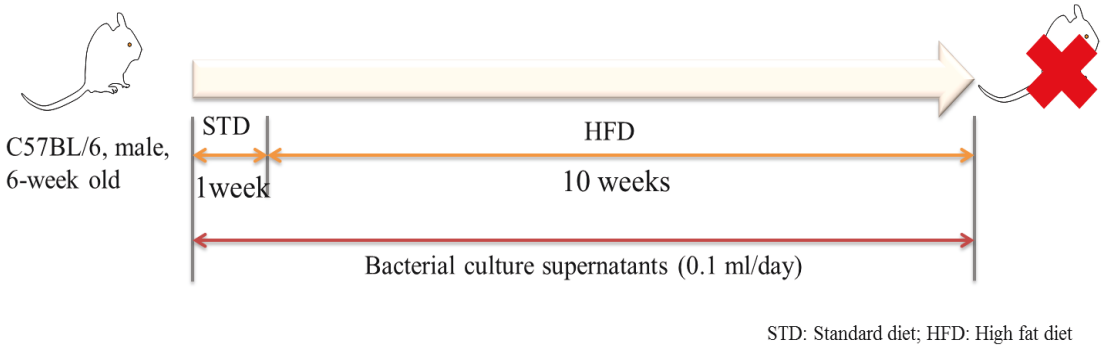

Figure 4.1 Experimental flow

The mice were fasted for 24 hours before harvesting blood from heart for serum biochemical analysis. Tissues were collected immediately, snap-frozen in liquid nitrogen and stored at -80°C for later analysis. All experiments were approved by the Ethical Committee of Waseda University.

4.2.2 Real-time PCR

Total RNA was isolated from small intestine and liver by using Qiagen RNeasy mini kit after homogenized by a homogenizer (Microtec, Chiba, Japan). RNA concentration and purity were determined by a NanoDrop^{TM} spectrophotometer (Thermo). Reverse transcription (RT) reaction and real-time PCR reactions were same in the manner with that mentioned in Chapter 3. All primers are under condition of 40 cycles of 95°C for 15 sec, 60°C for 60 sec, and 4°C hold. The RT products of colon were diluted 5 times. The primers used in this chapter are listed in **Table 4.1**.

Table 4.1 Primers used in this chapter

Gene	Forward	Reverse	Reference
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	
Fxr	TCCAGGGTTTCAGACACTGG	GCCGAACGAAGAAACATGG	
Ibabp	CAGGAGACGTGATTGAAAGGG	GCCCCCAGAGTAAGACTGGG	
Ostα	TGTTCCAGGTGCTTGTCATCC	CCACTGTTAGCCAAGATGGAGAA	
Fgf15	ACGTCCTTGATGGCAATCG	GAGGACCAAAACGAACGAAAT T	
Cyp7a1	AGCAACTAAACAACCTGCCAGTAC TA	GTCCGGATATTCAAGGATGCA	[13]
Cyp7b1	TAGCCCTCTTTCCTCCACTCATA	GAACCGATCGAACCTAAATTCCT	
Cyp8b1	GGCTGGCTTCCTGAGCTTATT	ACTTCCTGAACAGCTCATCGG	
Ntcp	ATGACCACCTGCTCCAGCTT	GCCTTTGTAGGGCACCTTGT	
Ibat	ACCACTTGCTCCACACTGCTT	CGTTCCTGAGTCAACCCACAT	

4.3 Results and discussion

4.3.1 Beneficial effects of daily administration of bacterial metabolites in diet-induced obese (DIO) mice

4.3.1.1 Mice body weight gain

To investigate if two FXR-stimulatory bacterial metabolites possess anti-obese activities *in vivo*, mice were fed with HFD supplemented with the culture supernatants of *B. dorei* or *E. limosum* every day for 11 weeks. Diet was changed every three days and diet intake was recorded. The groups that received HFD showed more diet intake compared to STD group due to the more HFD waste. However, the group that fed with *E.limosum* culture supernatant had lower diet consume. HFD dramatically elevated mice body weight compared to mice that received STD (**Figure 4.2B, C**). Since 6 weeks of administration of *B. dorei* derived-metabolites (the period of HFD intake, 5 weeks), the mice showed lower body weight compared with mice that received PBS only (**Figure 4.2B**), indicating that *B. dorei* cultural metabolites may help mice to be resistant to the body weight gain. In contrast, the mice that received *E. limosum* did not show any changes in body weight gain (**Figure 4.2B, C**). Take the diet intake and body weight together, the energy store might be more efficient that energy expenditure in mice that received *E. limosum* culture supernatant.

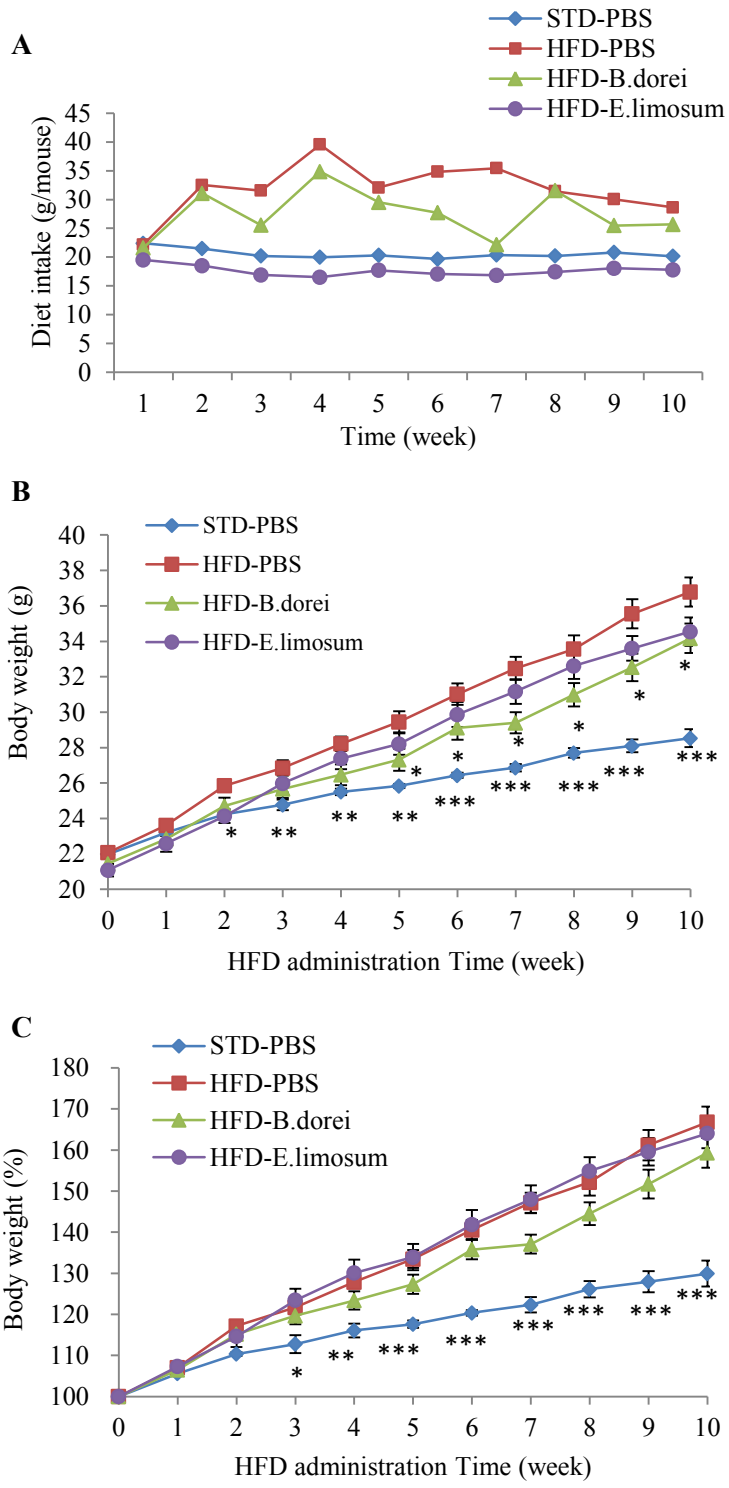

Figure 4.2 Changes in mice given bacterial culture supernatants. (A) Diet intake. (B-C) Mice body weight changes. Mice received intragastric administration of culture supernatants of *B. dorei* or *E. limosum* for 11 weeks. Diet was changed to HFD from second week. *P<0.05, **P<0.01, ***P<0.001. Compared to HFD-PBS group by Student's T test. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

Meanwhile, the mice body weight was compared among four groups by using ANOVA method (**Table 4.**2). HFD increased mice body weight compared with STD (p=0.000). When mice received HFD, *B. dorei* culture supernatants reduced mice body weight gain compared to mice fed with PBS (p=0.011). The mice received *E. limosum* culture supernatants did not affect mice body weight significantly (p=0.081).

Table 4.2 Mice body weight comparison by ANOVA method

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HFD-PBS	STD-PBS	3.84545*	0.90522	0.000	2.0617	5.6292
	HFD-*B. dorei*	1.88939*	0.73911	0.011	0.433	3.3458
	HFD-*E. limosum*	1.29697	0.73911	0.081	-0.1594	2.7534

*. The mean difference is significant at the 0.05 level by one-way ANOVA with LSD posthoc comparison.

At the end of the study, mice were sacrificed after 24 hours fasting, liver weight of each mouse was weighed. According to **Figure 4.3**, HFD feeding mice showed increased liver weight compared to STD group. Long-term administration of each bacterial metabolite had a reducing effect of liver weight in DIO mice. Especially, administration of *E. limosum*-derived metabolites led to a significant reduction in liver weight compared with the PBS control group. On the other hand, DIO mice treated with *B. dorei*-derived metabolites tended to reduce liver weight compared with the PBS control group, but this was not statistically significant.

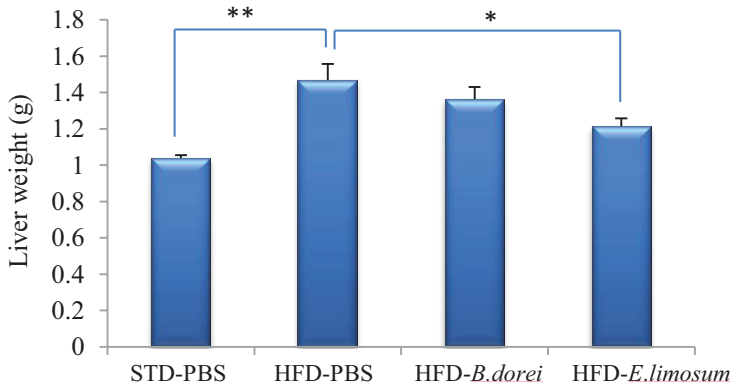

Figure 4.3 Liver weight. Mice received intragastric administration of culture supernatants of *B. dorei* or *E. limosum* for 11 weeks. Diet was changed to HFD from second week. *P<0.05, **P<0.01. Compared to HFD-PBS group by student's T test. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

The metabolic effects of *Fxr* activation by two bacteria in mice were evaluated first. The mice receiving the cells of *B. dorei* or *E. limosum* twice per week were sacrificed after bacteria gavage for 11 weeks. The mice body weight was significantly indistinguishable from the PBS-treated mice, which are shown in **Appendix Figure 1**. In addition, the liver weight did not show significant differences compared to PBS group.

4.3.1.2 Serum biochemical analysis

According to previous studies, the level of serum lanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, glucose and triglycerides were higher in HFD groups as compared with the mice given normal diet after 8 weeks administration[14]. These factors are involved in fat and glucose metabolism, and the elevated levels of them in general signify some form of hepatic damage or injury. Thus, in this study, I hoped the levels of these factors would be down-regulated by introduction of culture supernatants of two bacteria. Blood was collected from heart after anesthesia and serum were separated for biochemical analysis by a SPOTCHEMTM EZ SP-4420 analyzer (Arkray, Tokyo, Japan).

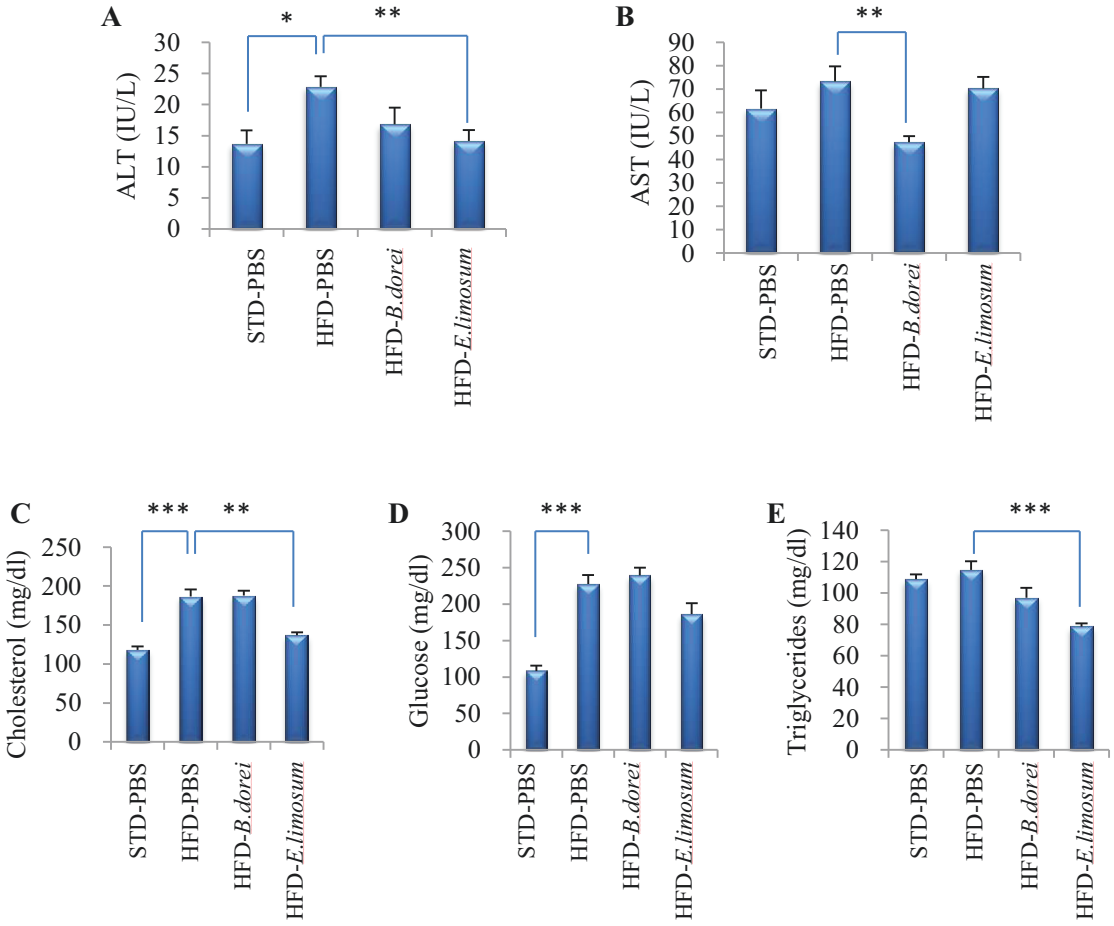

Figure 4.4 Serum biochemical analysis. Blood was collected from heart after fasting for 24 hours and serum was separated for analysis. *P<0.05, **P<0.01, ***P<0.001. Compared to HFD-PBS group by Student's T test. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

In the HFD control group, C57BL/6 mice had increased activities of liver functional markers, including serum ALT, cholesterol and glucose, indicating pathological changes in HFD mice. However, significant differences of AST and triglycerides were not detected compared to mice that received STD (**Figure 4.4**). The levels of ALT and AST were down-regulated when mice received *B. dorei* derived culture supernatant for 11 weeks. The mice fed with *E. limosum* derived culture supernatant reduced levels of ALT, cholesterol and triglycerides.

4.3.1.3 Histology assessment of liver

Feeding mice with a HFD always induce impaired liver damage in the liver. In order to detect pathological changes, liver samples were fixed in formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE, **Figure 4.5**). Marked fat accumulation and histology were observed when mice were fed with HFD, which was characterized by macrovesicular steatosis with large and small fat droplet (green arrow), mixed inflammatory cells infiltration (blue arrow) and hepatocyte ballooning (red arrow). Treatment with *B. dorei* derived culture supernatants slightly alleviated the severity of liver steatosis. But serious inflammation was found the one mouse with *B.dorei* culture supernatants administration (**Appendix Figure 15**). *E. limosum* treatment did not enhance liver steatosis.

HFD feed mice presented hepatic fibrosis as well. With HE stain, it is difficult to find this pathological changes. Picrosirius red stain was used to observe collagen in liver. As illustrated in **Figure 4.6**, hepatic fibrosis was found when mice received a HFD, that more collagen were found around and diffusing along vein. However, two bacterial culture supernatants did not alleviate the hepatic fibrosis. The detailed picrosirius red stain results of each mouse are shown in **Appendix Figure 17**.

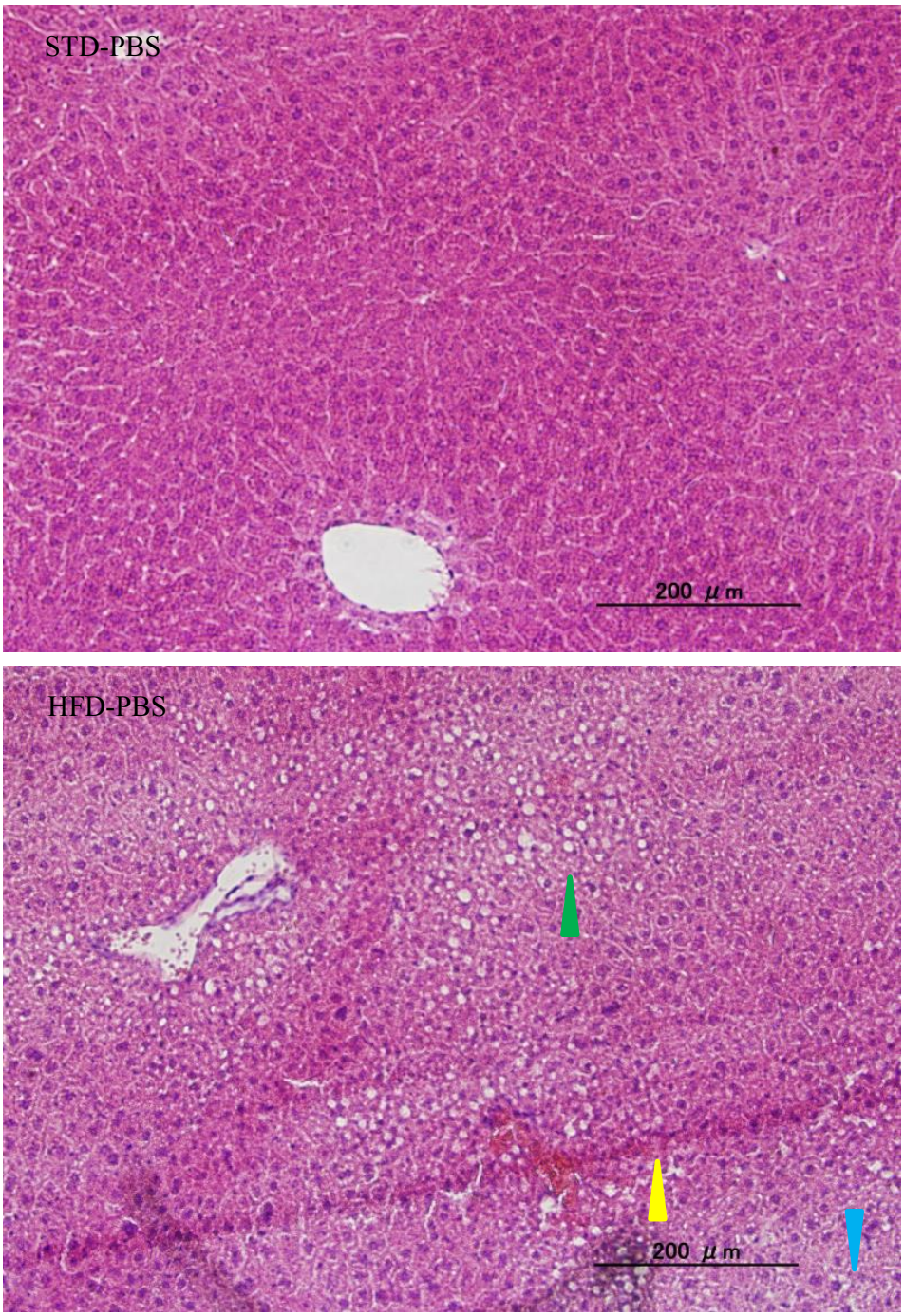

Figure 4.5 HE stain of liver tissue. Green arrow means large and small fat droplet, yellow arrow means mixed inflammatory cells infiltration and blue arrow means hepatocyte ballooning. *(Continued on the following page)*

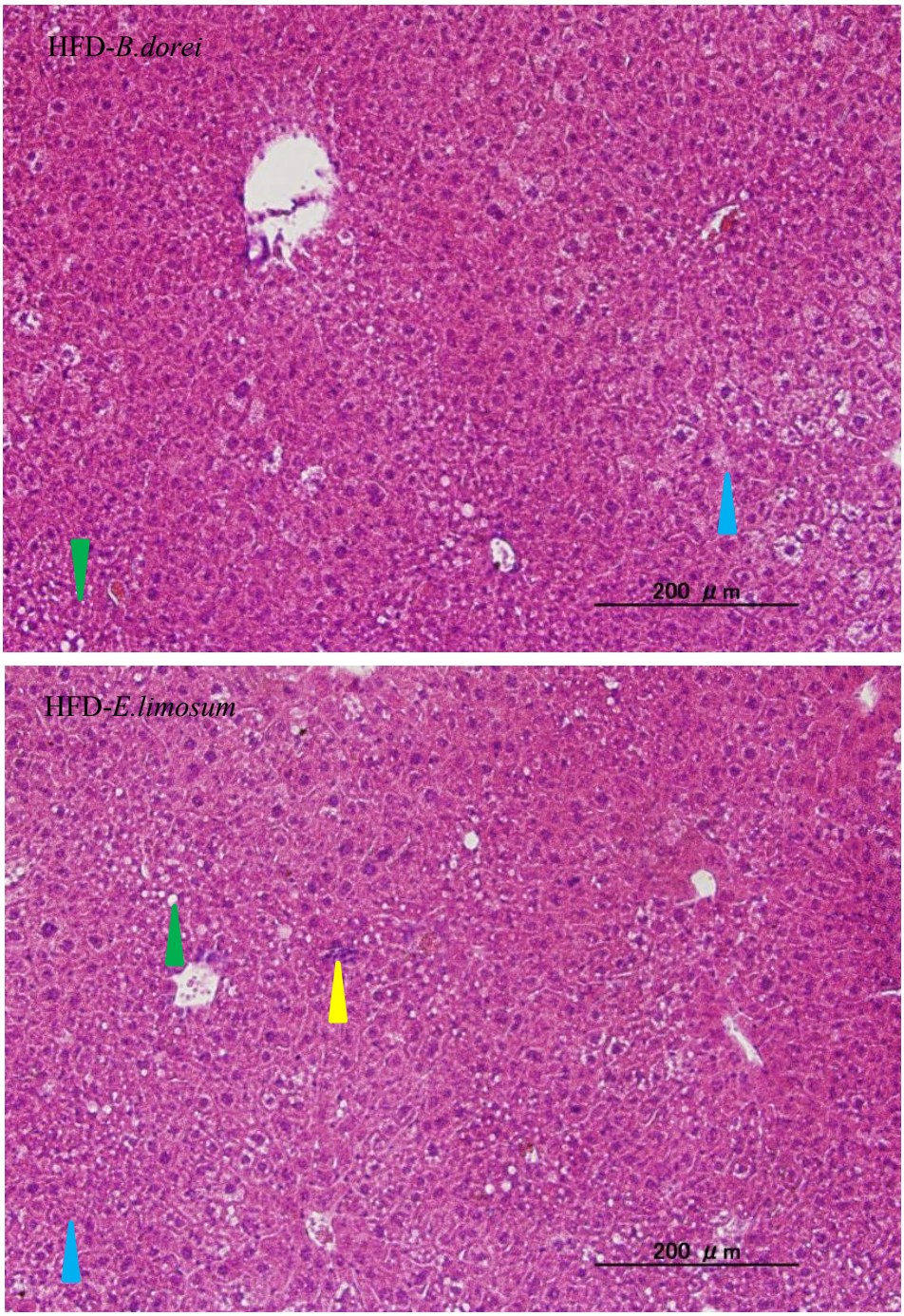

Figure 4.5 HE stain of liver tissue. Green arrow means large and small fat droplet, yellow arrow means mixed inflammatory cells infiltration and blue arrow means hepatocyte ballooning.

