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Infant Formula Feeding Increases Hepatic Cholesterol 7α Hydroxylase (CYP7A1) Expression and Fecal Bile Acid Loss in Neonatal Piglets

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Abstract

Background: During the postnatal feeding period, formula-fed infants have higher cholesterol synthesis rates and lower circulating cholesterol concentrations than their breastfed counterparts. Although this disparity has been attributed to the uniformly low dietary cholesterol content of typical infant formulas, little is known of the underlying mechanisms associated with this altered cholesterol metabolism phenotype.

Objective: We aimed to determine the molecular etiology of diet-associated changes in early-life cholesterol metabolism with the use of a postnatal piglet feeding model.

Methods: Two-day-old male and female White-Dutch Landrace piglets were fed either sow milk (Sow group) or dairy-based (Milk group; Similac Advance powder) or soy-based (Soy group; Emfamil Prosobee Lipil powder) infant formulas until day 21. In addition to measuring serum cholesterol concentrations, hepatic and intestinal genes involved in enterohepatic circulation of cholesterol and bile acids were analyzed by real-time reverse-transcriptase polymerase chain reaction and Western blot. Bile acid concentrations were measured by liquid chromatography–mass spectrometry in serum, liver, and feces.

Results: Compared with the Sow group, hepatic cholesterol 7α hydroxylase (CYP7A1) protein expression was 3-fold higher in the Milk group ($P < 0.05$) and expression was 10-fold higher in the Soy group compared with the Milk group ($P < 0.05$). Likewise, fecal bile acid concentrations were 3-fold higher in the Soy group compared with the Milk group ($P < 0.05$). Intestinal mRNA expression of fibroblast factor 19 (*Fgf19*) was reduced in the Milk and Soy groups, corresponding to 54% and 67% decreases compared with the Sow group. In the Soy group, small heterodimer protein (SHP) protein expression was 30% lower compared with the Sow group ($P < 0.05$).

Conclusions: These results indicate that formula feeding leads to increased CYP7A1 protein expression and fecal bile acid loss in neonatal piglets, and this outcome is linked to reduced efficacy in inhibiting CYP7A1 expression through FGF19 and SHP transcriptional repression mechanisms. *J Nutr* 2018;148:702–711.

Keywords: enterohepatic circulation, bile acid synthesis, cholesterol, cholesterol 7-α hydroxylase, nutrition, breastfeeding, microbiome

Introduction

The health benefits associated with breastfeeding are well established and include lower incidences of childhood necrotizing enterocolitis and diarrhea, enhanced immune responses to upper respiratory and ear infections, and neonatal weight gain (1, 2). Risks of obesity, type 2 diabetes, and cardiovascular disease are also lower in adults who were breastfed (2–4). These findings suggest that the richness of dietary components, bioactive compounds within breast milk, or both have long-lasting programming effects on offspring physiology. Therefore, the American Academy of Pediatrics recommends exclusive breastfeeding for at least the first 6 mo of life (1, 2). Although 70% of women initiate breastfeeding, many parents will ultimately choose infant formula as an acceptable alternative to breast milk, and consequently only 20% of US infants are exclusively breastfed until 6 mo of age (5).

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One well-defined nutritional difference between human breast milk and typical infant formulas is cholesterol content. In breast milk, cholesterol concentrations range from 0.25 to 0.46 mmol/L (6, 7). In contrast, cholesterol concentrations in dairy-based formulas range from 0.08 to 0.13 mmol/L, and soy-based formulas contain no cholesterol (8, 9). As such, cholesterol requirements necessary for growth and development in formula-fed infants are met by endogenous synthesis in the liver, and subsequent biliary secretion for intestinal uptake and recirculation. Several clinical studies have shown increased fractional cholesterol synthesis rates in formula-fed infants compared with those who received breast milk, which corresponded to lower serum total- and LDL-cholesterol concentrations than in their breastfed counterparts (8–11). At weaning, serum cholesterol concentrations and cholesterol synthesis rates normalize, and differences in cholesterol metabolism are minimal among breastfed and formula-fed infants throughout childhood and adolescence (10, 12–14). However, observational studies in adult populations have shown a positive association between breastfeeding and lower circulating cholesterol later in life, supporting the idea that early diet exposure influences long-term cholesterol homeostasis (15). The mechanisms underlying these programmed effects, or how early exposure to increased endogenous cholesterol biosynthesis (as observed in formula-fed infants) alters the development of normal cholesterol homeostasis, are not known.

The neonatal piglet is a good surrogate for certain aspects of infant physiology and nutritional requirements (16, 17). In addition, the cholesterol concentration in sow milk (~0.34 mmol/L) is similar to that in human breast milk, and piglets may be fed commercially available infant formulas immediately after birth, which more readily models the feeding paradigm of human infants (18). Similar to formula-fed infants, we and others have reported increased hepatic cholesterol synthesis, but lower circulating total-cholesterol concentrations in formula-fed piglets than in piglets fed sow milk. Surprisingly, we also reported a significant upregulation of hepatic cholesterol 7α hydroxylase (*Cyp7a1*) gene expression in association with both milk formula and soy formula feeding in neonatal piglets (19). CYP7A1 is the rate-limiting enzyme in cholesterol catabolism to bile acids, and hepatic expression is regulated through feedback mechanisms associated with bile acid and cholesterol uptake in the intestine (20–24). Therefore, it is possible that early postnatal formula feeding, which is uniformly low in cholesterol relative to breast milk, may alter ≥1 of these feedback mechanisms regulating CYP7A1 protein expression. To test this hypothesis, we fed neonatal piglets either a dairy-based formula (Milk group) or a soy-based formula (Soy group) for 20 d after birth and compared cholesterol and bile acid metabolism and regulatory pathways with those observed in piglets fed sow milk (Sow group).

Methods

Animal experiments. All piglets were housed in the animal facilities of the Arkansas Children's Hospital Research Institute. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Detailed descriptions of protocol and animal care have been previously published (25, 26). Multiparous Dutch Landrace Suroc sows with litter sizes of 8–11 were used. At birth, male and female piglets were allowed to suckle at the farm for 2 d, then stratified by sex and randomly assigned to continue sow feeding (Sow group; $n = 12$) at the farm or transferred to the Arkansas Children's Nutrition Center and fed either a cow milk–based formula (Milk group; $n = 12$) (Similac Advance powder; cholesterol concentration of 0.052 mmol/L; Ross Products, Abbott Laboratories) or a soy-based formula (Soy group; $n = 12$) (Enfamil Prosobee Lipil powder; cholesterol concentration of 0.00 mmol/L; Mead Johnson Nutritionals) for 21 d. Formula-fed piglets were trained to drink from small bowls on a fixed schedule—the first week was every 2 h, the second week was every 4 h, and the third week was every 6 h—to provide 1.047 MJ · kg^{-1} · d^{-1} until day 21. The composition for the infant formulas used in this experiment, and an estimation of sow milk, has been previously published (25, 27, 28). Sow, Milk, and Soy group piglets were killed by exsanguination after anesthetization, 6–8 h after the final feeding period. Tissue samples were snap-frozen in liquid nitrogen; feces were collected in the rectum. All of the samples were stored at −70°C until analysis.

Serum biochemistry. After being killed, blood was collected, left at room temperature for 15 min, and separated from the clotted material by centrifugation (2000 × *g* for 10 min at 4°C). Serum total cholesterol, HDL cholesterol, and LDL cholesterol were measured with the use of commercially available assays (IR060-R; Synermed; STA-391; Cell Biolabs, Inc.).

Real-time RT-PCR. Liver and ileum RNA was isolated and reversed transcribed to cDNA as previously described (19). Real-time RT-PCR analysis was carried out by using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems). Gene expression was analyzed by using the $2^{-\Delta\Delta ct}$ method relative to *Gapdh* gene amplification. Primer sequences are shown in **Supplemental Table 1**.

Tissue biochemistry. Liver (~100 mg) and feces (~50 mg) lipids were extracted by chloroform:isopropanol:NP-40 (7:11:0.1), and total, free, and esterified cholesterol concentrations were measured by using a commercially available assay (ab65359; Abcam). Nuclear and membrane protein fractions were obtained as previously described (29). Proteins (30 μg) were separated by SDS-PAGE by standard methods. Blotted proteins were incubated with anti–short heterodimer protein (anti-SHP; ab80417; AbCam) and anti-CYP7A1 (sc-14423; Santa Cruz Biotechnologies). Protein bands were detected by chemiluminesence and quantified as described (29).

LC-MS analysis of 7α-hydroxy-4-cholesten-3-one. The presence and quantification of 7α-hydroxy-4-cholesten-3-one (C4) was determined in fasting serum samples with the use of LC-MS. The internal standard, 7α-hydroxy-4-cholesten-3-one-d7 (C4-D7) at 9 nM final concentration was added to each serum sample (30 μL); lipids were extracted by using a modified Bligh and Dyer method as described previously (30). Extracts were reconstituted in 200 μL acetonitrile:water (80:20) with 0.1% formic acid spiked with external standard (lorazepam; 12 nM final concentration). Chromatographic separation was performed on an UltiMate 3000 UHPLC system (Thermo Scientific) by using an Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters) kept at 35°C. A flow rate of 260 μL/min was used—mobile phase (A) (0.1% formic acid in water) and (B) (0.1% formic acid in acetonitrile),

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Supplemental Tables 1–4 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.