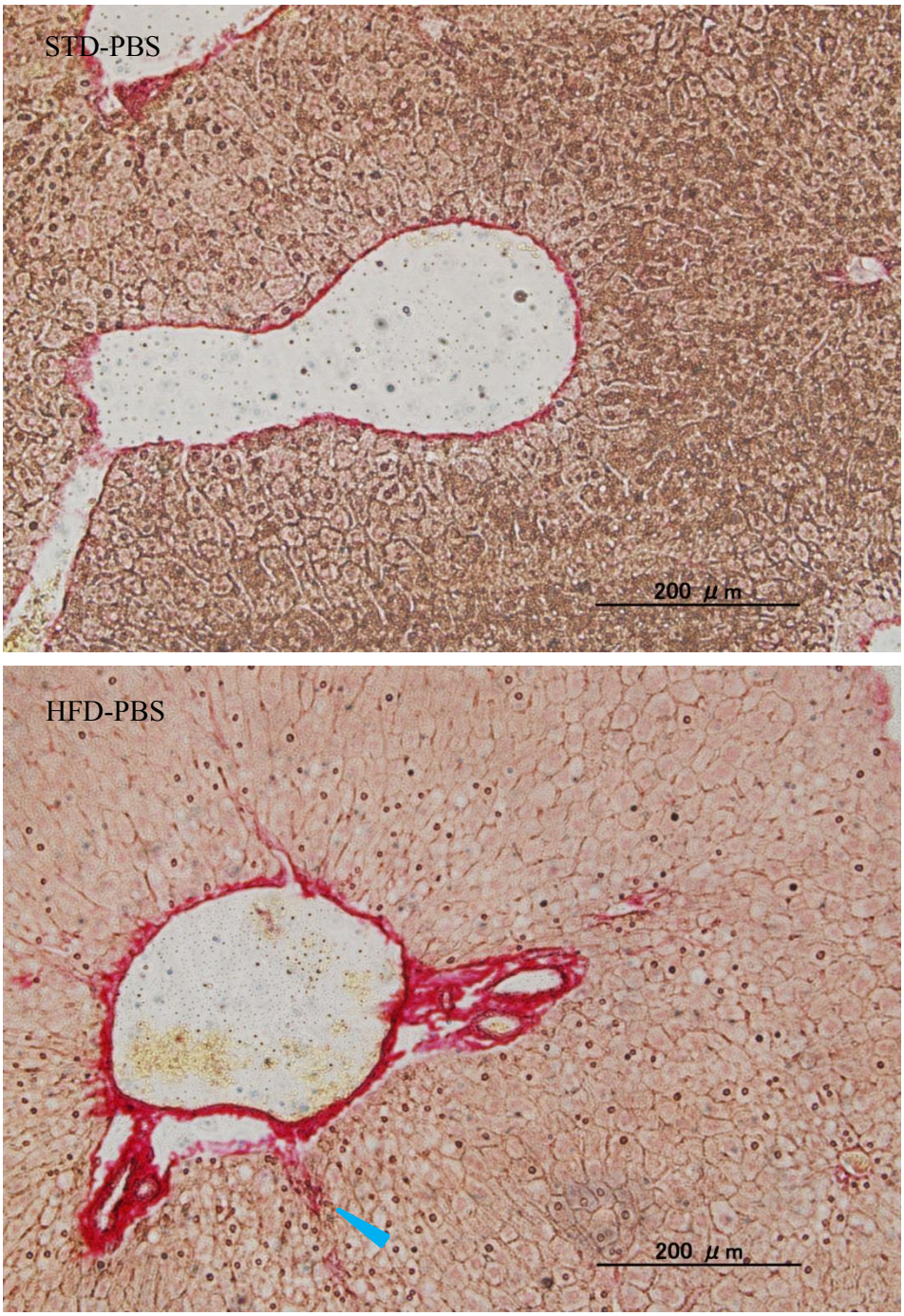

Figure 4.6 Picrosiriu red stain of liver tissue. Red color presents collagen, blue arrow means fibrosis.

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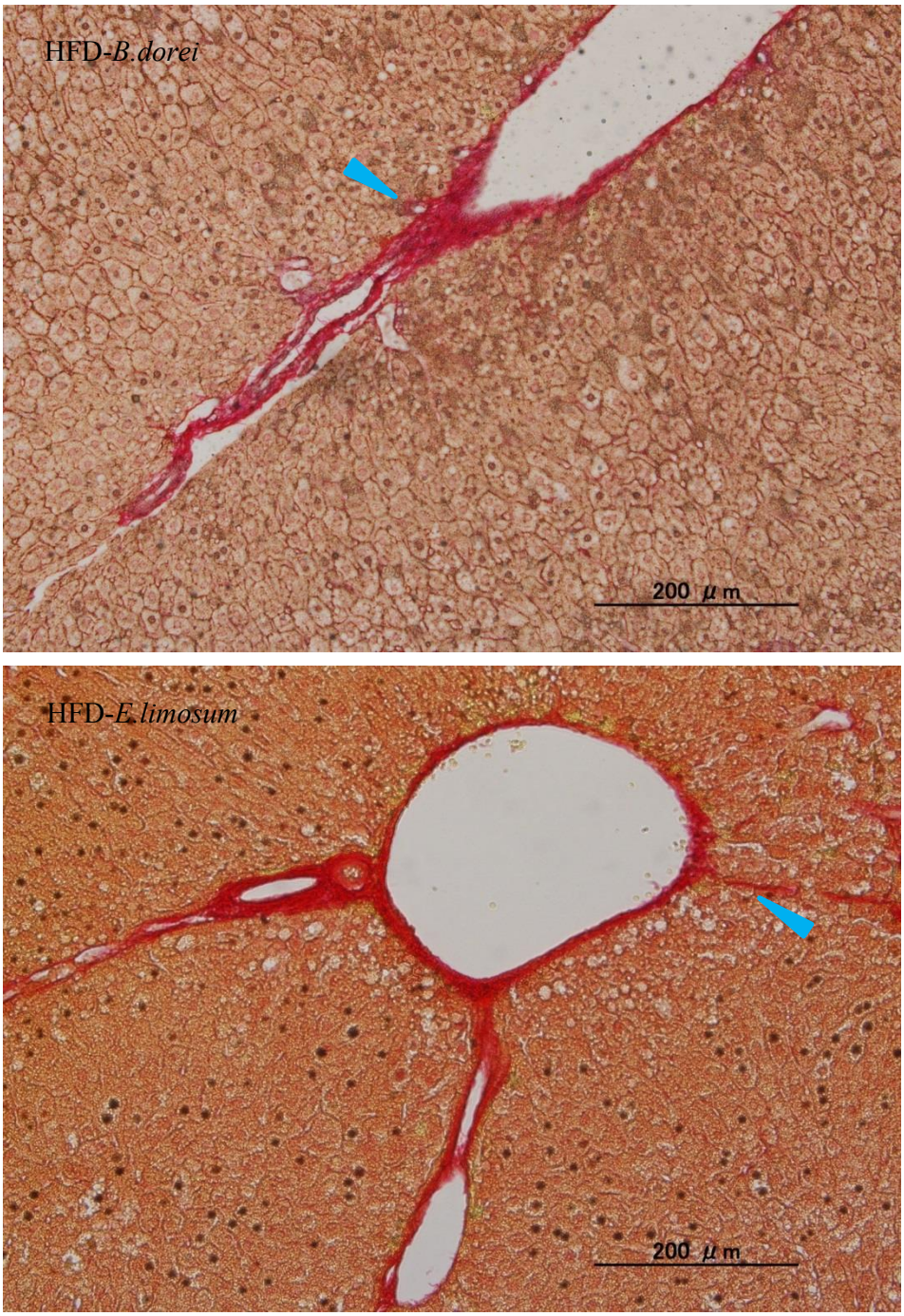

Figure 4.6 Picrosiriu red stain of liver tissue. Red color presents collagen, blue arrow means fibrosis.

4.3.1.4 Beneficial effects of two bacterial metabolites

Metabolic syndrome is resulted from the increasing prevalence of obesity. In order to investigate whether *B. dorei* and *E. limosum* FXR-derived metabolites conferred anti-obesity effects, they were daily administrated to DIO mice. HFD-fed mice treated with FXR-stimulating bacterial metabolites (*B. dorei*) helped mice to be resistant to obesity compared with control mice.

Although histological analysis did not clearly demonstrate the alleviation by two bacterial metabolites, *in vivo* administration of FXR-stimulating bacterial metabolites decreased the levels of serum biochemical markers for liver injury and lipid metabolism in DIO mice. The levels of ALT and AST reflect the presence of hepatic inflammation, steatosis and fibrosis. Since AST is highly expressed in the liver, heart, and muscle cells, the increased AST levels are usually a sign of liver disease, but also other diseases. Since ALT is an enzyme mostly produced in hepatocytes, the level of ALT in blood can be regarded as a biomarker to evaluate the degree of hepatocellular damage in obese mice chronically fed HFD. Many studies have shown the decreased levels of AST and ALT indicate ameliorate liver dysfunction [15, 16]. The reduced levels of ALT and AST in this study may reveal that *B. dorei* derived metabolites are effective in suppressing the development of HFD-induced fatty liver diseases.

The increased levels of triglycerides and cholesterol often reflect the dyslipidemia in DIO mice. This study demonstrated that the levels of serum cholesterol and triglycerides could be down-regulated by two FXR-stimulatory bacterial metabolites, which were confirmed by the fat droplet decrease in microscopic observation. It was reported that *Lactobacillus plantarum* strain K21 alleviated bodyweight gain, decrease cholesterol and triglyceride levels and mitigate liver damage in DIO mice [15]. In addition, the decreased concentration of triglycerides and cholesterol reveals the depression of nonalcoholic fatty liver disease (NAFLD) [17].

Taken together, two FXR-stimulatory bacterial metabolites are potentially used for metabolic diseases induced by diet.

4.3.2 Bacterial metabolite-induced modulation of FXR target genes

4.3.2.1 Gene expression profiles in the ileum

To investigate the role of two bacterial culture supernatants on bile acids homeostasis in diet induced obesity mouse model, total RNA were obtained from the ileum, the colon and the liver for real-time PCR analysis.

Upon HFD administration, the expression of *Shp* mRNA was upregulated in the ileum (**Figure 4.7B**), whereas other genes were not affected. When mice were fed with *B. dorei* or *E. limosum* derived culture supernatants, the levels of *Fxr* increased by 1.6-fold and 2.1-fold respectively (**Figure 4.7A**), which confirmed the *in vitro* findings that two bacterial metabolites stimulate FXR. Transporter *Ibat* was up-regulated with 1.9-fold and 2.5-fold compared to DIO mice (**Figure 4.7C**). In addition, *E. limosum* derived metabolites down-regulated target gene *Shp* expression, while *B. dorei* derived metabolites up-regulated *Shp* with 1.5-fold in the ileum (**Figure 4.7B**). Gene *Fgf15*, *Ibabp* or *Ostα* were unaffected by two bacterial culture supernatants (**Figure 4.7D-F**). These results revealed gene-selective regulation of two bacterial culture supernatants.

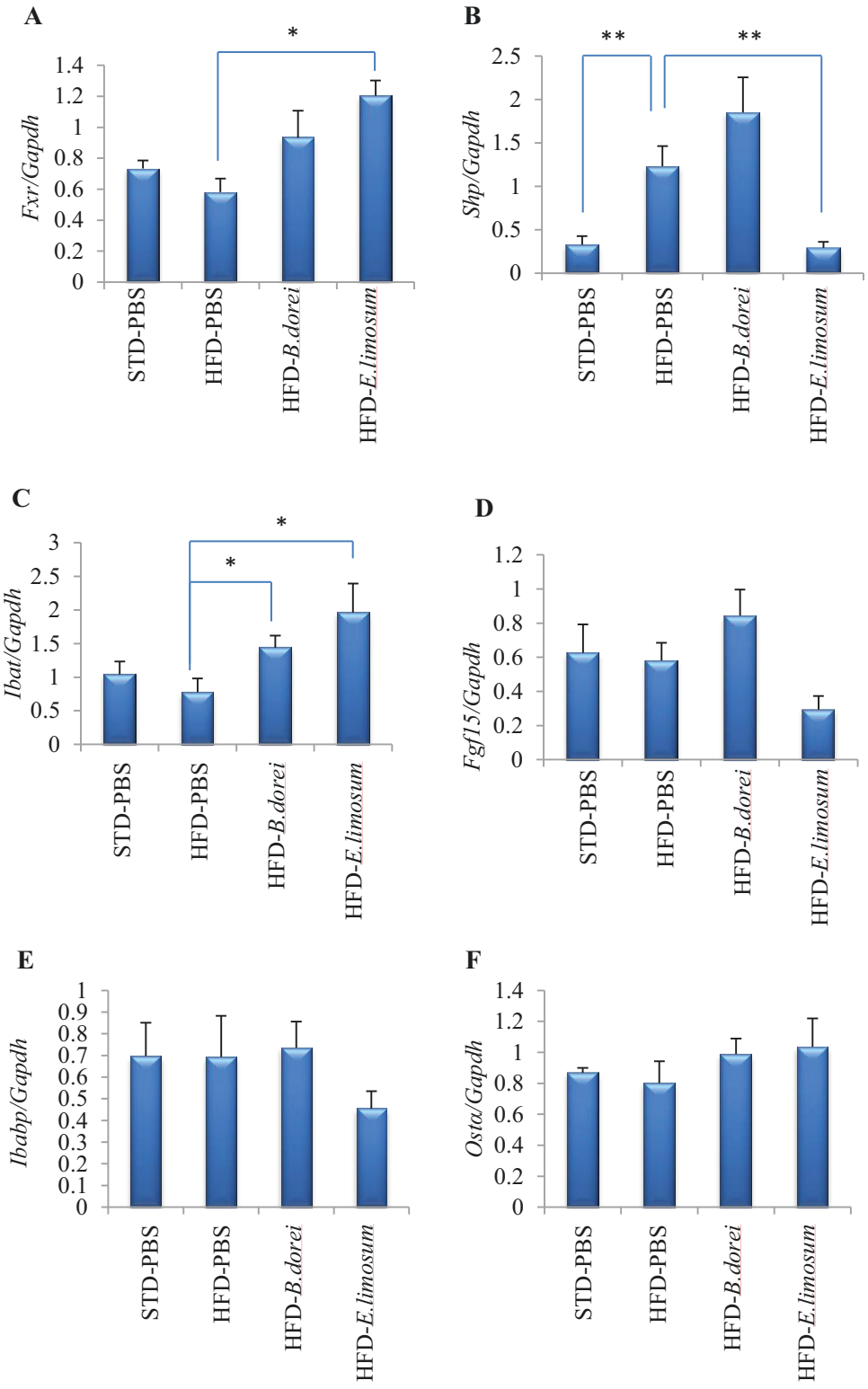

Figure 4.7 Gene expressions in the ileum. *P<0.05, **P<0.01. Compare to HFD-PBS group by Student's T test. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

The ileal *Ibat* was down-regulated by *E. limosum* derived metabolites, which could be explained by the up-regulation of ileal *Shp* expression. Previous studies stated that the negative feedback regulation of ileal *Ibat* was mediated by FXR induced *Shp* expression in the intestine [18, 19]. On the other hand, the reduced expression of *Ibat* in the ileum, may result the inhibition of bile acids reabsorption in the enterohepatic circulation. Thus, *E. limosum* might be useful in regulation of bile acids excess excretion in the liver. Actually, the expression of *Ibat* is still ongoing controversy and species different. *Ibat* in the rat is unaffected by bile salts [20], while it is under positive feedback regulation in humans [21]. Elegant studies have elucidated *Ibat* can be positive and negative feedback regulated by bile acids [22].

4.3.2.2 Gene expression profiles in the colon

Colon is another organ expressing *Fxr*. In fact, the colon adenocarcinoma cells SW480 was used as a basis for *Fxr* stimulatory bacteria screening *in vitro*. In the colon, HFD did not provide significant impact on *Fxr* activity. *Shp* expression level was up-regulated by 6-fold, *Ibabp* and *Fgf15* were reduced with 2.7-fold and 5-fold, respectively. However, the differences of three gene expression levels were not significant, due to the big error. Other *Fxr* target genes were unaffected either. When fed with HFD, the bacterial culture supernatants derived from *B. dorei* or *E. limosum* did not affect the expression of *Fxr* and its target genes (**Figure 4.8**), which are similar to the results when mice received bacterial cells administration (**Appendix Figure 5**).

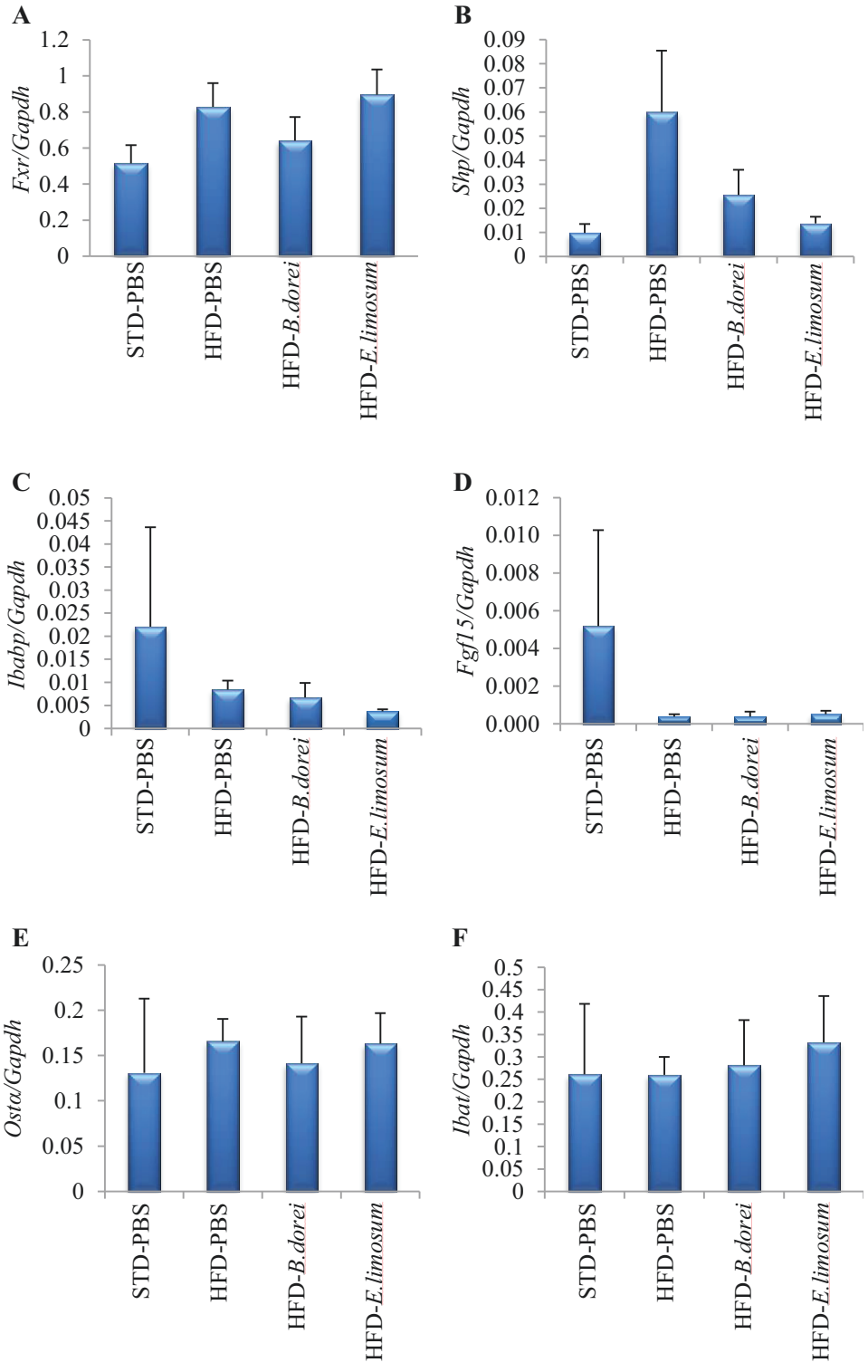

Figure 4.8 Gene expressions in the colon. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

4.3.2.3 Gene expression profiles in the liver

Fxr is highly expressed in the liver, where it regulates bile acids distinct transcriptional networks (**Figure 4.9**). Thus, whether genes involved in bile acids in the liver were regulated by treatment with two bacteria was confirmed. In the liver, HFD administration induced significant increase of gene *Shp* by 1.8-fold and *Bsep* by 1.5-fold, while *Fxr* was not affected. However, *Fxr* expression was elevated when mice were fed with two bacteria derived culture supernatants, which showed increase of 1.3-fold and 1.4 fold, respectively (**Figure 4.10A**). The mice fed with *E. limosum* derived culture supernatant displayed significant *Shp* reduction and increased *Ntcp* expression (**Figure 4.10B, D**). On the other hand, *B. dorei* culture supernatant did regulate *Fxr* target genes involved in bile acids metabolism in the liver.