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Abbreviations used: *Bsep*, bile salt export pump; BSH, bile salt hydrolase; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α hydroxylase; C4, 7α-hydroxy-4-cholesten-3-one; C4-D7, 7α-hydroxy-4-cholesten-3-one-d7; DCA, deoxycholic acid; FGF19, fibroblast factor 19; FXR, farnesoid X receptor; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; *Hmgr*, Hmg-CoA reductase; LCA, lithocholic acid; LXR, liver X receptor; Milk, dairy-based formula; *Npc1l1*, Niemann-Pick C1-like 1; SHP, short heterodimer protein; Sow, sow milk; Soy, soy-based formula; *Srebp2*, sterol regulatory element-binding protein 2.

with the use of an 11-min elution gradient: hold 32% B for 1 min, ramp to 97% B for 2 min, hold 97% B for 5 min, return to 32% B for 2 min, and hold 32% B for 1 min. Identification was carried out on a Q Exactive high-resolution accurate mass spectrometer (Thermo Scientific); data were acquired by positive heated electrospray ionization (HESI+) t-SIM scan and analyzed by using Xcalibur 4.0 and TraceFinder 3.3 software (Thermo Scientific). Nitrogen as sheath, auxiliary, and sweep gas was set at 46, 11, and 2 units, respectively. Spray voltage, capillary temperature, and source heater were at 3.5 kV, 250°C, and 400°C, respectively; t-SIM conditions: resolution, 35,000 FWHM (Full width at half maximum); AGC target, 1e6 ions; maximum injection time, 200 ms; targeted masses, 401.34141 and 408.38534 *m/z*; and isolation window, 4.0 *m/z*. Peak area measurements normalized to the external standard were used to quantitate C4 and C4-D7. Calibration curves, 0–65 nM, showed linearity $>r^2 = 0.997$. The limit of detection for C4 and C4-D7 is 0.97 nM and 0.58 nM, respectively. The limit of quantitation for both compounds is 1.9 nM. C4 concentrations were corrected by using percentage recovery of the internal standard, D7-C4.

LC-MS analysis of bile acid concentrations. The presence and quantification of 21 bile acids were determined in the serum, liver, and feces with the use of an LC-MS method adapted from García-Cañaveras et al. (31). Serum samples (50 μL) were prepared as previously described (31). Liver tissue (~100 mg), fecal contents (~20 mg), and quality controls (mixed bile acid solution, final concentration of 1.8 μM) were extracted with 600 μL cold methanol plus 200 μL recovery standard solution (12 μM); samples were homogenized twice with the use of a Precellys 24 homogenizer with 2.33-mm metal beads, (Bertin Corp.). Homogenates were centrifuged at 3000 × *g* at 4°C for 5 min; supernatant was collected and extracted again with 400 μL MeOH. For each sample, supernatants were pooled, separated into aliquots, and stored at −70°C. Aliquots, 100 μL for a 75× dilution and 400 μL for a 3× dilution, were evaporated to dryness under a nitrogen stream, and reconstituted in 50% methanol plus external standard (300 nM lorazepam, final concentration). Chromatic separation was performed on an Ultimate 3000 UHPLC system fitted with a Hypersil GOLD C18 reversed-phase column (50 × 2.1 mm, 1.9 μm column). Identification was carried out on a Q-Exactive high-resolution accurate mass spectrometer, and data were acquired by an ESI-Full-MS scan, and analyzed by using Xcalibur 4.0 and TraceFinder 3.3 software. Nitrogen as sheath, auxiliary, and sweep gas was set at 50, 13, and 3 units, respectively; resolution, 70,000 FWHM (Full width at half maximum); AGC target, 3e6 ions; maximum injection time, 200 ms; scan range, 50–750 *m/z*; spray voltage, 3.50 kV; and capillary temperature, 320°C. Individual bile acids were identified by exact mass and retention time as shown in **Supplemental Table 2**. Peak area measurements normalized to the external control were used to quantitate; calibration curves, 0–5000 nM, showed linearity >0.99. Fecal, liver, and serum bile acid concentrations were corrected by using percentage recovery values of the appropriate internal standard and tissue weight when appropriate. Quantitative assessment of individual hepatic and serum bile acids is summarized in **Supplemental Tables 3** and **4**.