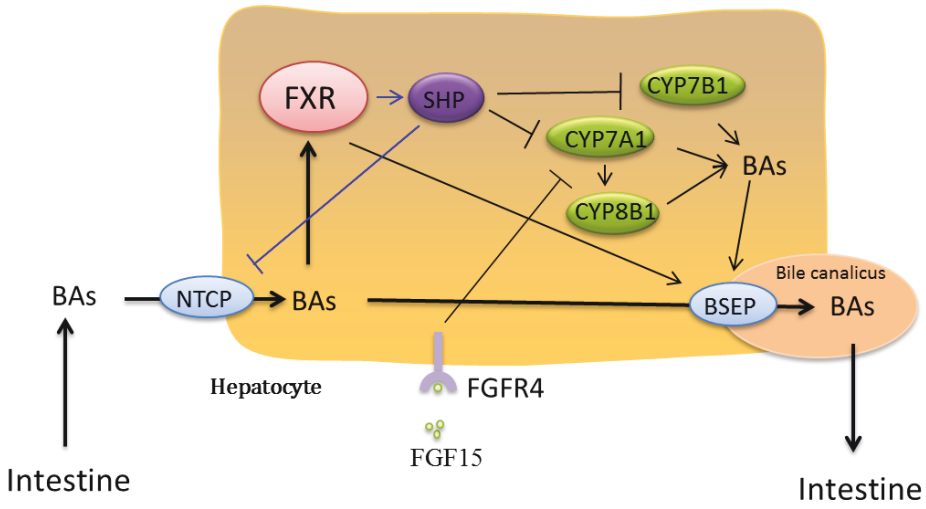

Figure 4.9 Bile acids pathway in the mouse liver

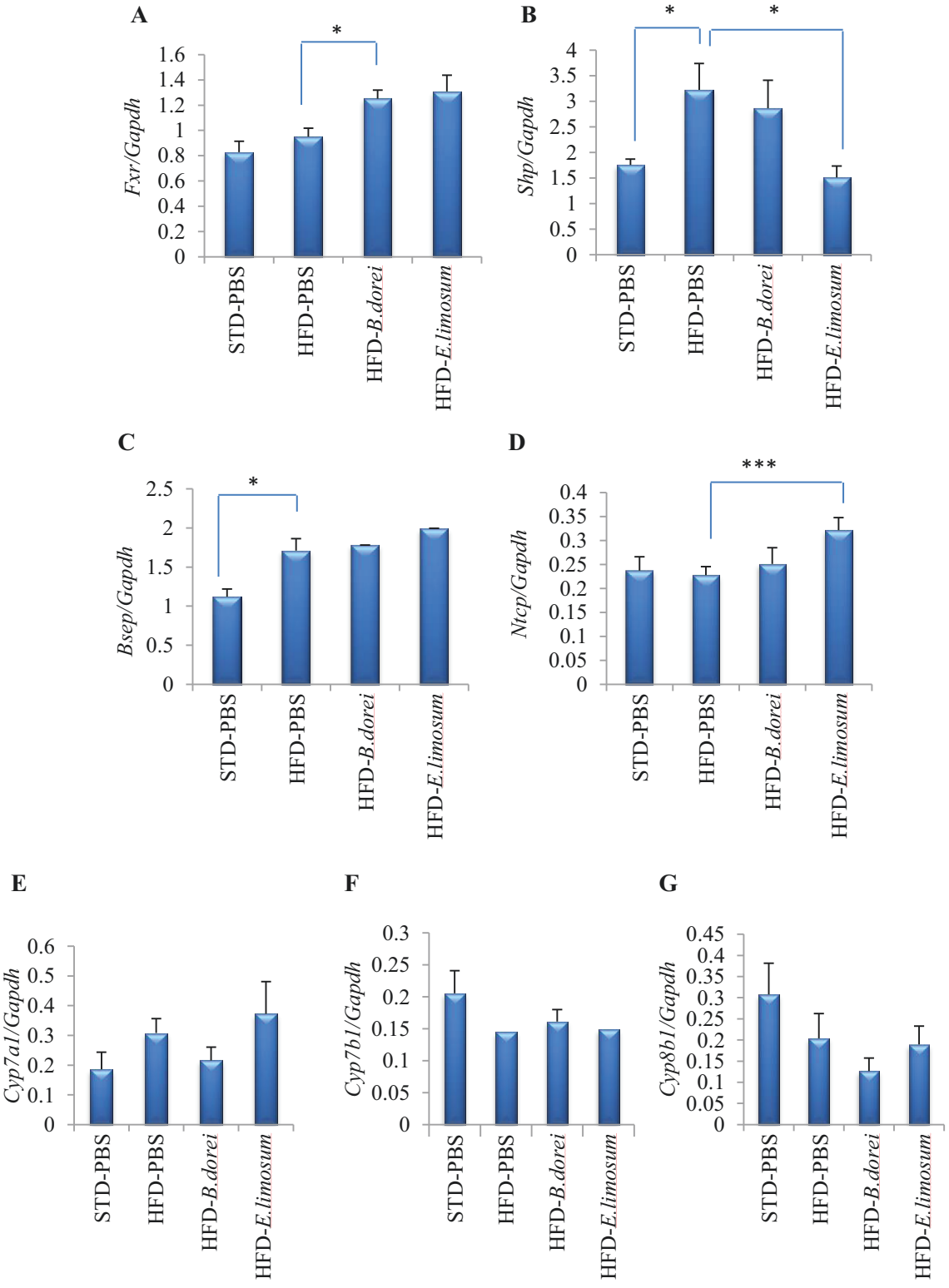

Figure 4.10 Gene expressions in the liver. *P<0.05, ***P<0.001. Compared to HFD-PBS group by Student's T test. Values are mean ± SEM. n=6 (n=3 in STD-PBS group).

As well as the gene-selective role in the ileum, the gene specific regulation was discovered in the liver. My results displayed that treatment with *E. limosum* elevated the expression of *Ntcp*, which is responsible for the bile acids uptake from the intestine. In turn, the re-absorbed bile acids induced *Fxr* activity. However, instead of *Shp* induction, the level of *Shp* was down-regulated by *E. limosum*, indicating the 'anatagonist' role of *E. limosum* derived culture supernatant. On the other hand, other genes were not affected, revealing the culture supernatant of *E. limosum* might act as *Fxr* selective modulators in the liver.

4.3.2.4 Two bacterial metabolites regulate bile acids metabolism

By checking the expression profiles of *Fxr* and *Fxr* target genes in the ileum, colon and liver, I showed that FXR activation in the intestine and liver directly induces the expression of genes regulating the transport of bile acids (BAs) (e.g. *Bsep*, *IBABP*, *OSTα*), while the genes involved in the synthesis (e.g. *Cyp7a1*, *Cyp8b1*) and re-absorption (e.g. *Ibat*, *Ntcp*) of BAs are repressed through SHP and FGF15/19 induced by FXR activation [23]. Two bacterial metabolites enhanced the *Fxr* gene expression in the intestine and liver, and ileal *Shp* gene expression tended to be increased by treatment with the metabolites derived from *B. dorei*. However, the expression of most FXR target genes was not positively regulated by intragastric administration of the bacterial supernatants. Rather, *E. limosum*-derived metabolites significantly repressed ileal and hepatic expression of *Shp* and up-regulated the expression of *Ibat* and *Ntcp*.

When treated with *E. limosum* metabolites, the repression of *Ibat* in the ileum may result less bile acids reabsorption from basal lamina, in turn resulting unchanged levels of *Ibabp, ostα* and *Fgf15*. One mechanism might be that the reduced reabsorption level of bile acids may give neuro-information to activate transporter *Ntcp* to uptake bile acids from the intestine. For the other, the small metabolites of *E. limosum* might get into the portal vein, and arrive in the liver. Then, these small metabolites can act as selective modulators for

hepatic *Fxr* activation, contributing to the repression of hepatic *Shp* inhibition and *Ntcp* elevation.

In vitro data showed that the FXR-stimulatory potential of *B. dorei* metabolites was stronger than *E. limosum*. However, the administration of *E. limosum* derived metabolites is more likely to affect the expression levels of FXR target genes *in vivo*. Moreover, target genes *Fgf15*, *Ibabp* and *Ostα* were not altered when introduction of two bacterial culture supernatants, which was not consistent with the results *in vitro*, indicating the differences between *in vitro* and in *vivo*. The difference between *in vitro* and *in vivo* experiments might be affected by the colonization and metabolic activities of FXR-stimulatory bacteria in the ileum. Previous studies showed that the composition of BAs, which are strongly affected by gut microbiota, modulated FXR-mediated gene expression *in vivo* [13, 24-26]. Thus, the alteration in gut microbiota by FXR-stimulating bacterial metabolites might also influence the regulation of FXR target gene expression. In future study, we need to investigate whether BAs metabolism and gut microbiota are influence by the administration of FXR-stimulatory bacteria or metabolites.

Fxr activity increased in both liver and intestine, when mice received two bacterial culture supernatants. Although I concluded that two bacterial culture supernatants directly induced FXR activity *in vitro*, it is insufficient to conclude they induced *Fxr* activity *in vivo* as well based on present results. In this study, the ordinary mice instead of *Fxr* deficiency mice were used to evaluate two bacterial culture supernatants. Thus, it is failed to emphasize the specific *Fxr* stimulatory role of two bacterial metabolites. Some other receptors are also reported to influence BAs composition and formation. For example, activation of LXRs inhibits the expression of *Cyp7a1* and may affect the sensitivity to BAs toxicity [27]. PPARα activation alters synthesis, conjugation and transport of bile acid, which is summarized in this view [28]. Meanwhile, FXR ligands often can activate not limit to one receptor. FXR agonist GW4064 as well as CDCA induce significant induction of PPARα mRNA levels [29]. These receptors have close cross talk between each other by

affecting same genes. Thus, two bacterial culture supernatants might interact with other receptors, contributing to the FXR target genes expressions *in vivo*.

In order to investigate the role of FXR activation by two bacterial culture supernatants, FXR deficiency mouse model provides us a powerful way. Within the FXR deficiency mouse model, the gene expression profiles and physiological changes resulted by FXR activation might be easier understood. The probiotics #VSL3 regulated FXR target gene expressions via FXR activation by using FXR deficiency mouse model [25]. Besides the fully FXR null mouse model, the tissue specific FXR deficiency mouse model is also widely used to characterize the role of FXR. The roles of FXR in the liver and intestine were demonstrated to be differential and complementary in regulating bile acid homeostasis by using liver specific and intestine specific FXR null mouse [27]. The regulation of *Cyp7a1* expression is though intestinal FXR-*Fgf15* signaling pathway by using intestine selective FXR null mice [27, 28]. Due to the reason that the intestine is the predominant place that bacterial metabolites have action on FXR, the intestinal specific FXR deficiency mouse model might give more precise data about FXR activation.

When two bacteria were introduced to mice at frequency of twice per week, differences were not detected (The whole data are shown in **Appendix**), which may contribute to the inadequate administration of bacteria. On the other hand, neither of two bacteria was detected in the mice intestine by using next generation sequence method (Data not shown), which may be explained by two aspects. One, two bacteria cannot get through stomach due to the low pH environment. However, they do affect the intestinal microbiome distribution. Another possible reason is that their populations were too small to be detected.

4.3.3 Two bacterial metabolites are involved in metabolic syndrome

Intestinal FXR signaling might be a drug target for obesity and metabolic complications (e.g. non-alcoholic fatty liver disease). In this study, HFD-fed mice treated

with FXR-stimulating bacterial metabolites (*B. dorei*) helped mice to be resistant to obesity compared with control mice. Furthermore, *in vivo* administration of FXR-stimulating bacterial metabolites decreased the levels of serum biochemical markers for liver injury (i.e. ALT, AST) and lipid metabolism (i.e. cholesterol, triglyceride) in DIO mice.

At present, there are contradictory reports on the role of intestinal FXR signaling in metabolic improvement. One potential mechanism is that the inhibition of intestinal FXR signaling improves obesity and insulin responsiveness in HFD-fed mice [24]. Previous reports showed that diet-induced weight gain or metabolic defects were suppressed in intestine-specific FXR-null mice [24]. It was also reported that the anti-obesity effect was associated with the inhibition of intestinal FXR signaling by the accumulation of tauro-β-muricholic acid (TβMCA), which is an endogenous antagonist of FXR [13]. The accumulation of TβMCA is caused by the depletion or decrease of gut microbiota that possess BAs deconjugation ability. However, intestinal FXR activation with the FXR agonist fexaramine enhanced energy expenditure mediated through the activation of β-adrenergic receptor signaling in adipose tissues, resulting in metabolic improvement in DIO mice [26]. The intestinal-selective effect of FXR might be coordinated by the induction of intestinal endocrine hormone *Fgf15* (homolog of *FGF19* in humans) [13, 25-27, 29, 30]. We showed that the administration of bacterial cells or metabolites of *B. dorei* tended to enhance the expression of ileal *Fgf15* gene compared with the PBS-treated group although this was not statistically significant. This suggests that the concentration of FXR agonist in the supernatants or the frequency of bacteria might be inadequate to achieve metabolic improvement in DIO mice. Further studies are required to evaluate whether metabolic improvement is enhanced by the increased administration frequency of bacterial cells or administration of FXR agonists purified from bacterial culture supernatants.

FXR target gene *Shp* might also be involved in development of metabolic diseases, as previous study reported that the overexpression of *Shp* exacerbated obesity phenotype in DIO mice [31]. The $Shp^{-/-}$ mice studies showed that mice are resistant to diet induced obesity and insulin resistance [32]. Moreover, the study of Wang et al. implied that decreased basal expression of PGC-1α expression induced by high expression levels of *Shp* [33]. The inverse relationship between the expression of *Shp* and PGC-1α was found in another study [34]. By further investigation of anti-obesity effects of *Shp* loss in DIO mice, they suggested that the *Shp* loss led to the disruption between insulin sensitivity and fat accumulation [35]. Thus, in this aspect, *E. limosum* derived metabolites might increase energy expenditure at transcription level due to the inhibition of *Shp* expression and reduction of serum triglycerides. However, some reports also suggest that increasing FXR activity and *Shp* might be useful in hypertriglyceridemia though inhibition of SREBP-1c activity [36]. To conclude, the effects of *E. limosum* should be further investigated.

Intestinal FXR activation has the potential of curing intestinal bowel disease (IBD) as well as metabolic disorders [37, 38]. A previous report showed that intestinal inflammation was associated with a decrease *Fxr* expression levels in the inflamed intestinal mucosa of Crohn's disease and experimental colitis mice [37]. Polymorphisms in the FXR gene were not associated with IBD pathogenesis [39]. Thus, intestinal FXR activation could have a therapeutic effect on IBD. Interestingly, the expression level of *Fxr* mRNA in the ileum was enhanced by treatment with both FXR-stimulating bacteria screened in this study. In future experiments, we will evaluate whether *B. dorei* or *E. limosum* exerts a curative effect on intestinal inflammation using experimental colitis mouse models.

4.3.4 Characteristics of two bacteria

A great many of probiotic strains have been evaluated for their ability to lower serum AST and ALT [15], triglycerides and sterol [15, 40], decrease glucose intolerance [41], reduce adipocyte size [42] and improve insulin resistance [41]. It's well known that most of effects of probiotics are strain-specific and cannot be expanded to other probiotics that in the same species or genus.

B. dorei was recovered from human feces collected from one healthy, Japanese, 23-year-old male in 2006. Later, it is found that *B. dorei* is more common in patients with active coeliac disease [43]. In addition, high abundance of *B. dorei* was found in type 1 diabetes in Finlish children [44]. *B. dorei* is easily mistook by both gene and biochemical similarity to *Bacteroides vulgatus*. It is reported that *B. vulgatus* protects against *E. coli* induced colitis in IL-$2^{-/-}$ mice without understanding the mechanism [45]. However, a cholesterol-reducing *bacteroides* sp. strain D8 was most similar (>99.5%) to species *Bacteroides dorei.* By specifical analysis of physiological effect, they concluded this strain that can reduce cholesterol level is independent to *B. dorei* [46]. The strain used in my study has 99% similarity to *B. dorei* and *bacteroides* sp. strain D8 by 16S rRNA. Generally, at gene level, it can be viewed as *B. dorei*. But the position of the strain used should be carefully checked both at gene and physiological levels. However, in another way, the new findings in this study give this strain new property, which may provide the new research direction of *B. dorei.*

E. limosum, a commensal microorganism in the intestine, grows under strictly anaerobic conditions. *E. limosum* can accelerate the growth of intestinal epithelial cells and inhibition of IL-6, probably though the butyrate production [47]. Another study also gave the similar evidence that *E. limosum* can ameliorate experimental colonic inflammation due to butyrate [48]. They found the metabolite of *E. limosum* increased mucosal integrity and exhibited anti-inflammatory action modulation of mucosal defense system via TLR4.

Although *E. limosum* is not involved in bile acids metabolism process, other species, like *E. lentum,* participate in deconjungation of bile acids [49]. Another strain *Eubacterium* sp. strain VPI 12708 was reported to have inducible bile acid 7-dehydroxylation activity [50].

My results demonstrated that for the first time *E. limosum* is also involved in bile acids metabolism both *in vitro* and *in vivo*. Although the exact mechanisms are not elucidated, the findings may hold great promise and appear to be useful in the future.

4.4 Conclusion

B. dorei and *E. limosum* are assessed by using HFD-induced obesity mice. Treatment mouse with two bacterial culture supernatants for 11 weeks reduced mice body weight gain and liver weight. *B. dorei* metabolites treatment decreased the levels of serum ALT and AST; while ALT, cholesterol and triglycerides were down-regulated by *E. limosum* treatment. The genes involved in bile acids metabolism were tissue- and gene- specifically regulated by two bacterial culture supernatants. Both of intestinal and hepatic *Fxr* were activated by two bacterial metabolites. Although administration of *B. dorei* derived culture supernatants increased transporter *Ibat* expression in the ileum, the genes were unaffected in the colon or liver. Transporter *Ibat* was increased via intestinal *Shp* inducement, and *Ntcp* was upregulated through hepatic *Shp* inhibition when mice received *E. limosum* metabolites. However, histology assessment of liver tissues did not show distinct differences.

4.5 Reference

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Chapter 5

General conclusions and perspectives

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5.1 General conclusion

In this study, a stable FXR reporter gene system was obtained for FXR modulators screening by transfection of a FXR expression plasmid and a FXRE-*luc* reporter vector into colon cancer cell line SW480. The induction of FXR target genes by screened FXR stimulatory bacteria was determined by real-time PCR. In addition, a high fat diet (HFD)-induced obese mouse model was used to evaluate the intestinal FXR stimulatory potential of FXR stimulatory bacteria.

A luciferase assay with the FXR reporter cell line demonstrated that the FXR-stimulatory activity of most bacterial cell samples was less than 2-fold. The culture supernatants of *Bacteroides dorei* and *Eubacterium limosum* induced FXR activity and selectively regulated FXR target expression in the FXR reporter system. Treatment with *B. dorei*-derived metabolites strongly induced ileal bile acid binding protein (*IBABP*) (8.4-fold) and organic solute transporter (*OST*) α (3.1-fold) compared with *E. limosum*-derived metabolites. Furthermore, administration of *B. dorei* derived metabolites showed significant reduction in body weight gain, and both two bacterial metabolites reduced liver weight in obese mice compared to PBS-treated controls. Administration of each bacterial metabolites improved in serum levels of obesity-related metabolic biochemical markers such as ALT, AST, total cholesterol, and triglyceride. Furthermore, two bacterial metabolites enhanced the *Fxr* gene expression in the intestine and liver, and ileal *Shp* gene expression tended to be increased by treatment with the metabolites derived from *B. dorei*.

The findings of this study expand our current knowledge of FXR modulators and bile acids metabolism alteration by bacteria in HFD fed mice. They may provide a new direction to clarify both FXR action pathway and molecular mechanisms of microbe-host interactions. Probiotics are currently used as therapeutic options for many diseases, and thus *B.dorei* and *E.limosum* could be applied as a therapy for bile acids disorders through intestinal specific activation.

5.2 Perspectives

This study for the first time discovered culture supernatants of *B.dorei* and *E.limosum* can be FXR direct modulators by using a stable FXR reporter system. The *in vivo* assessment revealed the bile acid regulation by two bacteria is both gene and tissue specific. The findings of this study expand our current knowledge of FXR modulators and microbiome induced changes of bile acids metabolism in the ileum. They may provide a new direction to clarify both FXR action pathway and molecular mechanisms of microbe-host interactions. Probiotics are currently used as therapeutic options for many diseases, and thus *B.dorei* and *E.limosum* could be applied as a therapy for bile acids disorders through intestine specific activation which may have less toxicity than drugs.

Because the alteration in gut microbiota by FXR-stimulating bacterial metabolites might influence the regulation of FXR target gene expression, this study may be more reasonable if the bile acids composition was investigated. Thus, it is necessary to investigate whether BAs metabolism and gut microbiota are influence by the administration of FXR-stimulatory bacteria or metabolites. On the other hand, I have not identified any bioactive molecules from culture supernatants yet. The cocktail of bacterial culture supernatants make FXR activation much more complicated. Moreover, it is still indispensable to use tissue-specific *Fxr* deficiency mice model to emphasize roles of two bacteria *in vivo* in future work.

Appendix

The results of bacterial cells administration

Mice body weight gain

The metabolic effects of *Fxr* activation with two bacteria in male C57BL/6J mice were evaluated first. The mice receiving PBS, *B. dorei* or *E. limosum* were sacrificed after bacteria gavage for 11 weeks. The mice body weight did not show significant differences when administration of *B. dorei* or *E. limosum*, which are shown in **Figure 1A, B**. At the end of the study, mice were sacrificed after 24 hours fasting, liver weight of each mouse was weighed. From **Figure 1C**, the liver weight of groups given *B. dorei* or *E. limosum* was lower than the control group without significant differences.

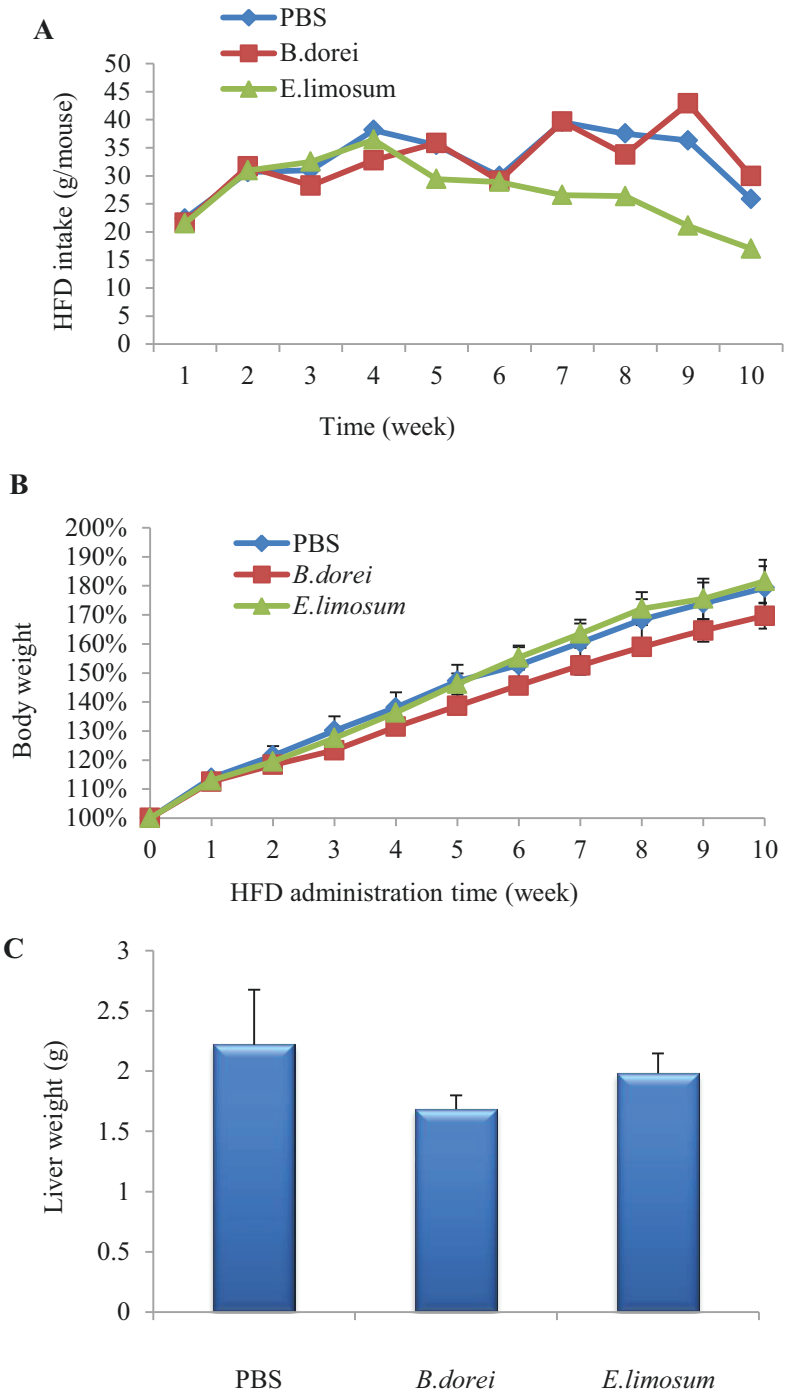


Figure 1 Changes in mice given bacterial cells. (A-B) Mice body weight changes. (C) Liver weight changes. Mice were intragastric administration of *B. dorei* or *E. limosum* for 11 weeks, diet was changed to HFD from second week. Values are mean ± SEM. n=6.

Serum biochemical analysis

Blood was collected from heart after anesthesia and serum were separated for biochemical analysis. However, differences were not detected in two groups given our candidate bacteria compared with control group (**Figure 2**).

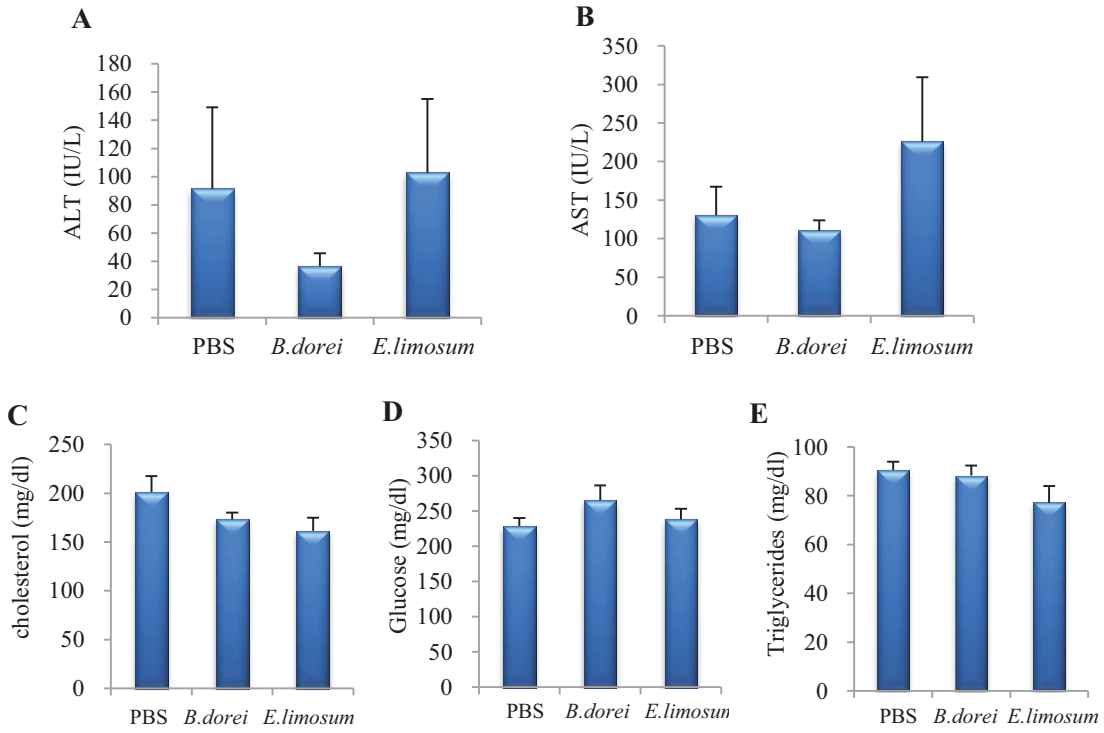


Figure 2 Serum biochemical analysis. Blood was collected from heart after fasting for 24 hours and serum was separated for analysis. Values are mean ± SEM. n=6.

Gene expression in the ileum

Consistent with in vitro results, treatment of *B. dorei* and *E. limosum* induced *Fxr* activity with 1.9 fold and 2.2 fold, respectively, compared to PBS treatment group (**Figure 3A**). *Fxr* targets OSTα was upregulated with 1.4 fold and 1.5 fold (**Figure 3C**), while *Ibat* was increased with 1.5 fold and 1.7 fold as well (**Figure 3E**). Gene *Ibabp* was only altered with 1.5 fold increasing by *E. limosum*. Genes *Fgf15* and *Shp* were

unaffected when either *B. dorei* or *E. limosum* was administrated. These results demonstrated a gene-selective regulation of two bacteria.

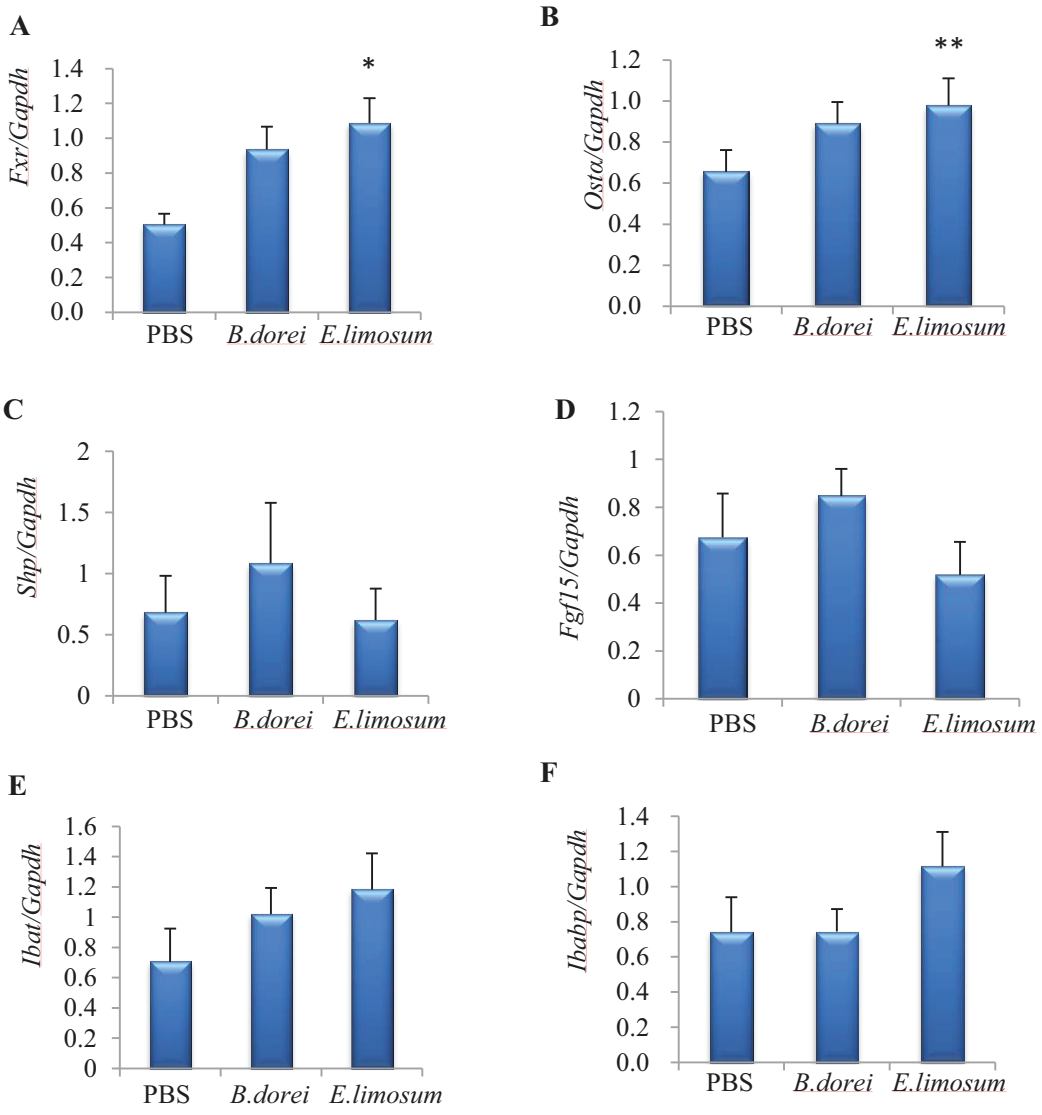


Figure 3 Gene expressions in the small intestine. Values are mean ± SEM; n=6. *P<0.05, **P<0.01, compared to PBS treatment group.

In the small intestine, about 95% of bile acids is reabsorbed via *Ibat* (*Asbt*) from apical side. In the enterocytes, *Fxr* activation increases the expression of *Ibabp* and two transporters, *Ostα* and *Ostβ*, which promote bile acids transport into the portal vein, and finally arrive in the liver. The bile acids function pathway in the small intestine is shown in **Figure 4**.

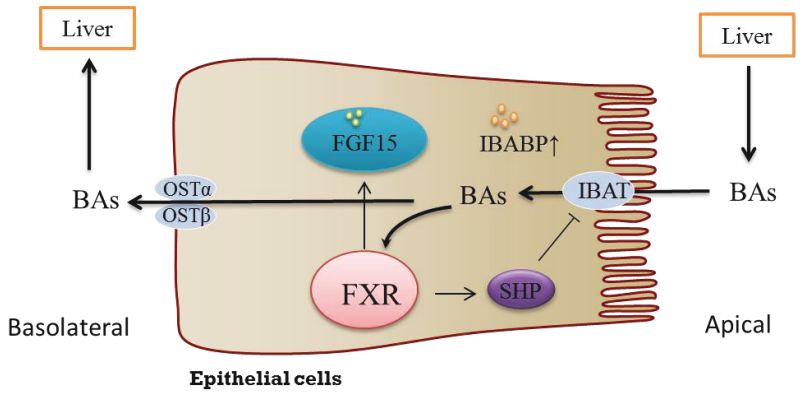


Figure 4 Bile acids pathway in the mouse ileum

According to the results, *Fxr* activity was up-regulated in the ileum by treatment with *B. dorei* and *E. limosum*. In the meantime, *Fxr* targets *Ostα*, *Ibabp* and *Ibat* were positively regulated compared to group given PBS. The increased *Ibat* expression would recycle more bile acids in the small intestine, while elevated *Ostα* would accelerate bile acids transfer to central vein. The results demonstrated that two bacteria may have an important role in protection of intestinal cells from high concentrations of bile acids through facilitating bile acids transport to the liver, which would be helpful in insufficient bile acids excretion in the liver.

It has been believed that *Ibat* is under negative feedback regulation in the mouse [1, 2]. Different from *Fxr* activation by bile acids, transporter *Ibat* was upregulated without statistical difference by two bacteria, and *Shp* expression was unaffected, indicating that a positive feedback regulation is independent of *Shp*. Actually, the expression of *Ibat* is still ongoing controversy and species different. *Ibat* in the rat is unaffected by bile salts [3], while it is under positive feedback regulation in humans [4]. Elegant studies have elucidated *Ibat* can be positive and negative feedback regulated by bile acids [5]. The finding may provide a new direction for *Ibat* function pathway study.

The classic activation of intestinal *Fxr* increases the expression and secretion of human FGF19 or mouse *Fgf15* into the portal vein, resulting bile acids synthesis inhibition in the liver. *Fgf15*, a key regulator involved in bile acids [6] and energy expenditure [7], was not

affected in this study. Many studies suggested that the increased Fgf15 expression lead to alterations in bile acids composition [6, 8, 9], and has an important role in fatty acids synthesis through suppression of the ability of insulin, attributing the effects of weight lost and inflammation reduction through Fxr-Fgf15 pathway [10]. The unaffected Fgf15 expression in this study would partly explain why the mice body weight and liver weight were not decreased significantly.

Gene expression in the colon

The gene expression results in the colon showed *B. dorei* deduced *Fxr* target gene *Ibabp* expression (**Figure 5B**). Gene *Fgf15* expressions were almost eliminated by administration of *B. dorei* and *E. limosum* (**Figure 5D**).

However, the fact that the total colon RNA quality is poor should not be ignored, which would contribute a lot to the gene expression profile in the colon. Acturally, 500 ng RNA was added to each reaction when apply RNA reverse PCR compared with 1000 ng RNA.

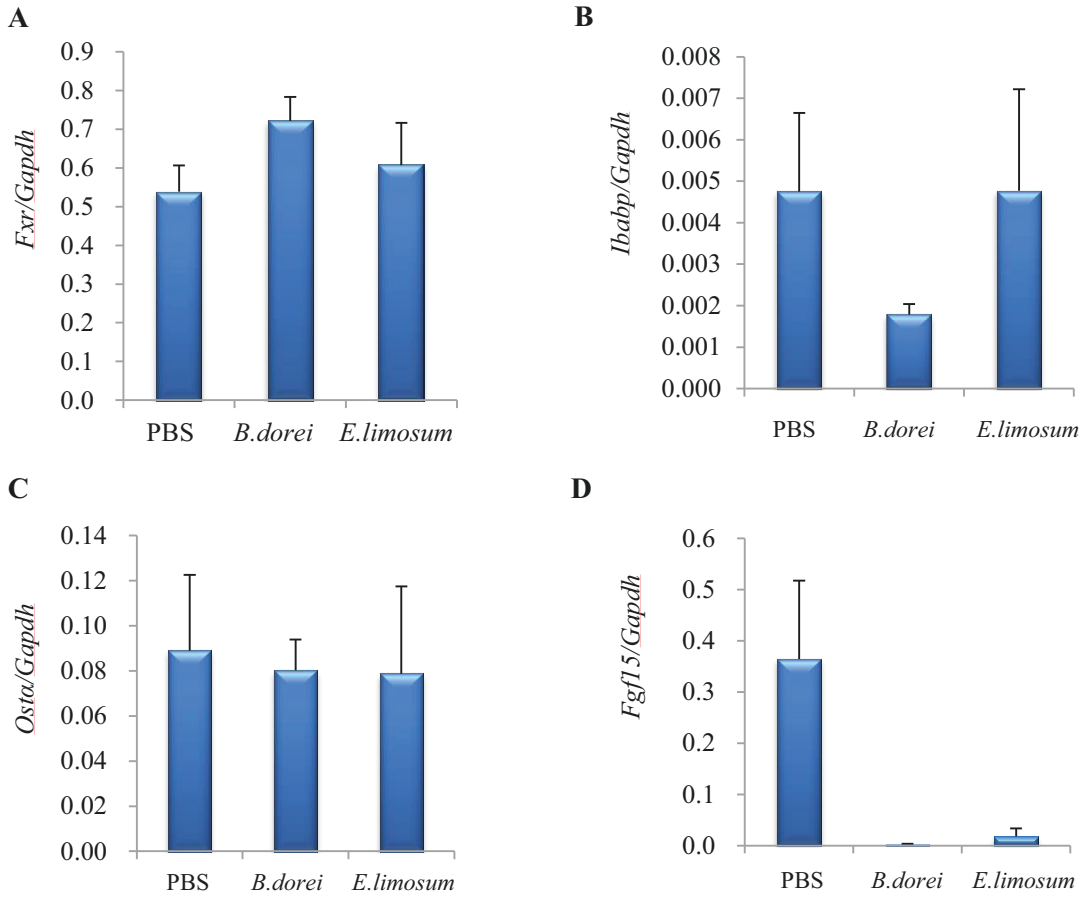


Figure 5 Gene expressions in colon. Values are mean ± SEM; n=4-6. *P<0.05, **P<0.01, compared to PBS group.

Gene expression in the liver

According to our results (**Figure 6**), the gene *Shp* was up-regulated with 3.5 fold (**Figure 6B**), while *Cyp7b1* expression was down-regulated with 2.3 fold (**Figure 6E**) in the group given *E. limosum* compared to the control group without significant differences. However, *Fxr* and other *Fxr* targets including *Bsep*, *Cyp7a1*, *Cyp8a1* and *Ntcp* were not affected. Administration of *B. dorei* did not altered *Fxr* and its target genes in the liver.

As well as the gene-selective role in the ileum, the gene specific regulation was discovered in the liver. My results displayed that treatment with *E.limosum* elevated SHP expression but without hepatic *Fxr* activation, which revealed *Shp* inducement is in an *Fxr*-independent manner. The similar result that *Shp* mRNA was highly induced when

PGC-1α was overexpressed in *Fxr*-null hepatocytes was reported, which indicates that *Shp* expression in an *Fxr*-independent manner [11]. The induction of *Shp* can in turn inhibit *Cyp*7b1 expression. A study gives the evidence that no significant difference was detected in *Cyp8b1* gene repression by bile acids between the *Fxr* deficiency mice and wild type mice indicating the suppression of *Cyp8b1* is *Fxr*-independent [12]. Thus, *E.limosum* would relieve bile acids accumulation in the liver.

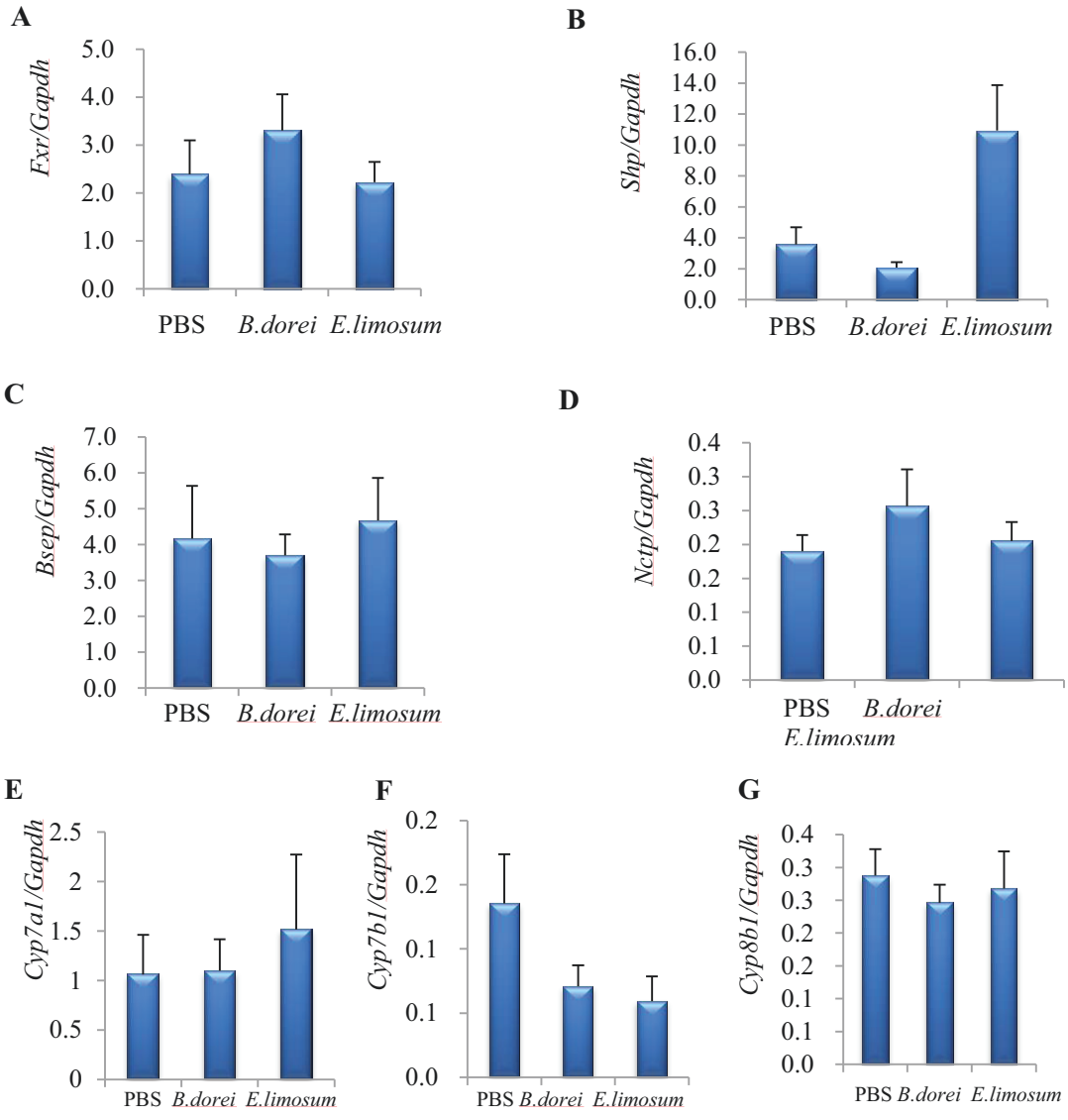


Figure 6 Gene expressions in the liver. Values are mean ± SEM; n=6.

Histology assessment of liver

HFD induced macrovesicular steatosis with large and small fat droplet (green arrow), mixed inflammatory cells infiltration (blue arrow) and hepatocyte ballooning (red arrow) in HE stain (**Figure 7**). Overall, there were a little more severe steatosis and inflammatory cell infiltration in group given *E. limosum*.

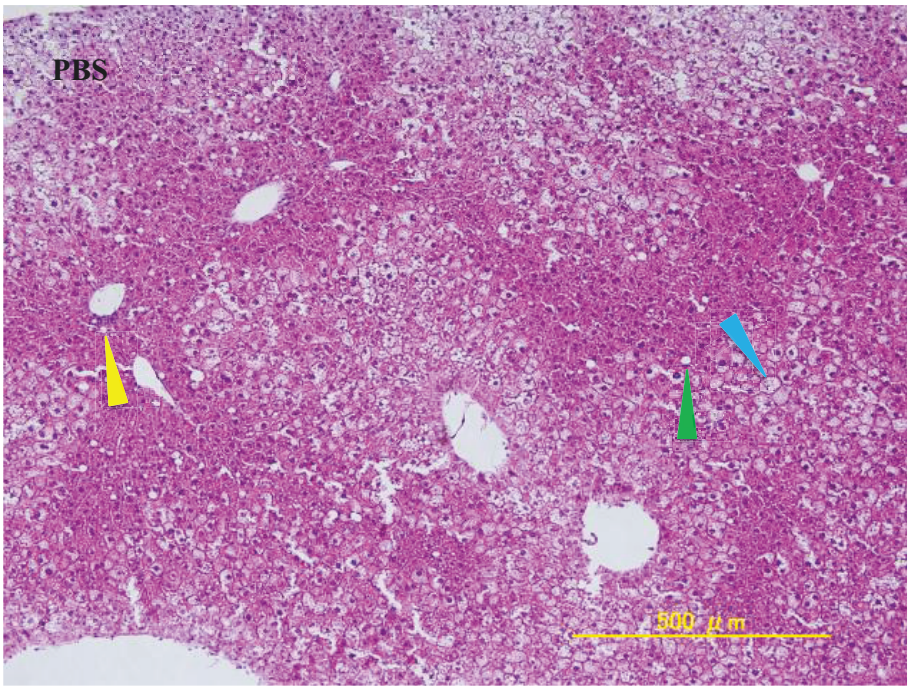


Figure 7 HE stain of liver. Green arrow means large and small fat droplet, yellow arrow means mixed inflammatory cells infiltration and blue arrow means hepatocyte ballooning. *(Continued on the following page)*

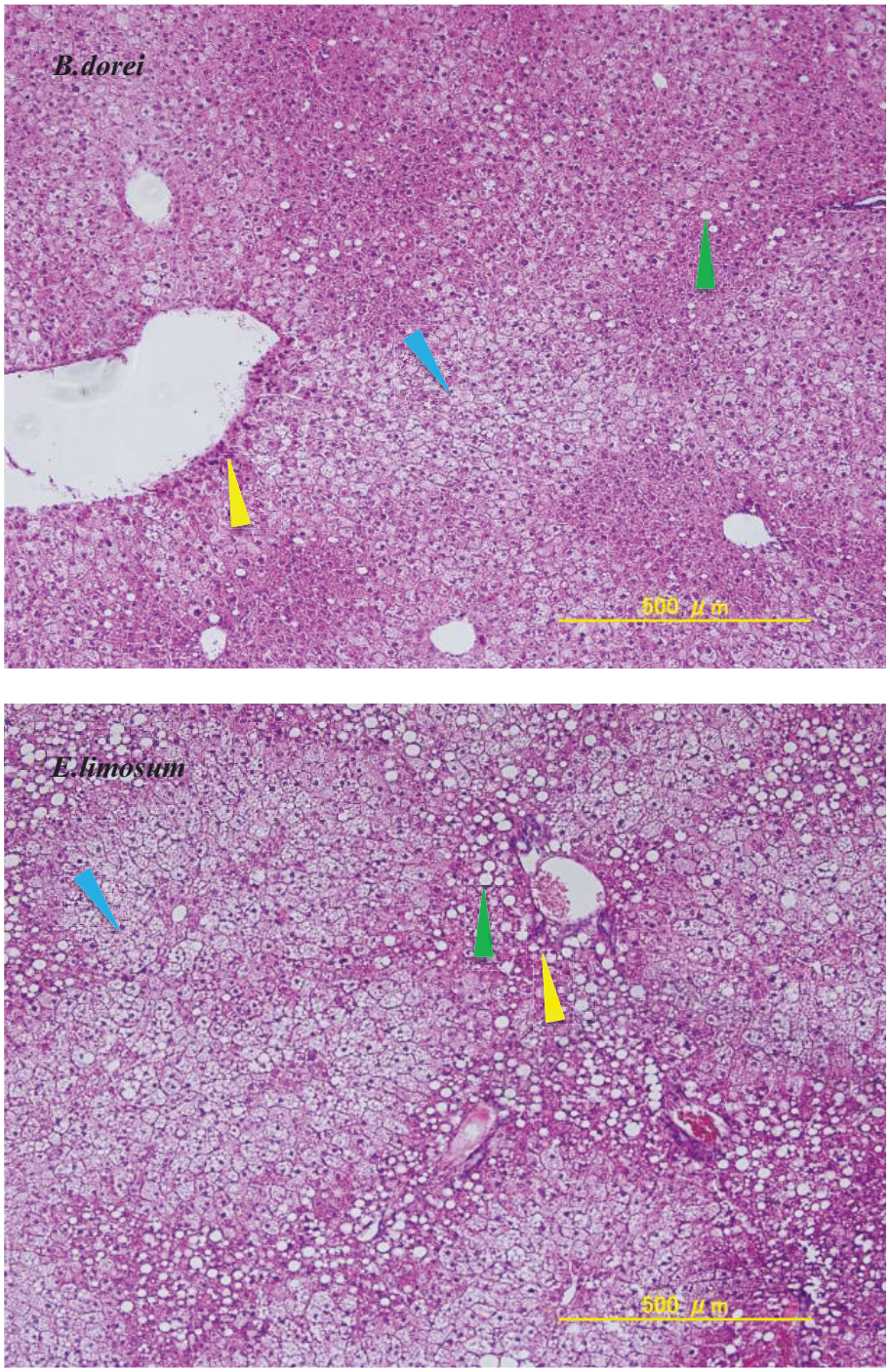
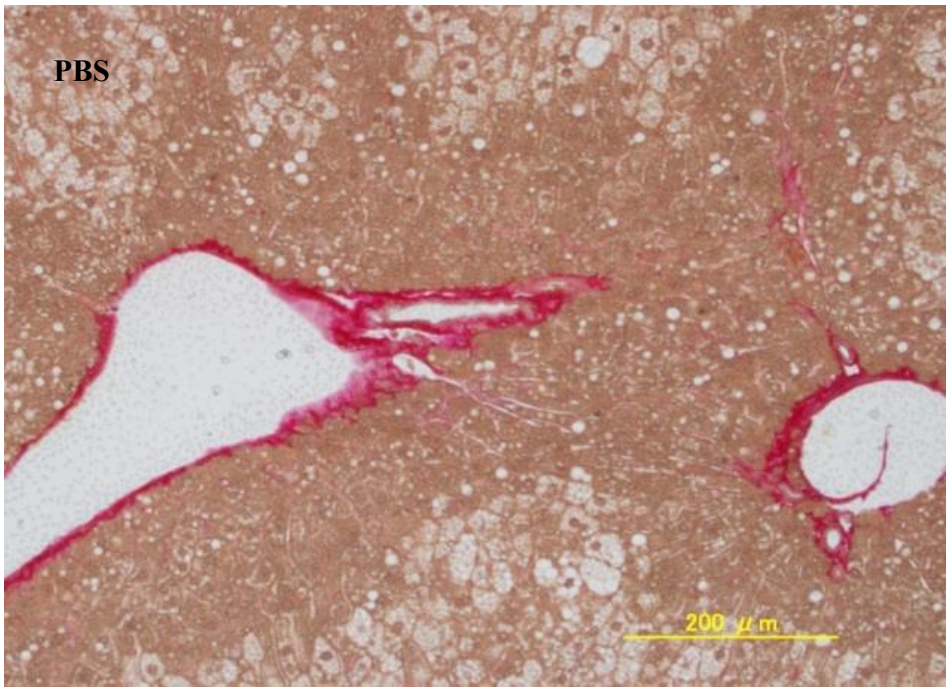


Figure 7 HE stain of liver. Green arrow means large and small fat droplet, yellow arrow means mixed inflammatory cells infiltration and blue arrow means hepatocyte ballooning.

HFD feed mice presented hepatic fibrosis as well. As illustrated in **Figure 8**, collagen was stained in red and all three groups showed hepatic fibrosis. More fibrosis severity was detected in the group that given *E. limosum* than other two groups.



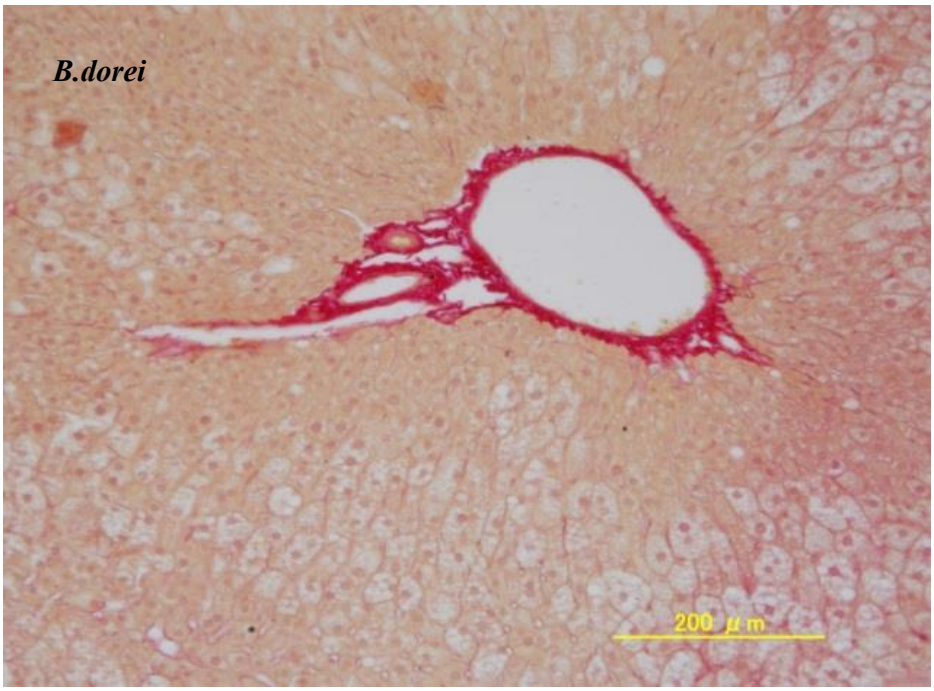


Figure 8 Liver picrosirius red stain. *(Continued on the following page)*

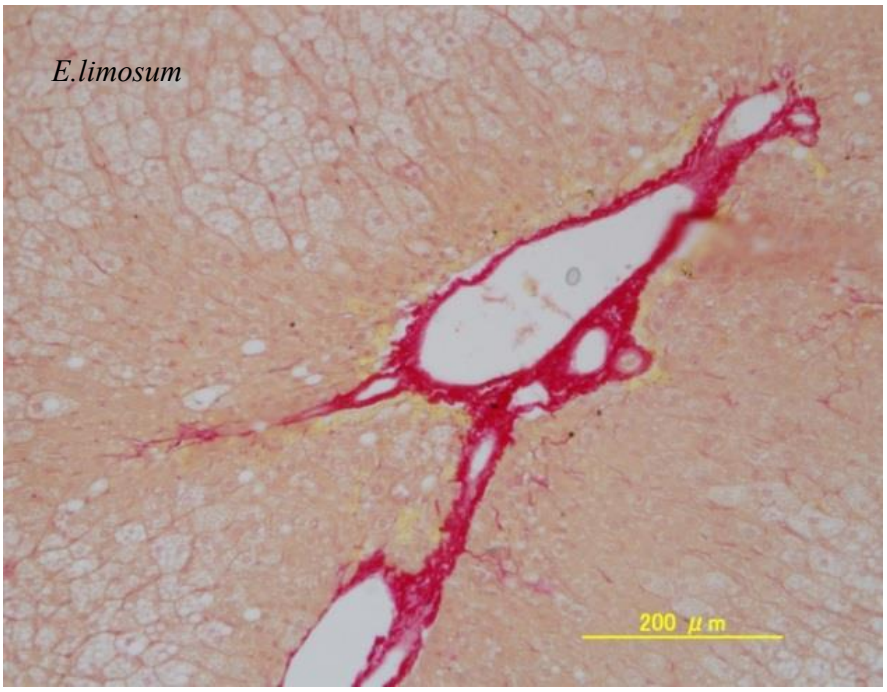


Figure 8 Liver picrosirius red stain.

Overall, although we can compare the differences among groups given different treatment, the results can be more reasonable if a control group that only fed standard diet is included. Considered not much positive results obtained, and long term *B. dorei* tended to help mice body weight gain loss, the promising achievements would be gained if mice receive increased bacteria quality.

Renference

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12. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. **Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis**. *Cell*. 2000,**102**:731-44.

Relevant results

Standard curve (Real-time PCR)

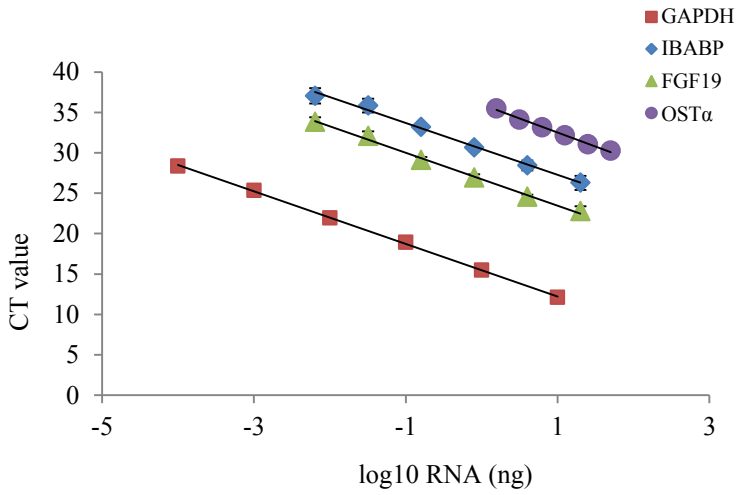


Figure 9 Primer standard curve in SW480 FXR reporter system

Table 1 Primer standard curve information in SW480 FXR reporter system

Gene	Equation	Coefficiency	Primer efficiency
GAPDH	y = -3.2552x + 15.473	0.9995	102.86%
IBABP	y = -3.2119x + 30.476	0.9934	104.81%
FGF19	y = -3.2742x + 26.744	0.9956	102.03%
OSTα	y = -3.458x + 35.973	0.9945	94.62%

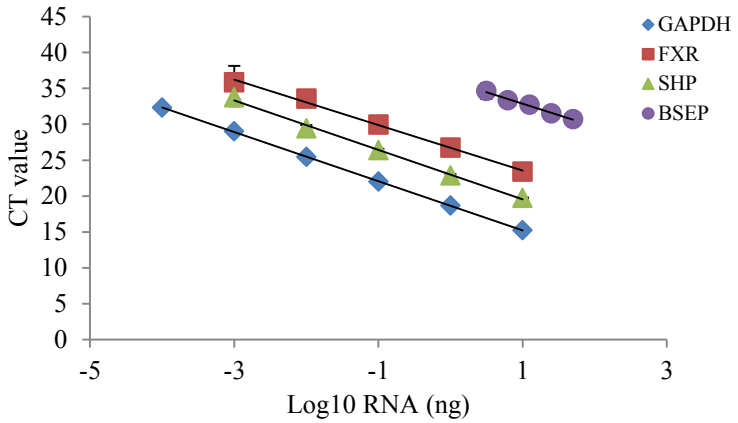


Figure 10 Primer standard curve in HepG2 cells

Table 2 Primer standard curve information in HepG2 cells

Gene	Equation	Coefficience	Primer efficiency
GAPDH	y = -3.4286x + 18.635	0.9999	95.73%
FXR	y = -3.1667x + 26.729	0.9956	106.91%
SHP	y = -3.453x + 22.975	0.9963	94.81%
BSEP	y = -3.209x + 36.088	0.9902	104.94%

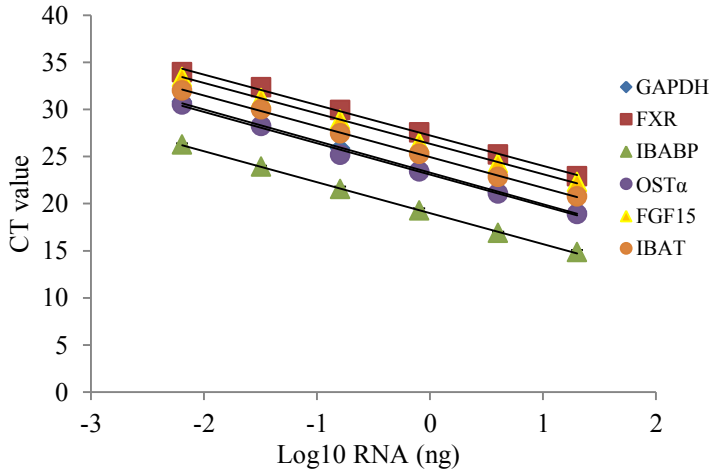


Figure 11 Primer standard curve in the ileum

Table 3 Primer standard curve information in the ileum

Gene	Equation	Coefficience	Primer efficiency
GAPDH	y = -3.356x + 23.296	0.9988	98.60%
FXR	y = -3.2265x + 27.226	0.8872	104.14%
IBABP	y = -3.2844x + 19.003	0.9996	101.59%
OSTα	y = -3.3246x + 23.083	0.9961	99.89%
GFGF15	y = -3.2265x + 26.347	0.9993	104.14%
IBAT	y = -3.2647x + 24.954	0.9992	102.44%

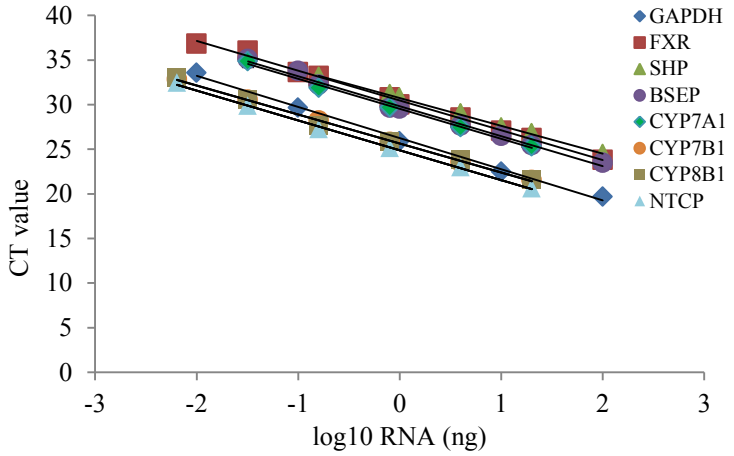


Figure 12 Primer standard curve in the liver

Table 3 Primer standard curve information in the liver

Gene	Equation	Coefficience	Primer efficiency
GAPDH	y = -3.4887x + 26.265	0.996	93.48%
FXR	y = -3.3368x + 30.46	0.996	99.38%
SHP	y = -3.1138x + 30.746	0.9981	109.48%
BSEP	y = -3.3578x + 29.836	0.9921	98.52%
CYP7A1	y = -3.3645x + 29.517	0.9959	88.08%
CYP7B1	y = -3.2674x + 25.63	0.9994	102.33%
CYP8B1	y = -3.2442x + 25.646	0.9951	103.35%
NTCP	y = -3.3451x + 24.896	0.9983	99.04%

The FXR stimulatory potentials by palmitic acid

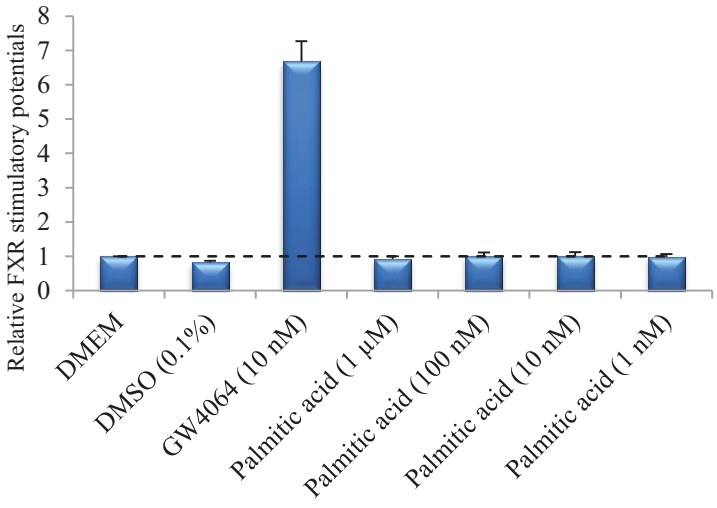
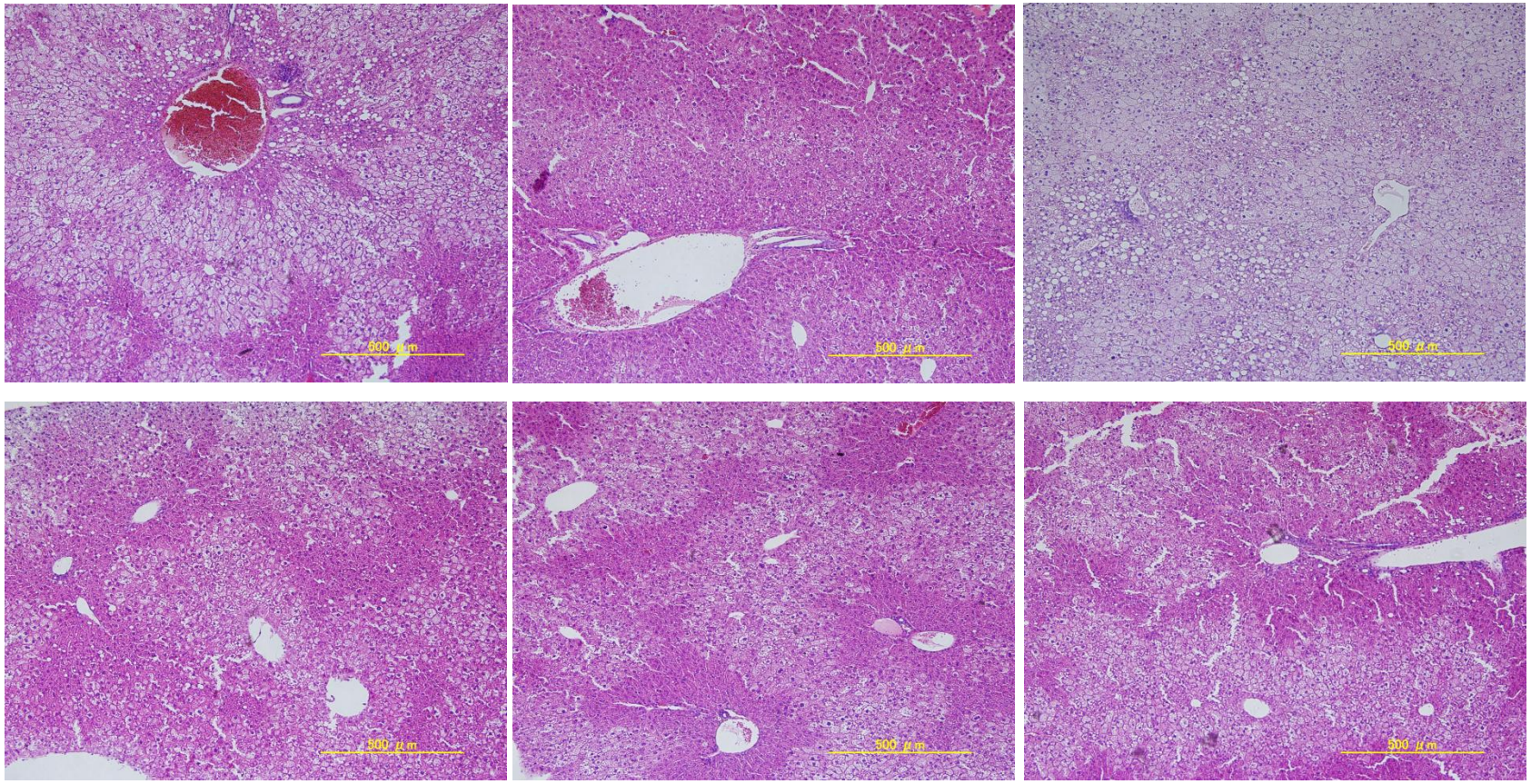


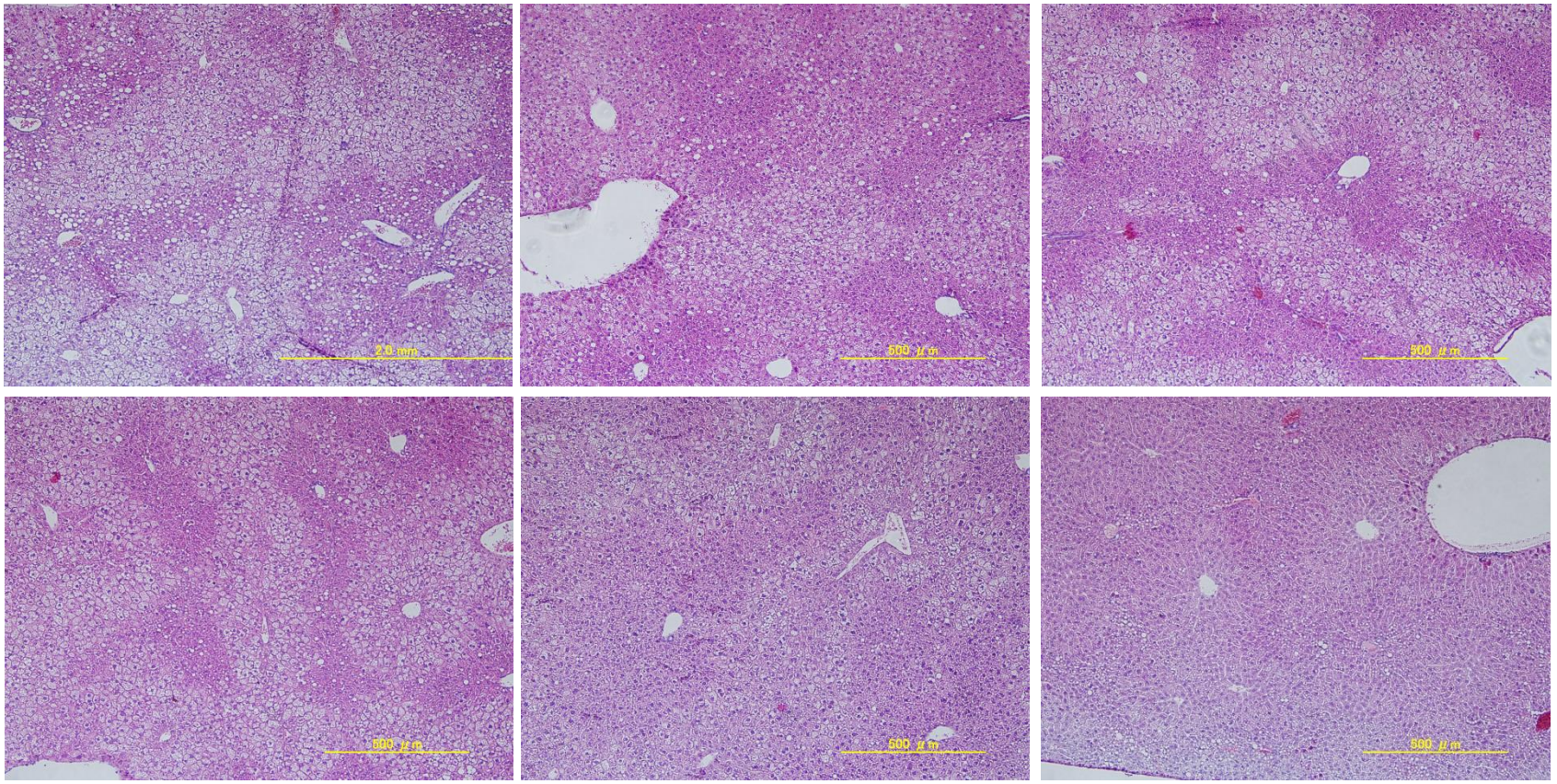
Figure 13 The Relative FXR stimulatory potentials by palmitic acid

HE stain



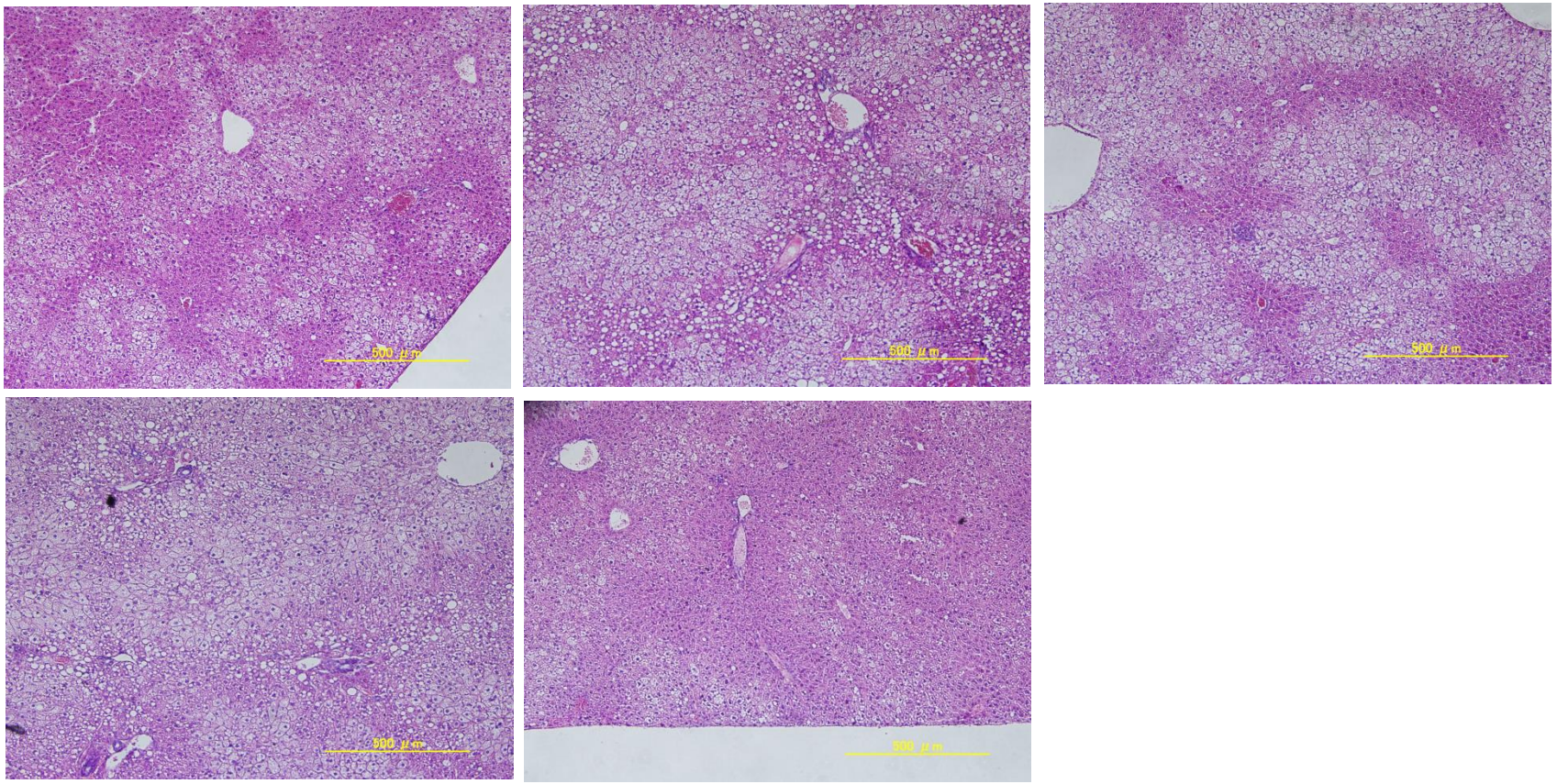
HFD-PBS

Figure 14 HE stain of liver tissue (Bacteria administration). The number means different mice. *(Continued on the following page)*



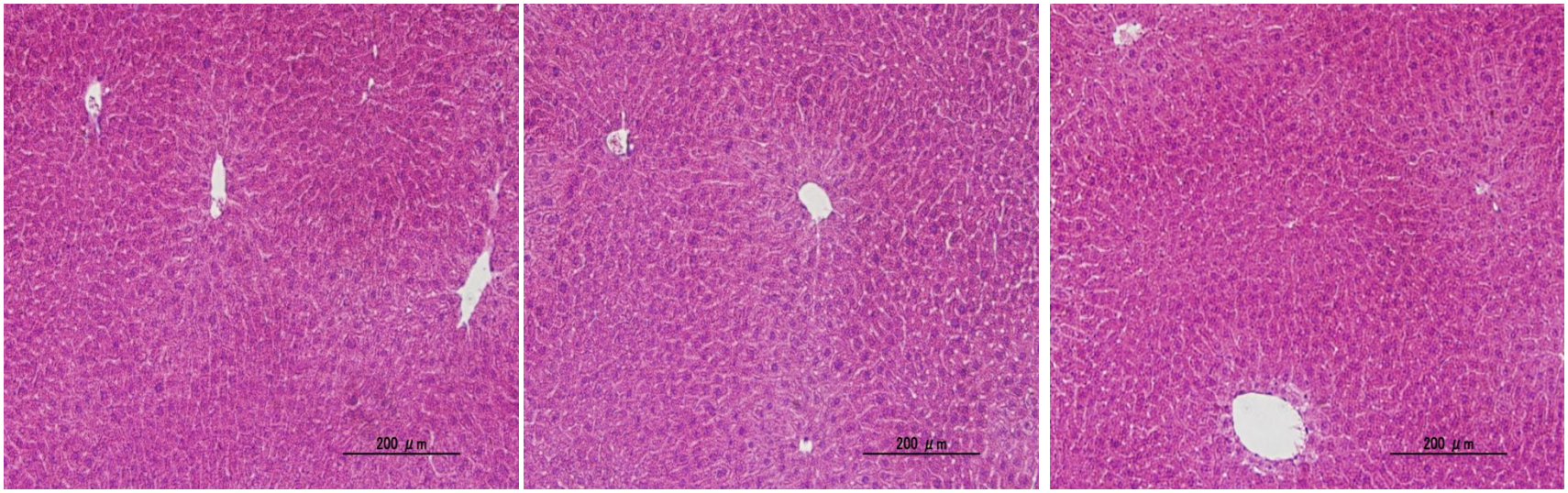
HFD-*B.dorei*

Figure 14 HE stain of liver tissue (Bacteria administration). The number means different mice. *(Continued on the following page)*



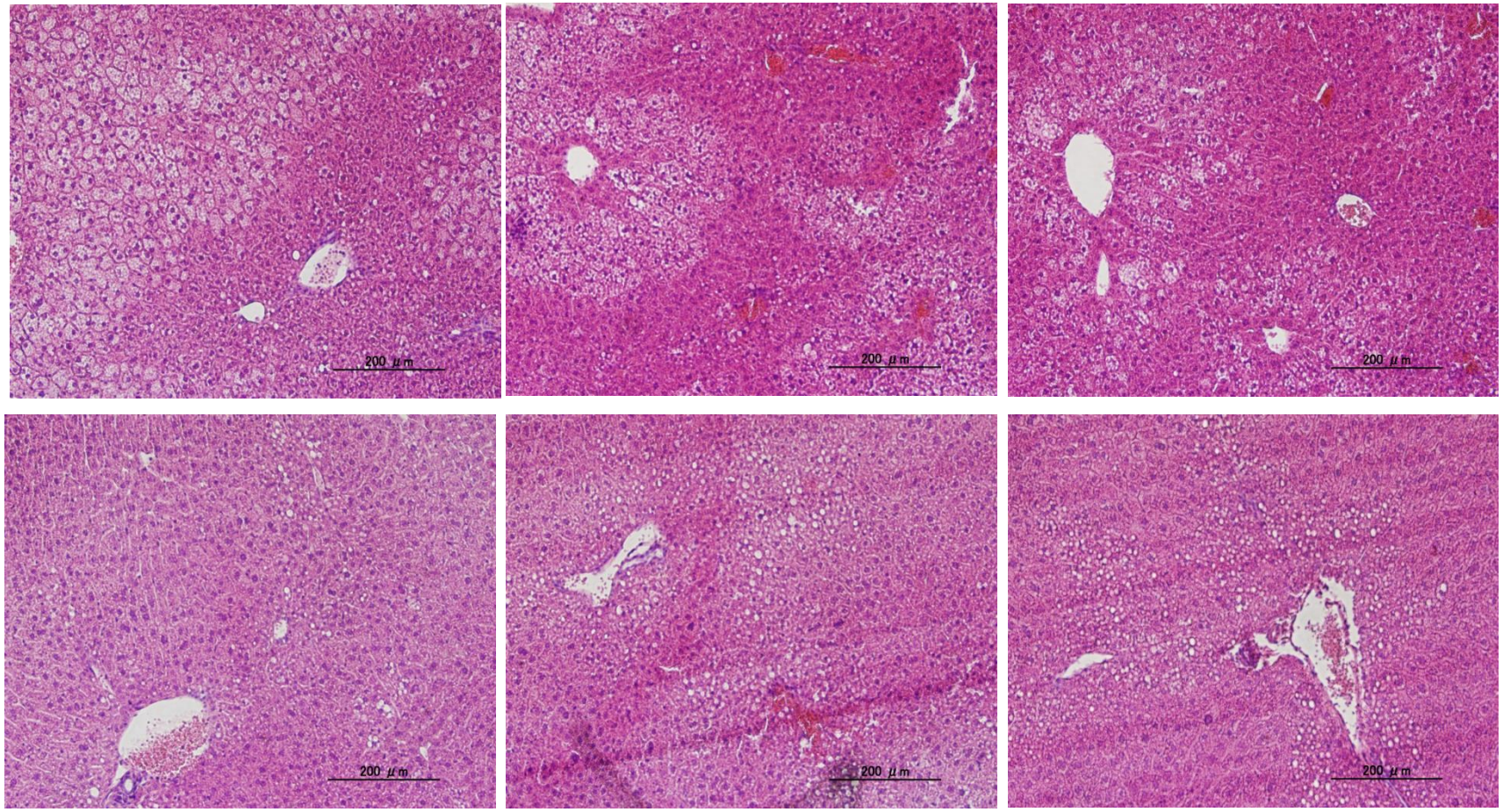
HFD-*E.limosum*

Figure 14 HE stain of liver tissue (Bacteria administration). The number means different mice.



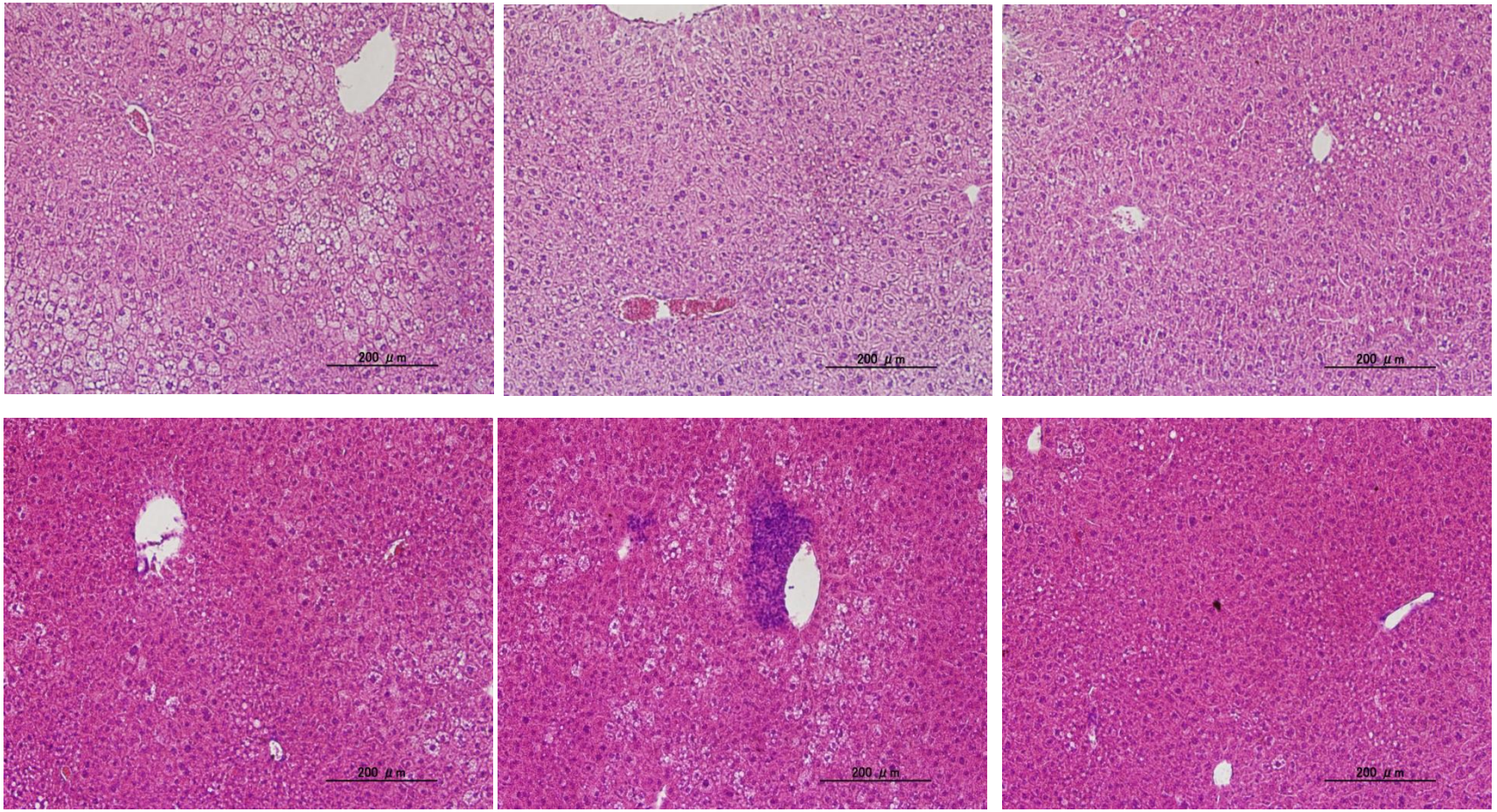
STD-PBS

Figure 15 HE stain of liver tissue (Bacterial culture supernatants administration). Representative images of each condition are shown in **Figure 4.5**. *(Continued on the following page)*



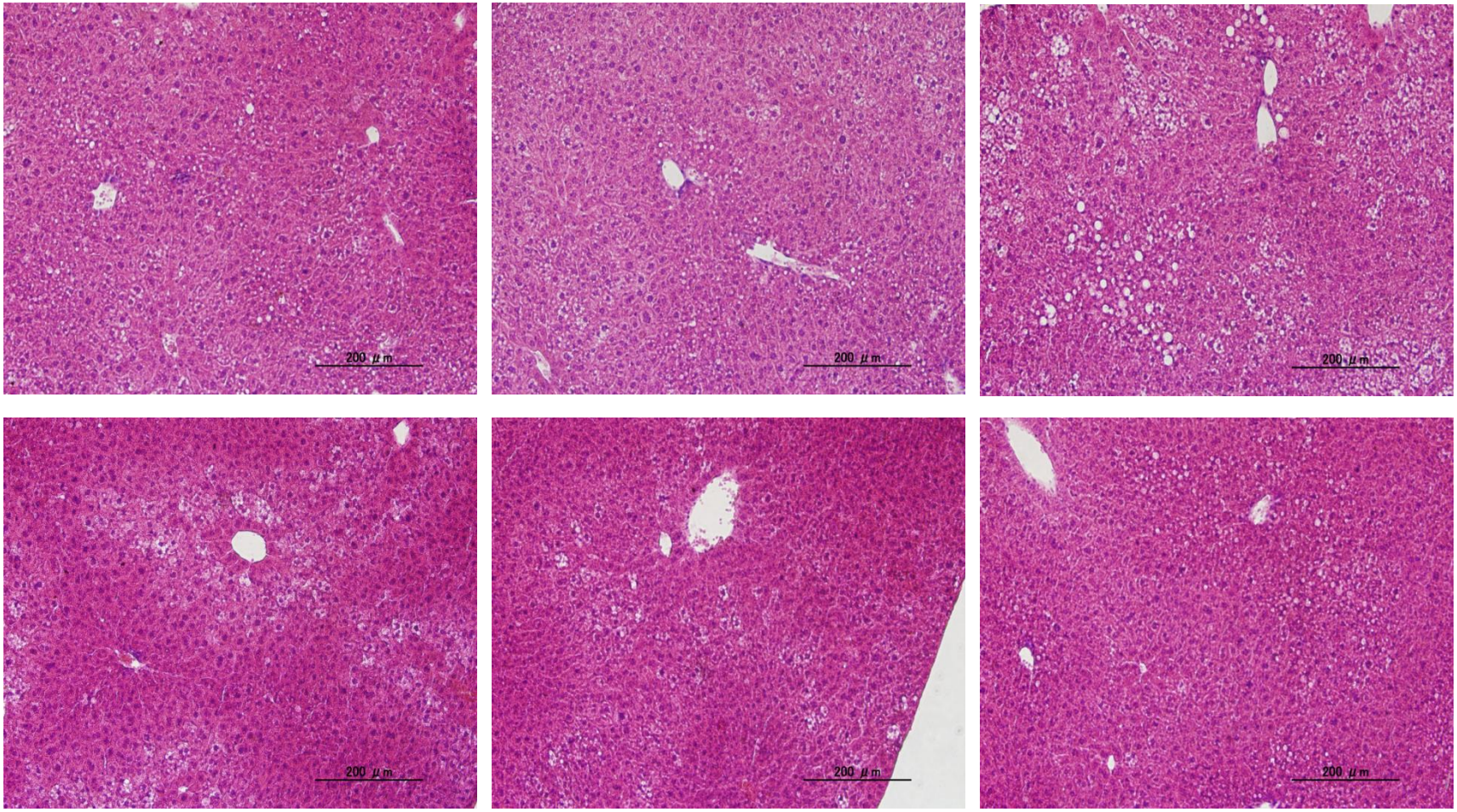
HFD-PBS

Figure 15 HE stain of liver tissue (Bacterial culture supernatants administration). Representative images of each condition are shown in **Figure 4.5**. *(Continued on the following page)*



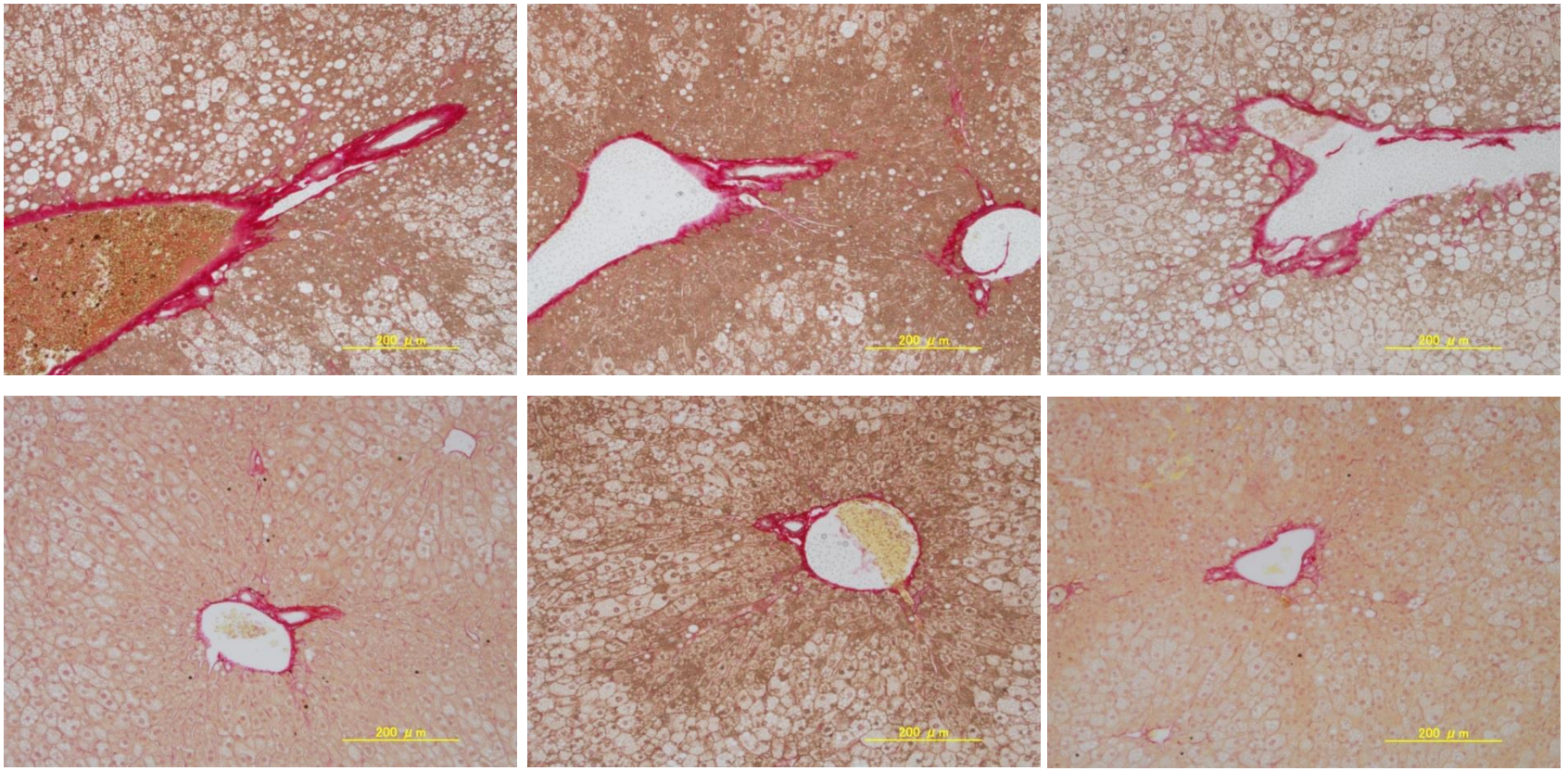
HFD-*B.dorei*

Figure 15 HE stain of liver tissue (Bacterial culture supernatants administration). Representative images of each condition are shown in **Figure 4.5**. *(Continued on the following page)*



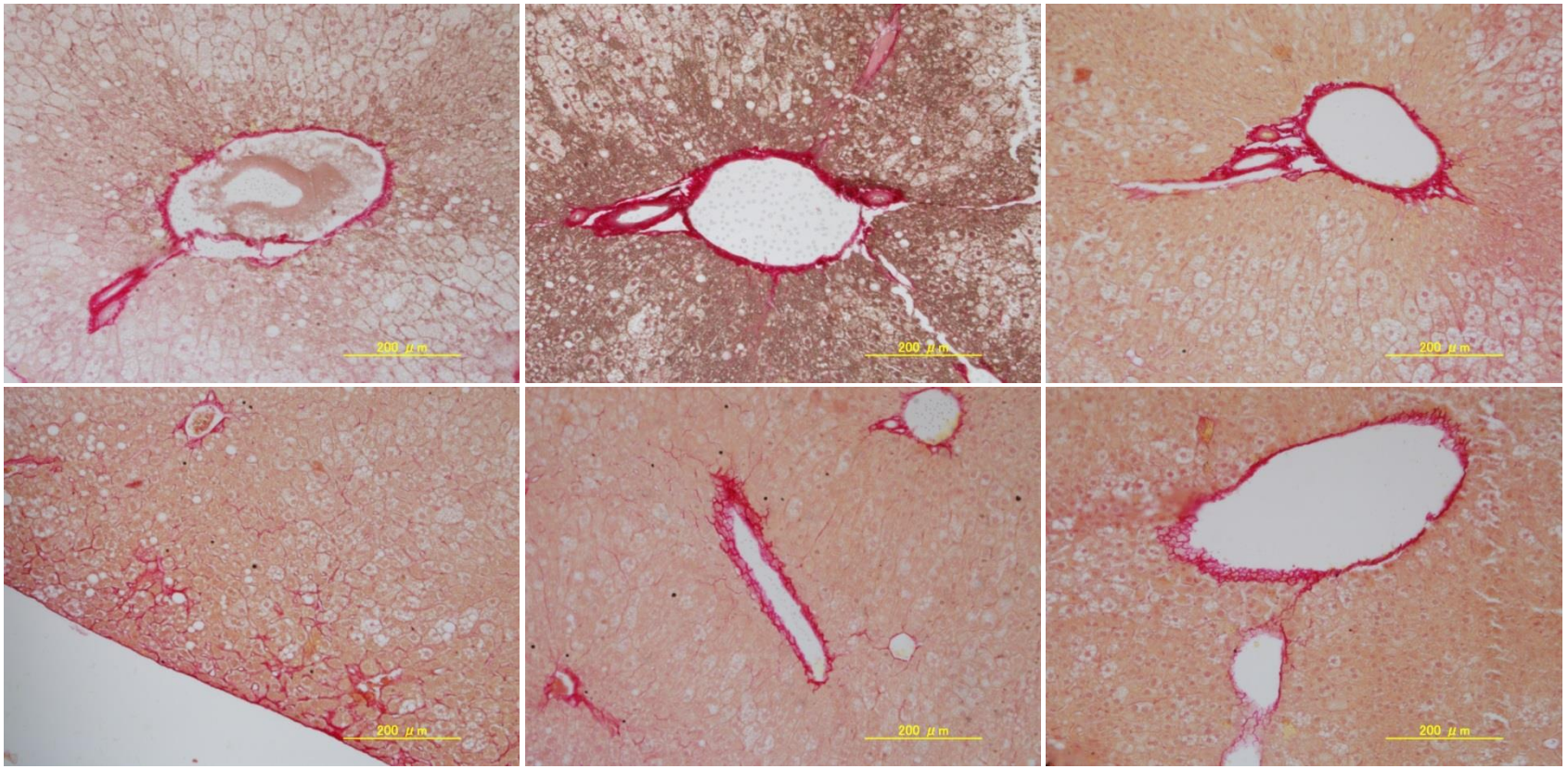
HFD-*E.limosum*

Figure 15 HE stain of liver tissue (Bacterial culture supernatants administration). Representative images of each condition are shown in **Figure 4.5**.



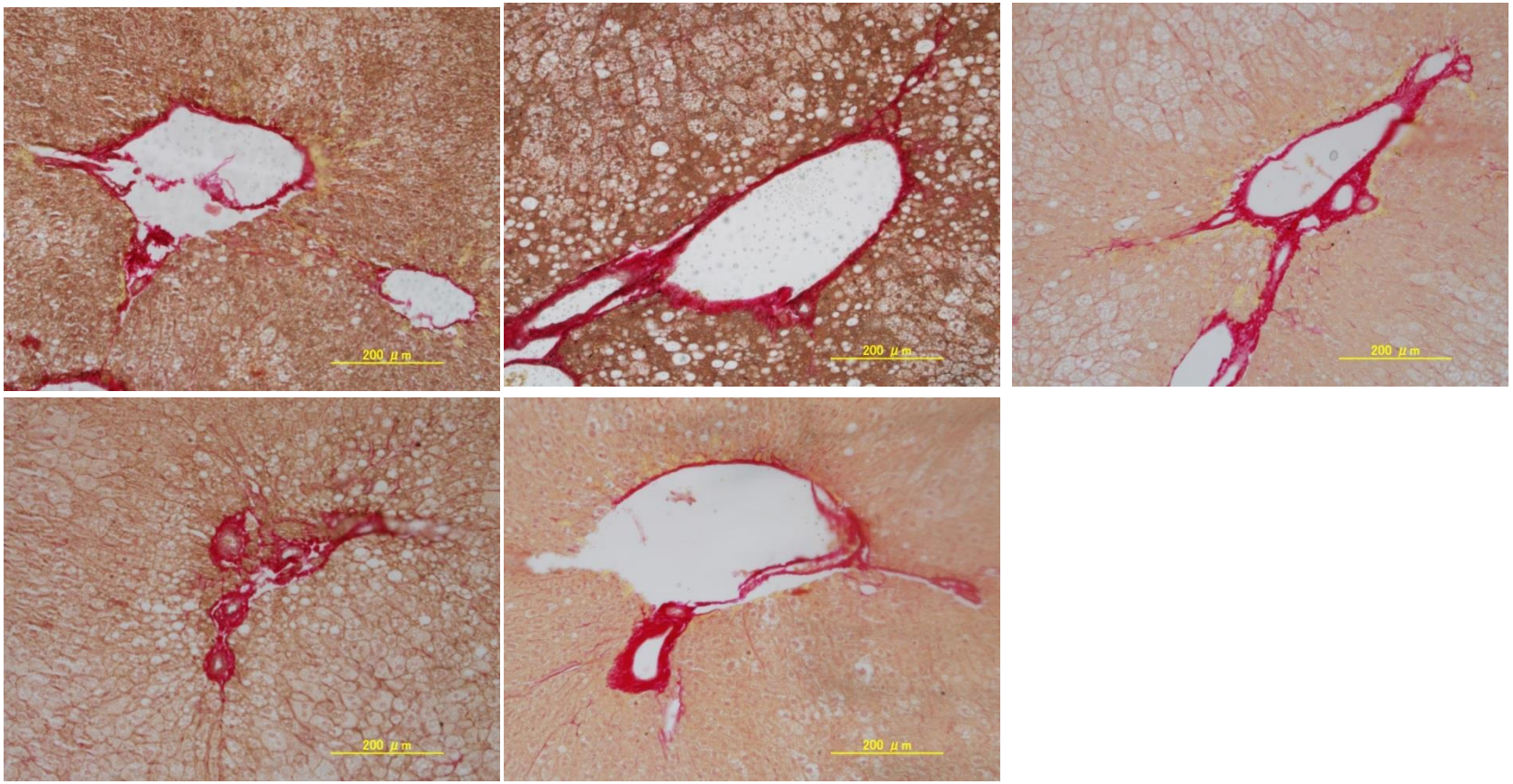
HFD-PBS

Figure 16 pocrosiris red stain of liver tissue (Bacteria administration). *(Continued on the following page)*



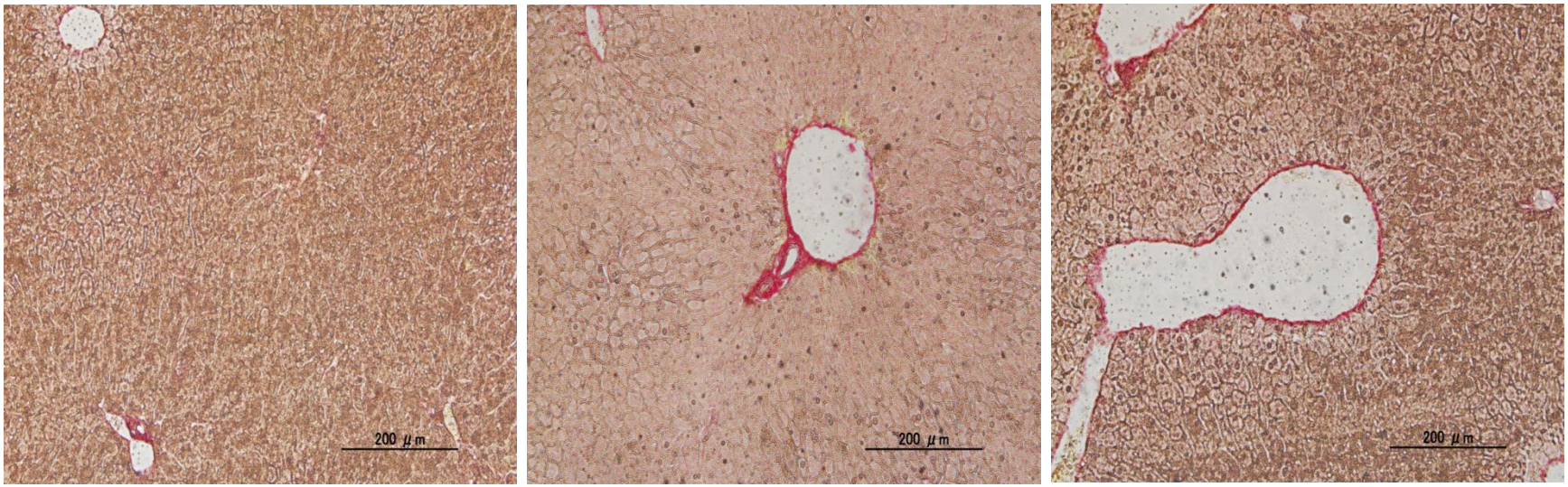
HFD-*B.dorei*

Figure 16 pocrosiris red stain of liver tissue (Bacteria administration). *(Continued on the following page)*



HFD-*E.limosum*

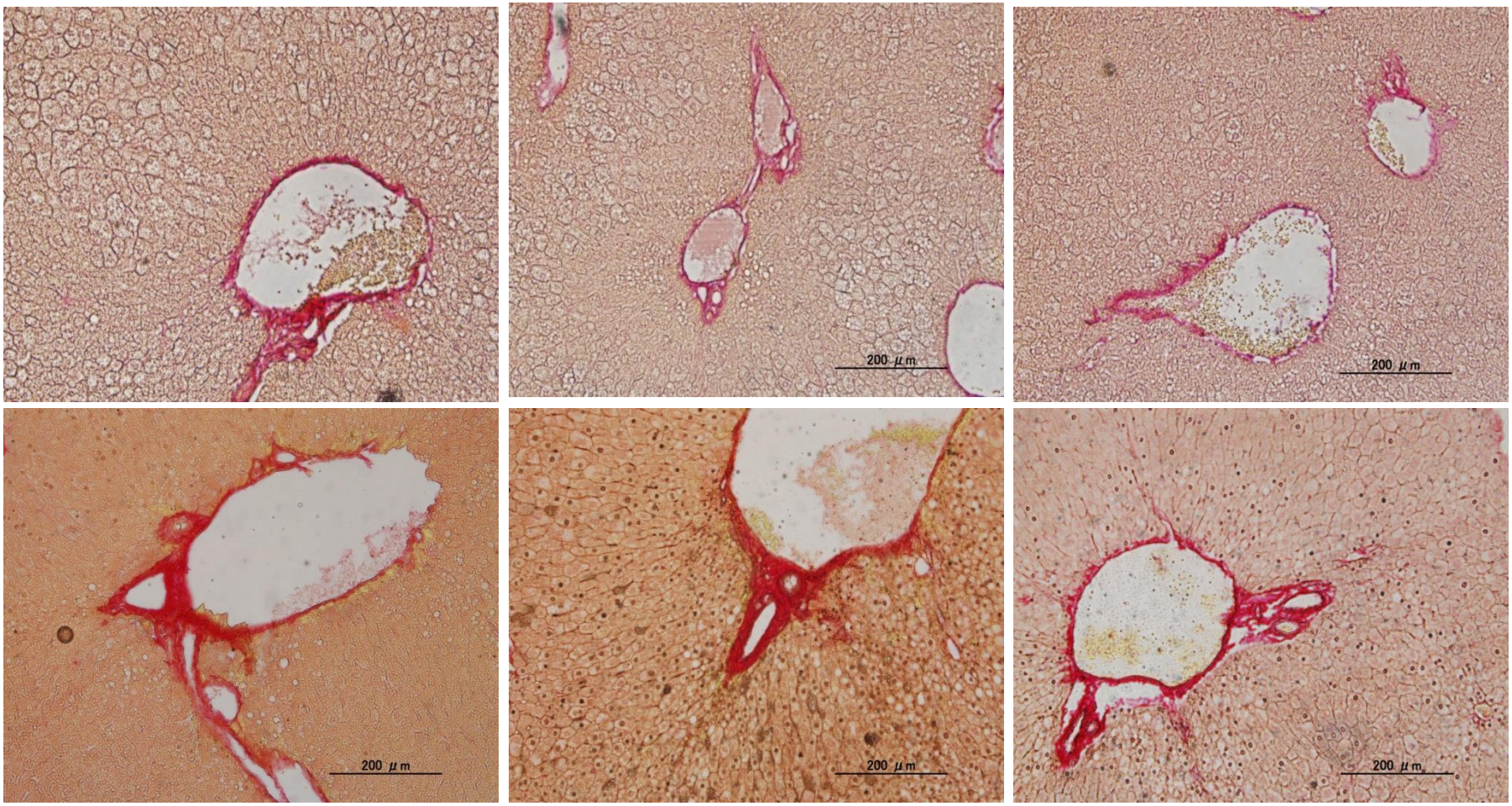
Figure 16 pocrosiris red stain of liver tissue (Bacteria administration).



STD-PBS

Figure 17 pocrosiris red stain of liver tissue (Bacterial culture supernatatns administration). Representative images of each condition are shown in **Figure 4.6**.

(Continued on the following page)



HFD-PBS

Figure 17 pocrosiris red stain of liver tissue (Bacterial culture supernatatns administration). Representative images of each condition are shown in **Figure 4.6**.

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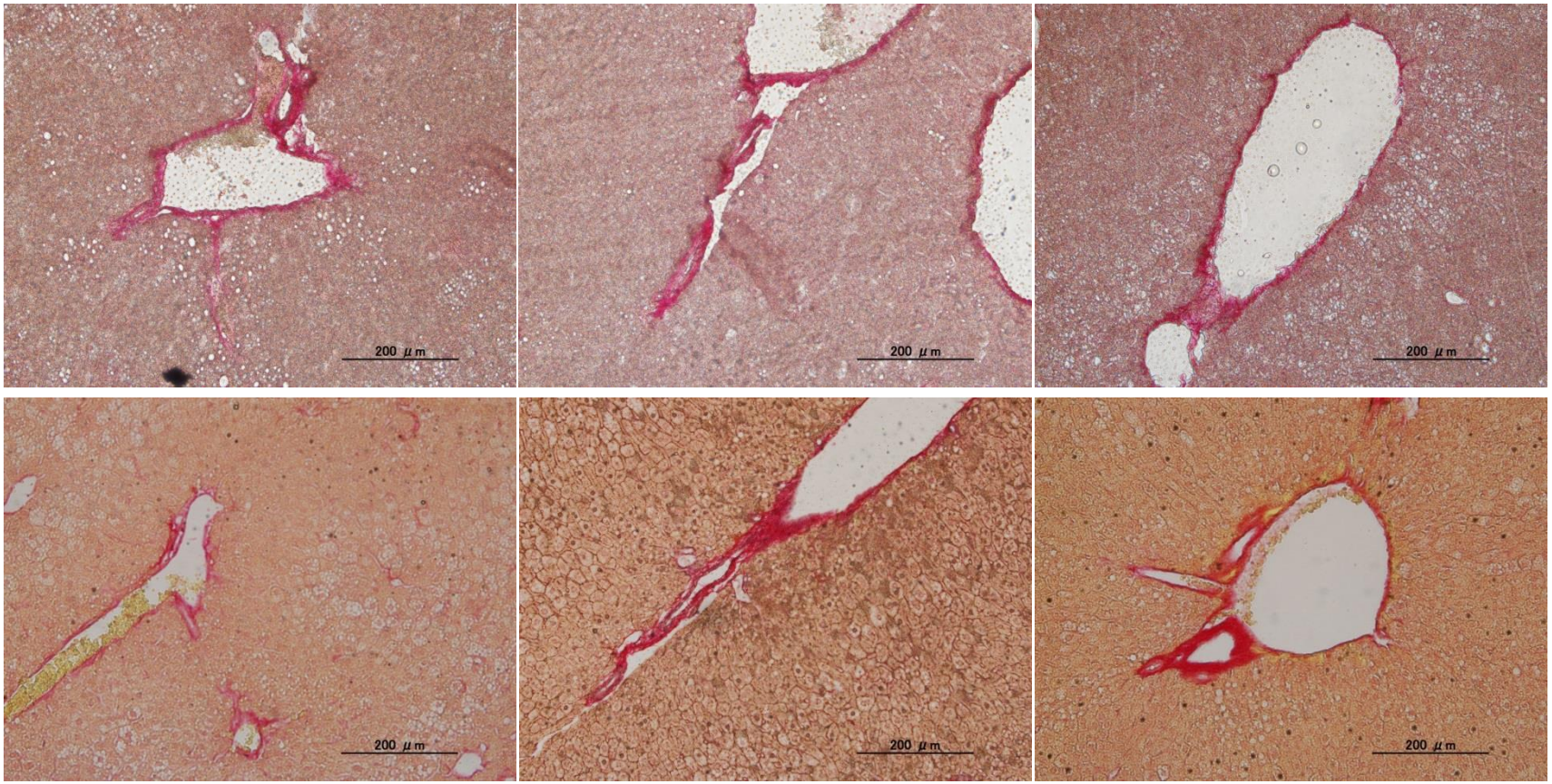
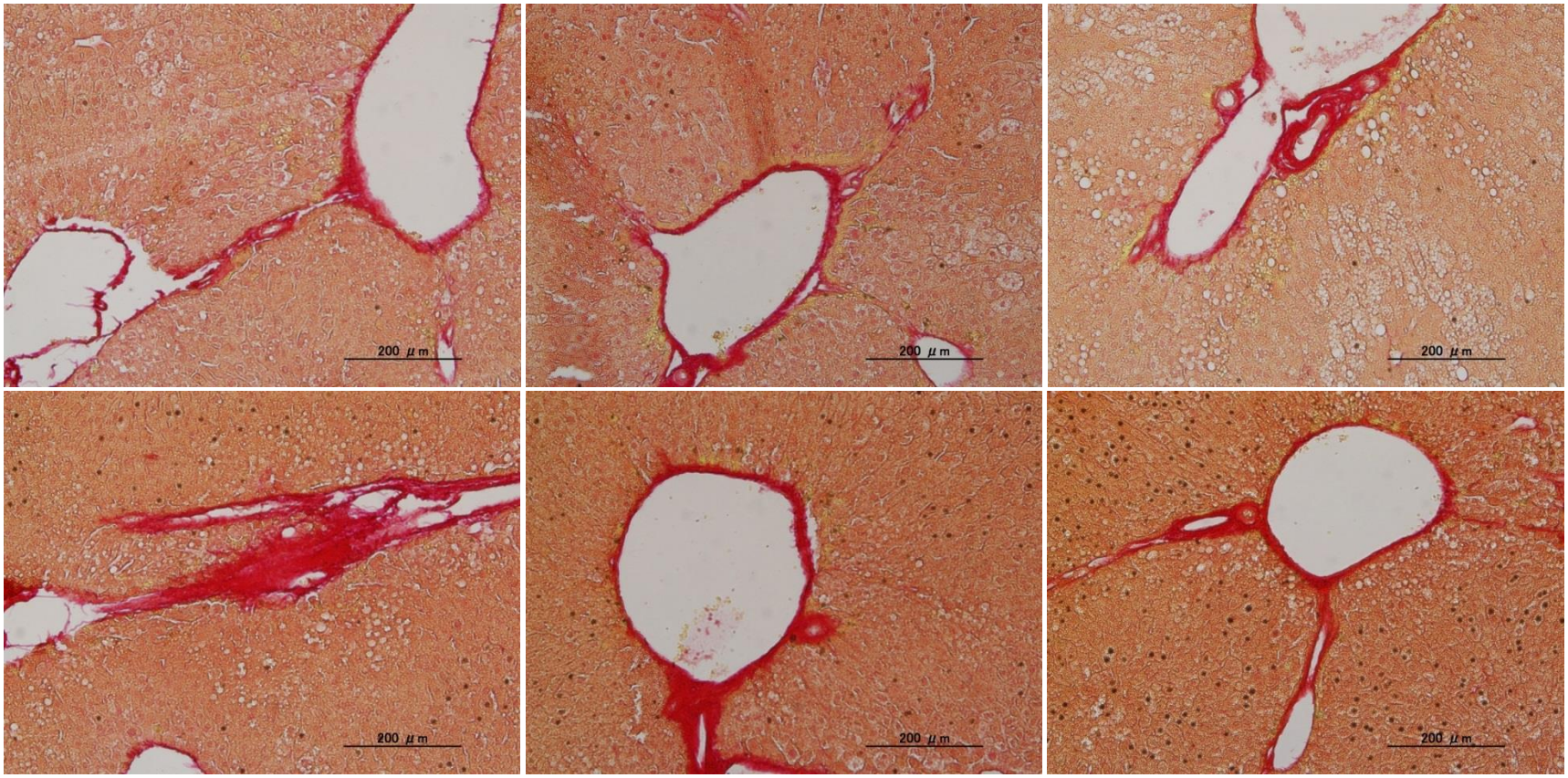


HFD-*B.dorei*

Figure 17 pocrosiris red stain of liver tissue (Bacterial culture supernatatns administration). Representative images of each condition are shown in **Figure 4.6**.
(Continued on the following page)



HFD-*E. limosum*

Figure 17 pocrosiris red stain of liver tissue (Bacterial culture supernatatns administration). Representative images of each condition are shown in **Figure 4.6**.

Synopsis of the thesis

Since the discovery of farnesoid X receptor (FXR) as a transcriptional sensor of bile acids, FXR has displayed a key role in regulation of bile acid homeostasis, cholesterol, glucose, lipid metabolism, hepatic regeneration, inflammation response. Therefore, FXR is a potential drug therapeutic target for a number of metabolic disorders. Up to now, many synthetic or natural extracts have been turned out to be FXR ligands or modulators, which have shown proofs in regulation of FXR relevant diseases. However, the underlying FXR function mechanism is still complicated. Indeed, many apparent discrepancies or even complete opposites often appeared due to the varied experimental methods. Consequently, FXR activation may control metabolic pathways though disparate modulations. FXR modulation has discrepant effects on certain metabolic pathways. The uses of FXR agonists for clinic treatment are still under estimate. The development of tissue-specific or gene-selective FXR modulators which can be easily absorbed after oral administration, may provide the appropriate applications to determine the physiological benefit of FXR modulators, and elucidate the complex FXR action pathways.

It is widely realized gut bacteria protect against obesity and insulin resistance, attenuate inflammation and restore colon homeostasis. Mouse studies indicated that there is a connection between gut microbiota and FXR function. Bile acid levels were reduced in the gallbladder and small intestine in the presence of gut microbiota compared to germ free mouse. Intestinal microbiome regulates bile acid homeostasis by altering bile acids composition resulting in FXR activation in intestine and liver. In addition, a recent study has shown a functional FXR activity is necessary for the probiotic VSL#3 to exert its activity on bile acid excretion and neo-synthesis in mouse. Asking how intestinal microbiome affects relevant diseases via FXR activation, the work would be easier by using individual bacterial strains due to complexity of whole gut microbiome.

Thus, our goal is to identify individual bacterial strains or their products which may act as FXR modulators. Meanwhile, by screening FXR stimulating bacteria, better understanding in FXR action pathways and mechanism of bacteria-host crosstalk can be expected.

In **chapter 1**, the background and previous studies related with this study were summarized, and the purpose of this study was described.

In **chapter 2**, in order to screen FXR modulators, a stable FXR reporter gene system was obtained. First, a FXR expression vector EX-T0601-M02 was transfected into human colorectal carcinoma cell line SW480. Then, a DNA fragment containing four copies of the FXR element (FXRE) from the phospholipid transfer protein promoter was ligated into a pGL4.27 vector to form pGL4-4×FXRE-*luc* vector. Finally, it was further transfected into SW480 FXR expression cells to construct FXR reporter cell line under G418 and hygromycin B selection. Luciferase activity of each colony was determined by administration of GW4064 as a FXR synthetic agonist. The stable FXR reporter cell colony was picked up based on high fluorescence and low background. Then, candidates for FXR reporter cells were exposed to different concentrations of GW4064 for characterizing agonist dose-response. The results showed that the level of FXR reporter activity increased in a dose-dependent manner. Furthermore, this study determined whether stimulation with FXR agonist is able to transactivate the FXR target genes in the FXR reporter cells. The results suggested that GW4064 induced the mRNA expression of ileal bile acid binding protein (IBABP), organic solute transporter α (OSTα) and fibroblast growth factor 19 (FGF19) in a dose-dependent manner. Taken together, the FXR reporter system was successfully obtained.

In **chapter 3**, by using the FXR reporter system constructed in chapter 2, a total of 38 bacterial strains derived from the intestine or dairy foods were evaluated to check whether they can be modulators for FXR activation. In order to understand the FXR activity inducement by different parts of bacteria, bacteria samples in the case of intact bacteria,

mechanical disrupted bacteria, heat-killed bacteria and bacterial culture supernatants were assessed by luciferase assay. The results presented that some bacterial cell forms slightly induced FXR activity less than two-fold compared to control group which only DMEM medium was added. Culture supernatants of *Bacteroides dorei* and *Eubacterium limosum* can intensely stimulate FXR activity.

To further determine if the culture supernatants of *B. dorei* and *E. limosum* intervene in bile acids homeostasis, expression levels of FXR target genes including IBABP, OSTα and FGF19 mRNA, were examined by real-time PCR in stable FXR reporter system. Culture supernatants of *B. dorei* and *E. limosum* at 10% level significantly induced FXR target gene IBABP and OSTα mRNA expression. But neither of these two culture supernatants affected FGF19 mRNA expression. These results reveled that both of two bacterial culture supernatants selectively modulated expression of FXR target genes involved in bile acids metabolism in stable FXR reporter cells.

Due to multiple possibilities of luciferase activity induction, the specificity of FXR activation by two bacterial metabolites was validated in cells with or without FXR expression. Two bacterial metabolites did not induce FXR activity in FXR null SW480 cells, indicating that the chemiluminescense activity stimulated by two bacterial metabolites is dependent on FXR. In addition, the levels of FXR target gene *Ibabp* and *Ostα* were very low compared with those in FXR containing cells, indicating that FXR target genes expression by two bacterial culture supernatants is dependent on FXR in SW480 cells.

To investigate whether two bacterial metabolites activate FXR target genes in other cell lines, I used two different cell lines (i.e. Caco-2 and HepG2), which endogenously express FXR . Two bacterial culture supernatants induced the *Ibabp* gene expression. The levels of *Ibabp* gene expression exhibited positive correlation with differentiation degree of Caco-2 cells. However, two bacterial metabolites did not induce the *Ibabp* gene expression in FXR null-SW480 cells. Also, the culture supernatant derived from *B. dorei* did not stimulate

FXR of a hepatocyte-derived cell line, HepG2 cells, by measuring the *Shp* gene that induced directly by FXR activation.

Activation of FXR by its ligands plays an important role in inflammatory processes as well. Before investigation of two bacterial culture supernatants, the anti-inflammatory effect of GW4064 was confirmed in the FXR reporter system. GW4064 repressed tumor necrosis factors α (TNFα) induced inflammatory cytokine interleukin 8 (IL8) mRNA expression at 2 μM. However, neither *B. dorei* nor *E. limosum* deduced TNFα induced IL8 expression when pretreated with culture supernatants for 18 hours. However, IL8 expression decreased when given lower concentration of bacterial culture supernatants, which implied that endotoxin contributing to IL8 expression may exist in the supernatants.

In **chapter 4**, to investigate whether two FXR-stimulatory bacteria confer the anti-obesity effect, the bacterial culture supernatants of *B. dorei* or *E. limosum* were administrated to high fat diet (HFD)-fed mice by the intragastric gavage for 11 weeks. The results showed HFD dramatically elevated mice body weight compared to standard diet (STD). Since 6 weeks of *B. dorei* derived culture supernatant administration, the mice showed lower body weight compared with mice that received PBS only, indicating that *B. dorei* cultural metabolites may help mice to be resistant to the body weight gain. However, the mice that received *E. limosum* did not show any changes in body weight. In the meantime, HFD feeding mice displayed increased liver weight compared to STD control group. *E. limosum* administration reduced mice liver weight, while *B. dorei* feeding mice showed a little lower liver weight without significant difference.

Serum biochemical analysis gave the results that increased activities of liver function markers, including serum alanine aminotransferase (ALT), cholesterol and glucose, indicating pathological changes in HFD feeding mice. The levels of ALT and aspartate aminotransferase (AST) were down-regulated when mice received *B. dorei* derived culture supernatant for 11 weeks. On the other hand, the mice received *E. limosum* derived culture

supernatant reduced levels of ALT, cholesterol and triglycerides. Thus, the results revealed that two bacterial metabolites might be effective in suppressing the development of HFD-induced fatty liver diseases.

To investigate the role of two bacterial culture supernatants on bile acids homeostasis in diet induced obesity mouse model, total RNA were obtained from the ileum, colon and liver for real-time PCR analysis. Upon HFD administration, the expression of *Shp* mRNA was up-regulated in the ileum, whereas other genes were not affected. When mice were fed with *B. dorei* or *E. limosum* derived culture supernatants, the levels of *Fxr* increased by 1.6-fold and 2.1-fold respectively, which confirmed the *in vitro* findings. Transporter *Ibat* was up-regulated with 1.9-fold and 2.5-fold. In addition, *E. limosum* down-regulated target gene *Shp* expression in the ileum. Gene *Fgf15*, *Ibabp* or *Ostα* were unaffected by two bacteria culture supernatants. These results revealed gene-selective regulation of two bacterial culture supernatants in the ileum.

In the colon, feeding of HFD did not provide significant impact on *Fxr* activity, while level of *Shp* was up-regulated by 6-fold, *Ibabp* and *Fgf15* were reduced with 2.7-fold and 5-fold, respectively. However, the differences of three gene expression levels were not significant, due to the big error. The culture supernatants derived from *B. dorei* or *E. limosum* did not affect the expression of *Fxr* and its target genes in the colon.

In the liver, HFD administration induced significant increase of gene *Shp* and *Bsep,* while *Fxr* level was not affected. However, *Fxr* expression was elevated when mice were fed with two bacteria derived culture supernatants, which showed increase of 1.3-fold and 1.4 fold, respectively. The mice fed with *E. limosum* derived culture supernatant displayed significant *Shp* reduction and increased *Ntcp* expression. On the other hand, *B. dorei* culture supernatant did not regulate *Fxr* target genes involved in bile acids metabolism in the liver.

Feeding mice with a HFD always induce impaired liver damage in the liver. With hematoxylin and eosin (HE) stain, marked fat accumulation and histology were observed

when mice were fed with HFD, which was characterized by macrovesicular steatosis with large and small fat droplet, mixed inflammatory cells infiltration and hepatocyte ballooning. Treatment with *B. dorei* derived culture supernatants slightly alleviated the severity of hepatic steatosis, while *E. limosum* treatment did not enhance liver steatosis. Picrosirius red stain was used to observe collagen in liver as well. More collagen was found around and diffusing along the vein when mice received a HFD. However, two bacterial culture supernatants did not alleviate the hepatic fibrosis.

To sum up in **chapter 5**, this study for the first time discovered culture supernatants of *B. dorei* and *E. limosum* can be FXR direct modulators by using a stable FXR reporter system. The *in vivo* assessment revealed the bile acid regulation by two bacteria is both gene- and tissue- specific. The findings of this study expand our current knowledge of FXR modulators and bile acids metabolism alteration by bacteria in HFD fed mice. They may provide a new direction to clarify both FXR action pathway and molecular mechanisms of microbe-host interactions. Probiotics are currently used as therapeutic options for many diseases, and thus *B. dorei* and *E. limosum* could be applied as a therapy for bile acids disorders through intestinal specific activation.

Publication lists

By Type	Theme, journal name, date & year of publication, name of author
Paper	
1	○Xianqin Zhang, Toshifumi Osaka, Satoshi Tsuneda. The metabolic products of two bacteria can modulate farnesoid X receptor activity. Nutrition&Metabolism. 2015.12:48, 1-14 DOI: 10.1186/s12986-015-0045-y.
Presentation 1	○Xianqin Zhang, Toshifumi Osaka, Satoshi Tsuneda. Exploring the intestinal bacteria involved in the regulation of immune-metabolism. Hindgut Club JAPAN symposium. 2014.12. Tokyo, Japan. (Poster presentation)
2	○Xianqin Zhang, Toshifumi Osaka, Satoshi Tsuneda. The interaction between host and commensal bacteria through the farnesoid X receptor. Japanese Society for Bacteriology. 2015.03. Gifu, Japan. (Poster presentation)

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