Statistical analysis. All of the statistical analyses presented were performed on $\log_2$-transformed data. The effects of diet, sex, and the interaction thereof were determined with the use of 2-factor ANOVA, and for all analyses tested, indicated a significant diet effect ($P < 0.001$) but no sex effect or interaction. Therefore, females and males from each diet group were analyzed together with the use of either 1-factor ANOVA followed by Student-Newman-Keuls post hoc analysis; when unequal variance was observed as determined by the Brown-Forsythe test, diet groups were analyzed by Kruskal-Wallis 1-factor ANOVA on Ranks. These analyses were performed by using the SigmaPlot software package 12.0. Mixed-effects linear regression for repeated measures was used to model postnatal weight as a function of feeding group. Restricted cubic splines for postnatal age were computed and interacted with feeding group in the model. Model was fitted via maximum likelihood estimation. Predicted means (marginal effects) and corresponding delta-method SEs were computed at each postnatal age and compared across feeding group by using Bonferroni-adjusted Wald tests. Data analysis was performed by using Stata version 15.1 (StataCorp). Diet-associated microbial differences in the small intestine of the Sow, Milk, and Soy groups have recently been published (25). Therefore, we measured associations between $\log_2$-transformed serum bile acid concentrations with duodenal bacterial genera that showed significant $\log_2$ fold differences between Sow and Milk or Soy groups as previously reported (25) using Pearson's pairwise correlation coefficients with the use of the open-source statistical software package R, version 3.3.

Results

Sow, Milk, and Soy group piglets have similar growth curves from postnatal day 2 to 20. During postnatal feeding, the weight gain did not differ by diet, as shown in **Supplemental Figure 1**. After being killed (day 21), body weights were also within normal limits and did not differ significantly by diet group or sex (26).

Formula feeding upregulates mRNA expression of cholesterol synthesis genes in the liver. As summarized in **Table 1**, we observed a 2- to 5-fold increase in mRNA expressions of the transcription factor sterol regulatory element-binding protein 2 (*Srebp2*) and its target genes, Hmg-CoA reductase (*Hmgr*) and LDL receptor (*Ldlr*), in the Milk and Soy groups relative to the Sow group ($P < 0.05$). In contrast to the apparent increase in hepatic cholesterol synthesis, fasting serum total- and LDL-cholesterol concentrations were significantly lower in Milk and Soy groups. Serum HDL cholesterol was lower in the Milk group than in the Soy and Sow groups (Table 1). Interestingly, hepatic total-cholesterol concentrations were 17% and 30% lower in Milk and Soy groups, respectively ($P < 0.05$). Hepatic free cholesterol was also significantly reduced in the Milk and Soy groups compared with the Sow group. Cholesterol ester content was reduced only in the Soy group when compared with the Milk and Sow groups (Table 1).

Formula feeding promotes bile acid synthesis by upregulating Cyp7a1 mRNA and protein expression and enzymatic activity. As seen in **Figure 1**A, B, *Cyp7a1* mRNA abundance and protein expression were significantly higher in the Milk and Soy groups compared with the Sow group and

TABLE 1 Hepatic expression of cholesterol synthesis genes and fasting serum and hepatic cholesterol concentrations in Sow, Milk, or Soy group piglets at postnatal day 21[1]

	Group		
	Sow	Milk	Soy
Srebp2 mRNA expression, relative to *Gapdh*	0.21 ± 0.03[a]	0.54 ± 0.06[b]	0.70 ± 0.06[c]
Hmgr mRNA expression, relative to *Gapdh*	0.04 ± 0.01[a]	0.54 ± 0.06[b]	1.00 ± 0.18[c]
Ldlr mRNA expression, relative to *Gapdh*	0.05 ± 0.01[a]	0.17 ± 0.01[b]	0.25 ± 0.02[c]
Total cholesterol, mmol/L	7.18 ± 0.85[a]	4.77 ± 0.34[b]	5.45 ± 0.31[b]
LDL cholesterol, mmol/L	5.10 ± 0.41[a]	2.95 ± 0.29[b]	3.01 ± 0.56[b]
HDL cholsterol, mmol/L	2.49 ± 0.15[a]	1.89 ± 0.08[b]	2.44 ± 0.23[a]
Total cholesterol, mg/g liver	2.43 ± 0.11[a]	2.03 ± 0.04[b]	1.76 ± 0.06[c]
Free cholesterol, mg/g liver	1.72 ± 0.09[a]	1.40 ± 0.03[b]	1.40 ± 0.05[b]
Cholesterol esters, mg/g liver	0.71 ± 0.04[a]	0.66 ± 0.05[b]	0.36 ± 0.03[c]

[1] Values are means ± SEMs, $n = 12$. Labeled means in a row without a common superscript letter differ, $P < 0.05$. *Hmgr*, Hmg-CoA reductase; *Ldlr*, LDL receptor; Milk; dairy-based formula; Sow; sow milk; Soy; soy-based formula; *Srebp2*, sterol regulatory element-binding protein 2.

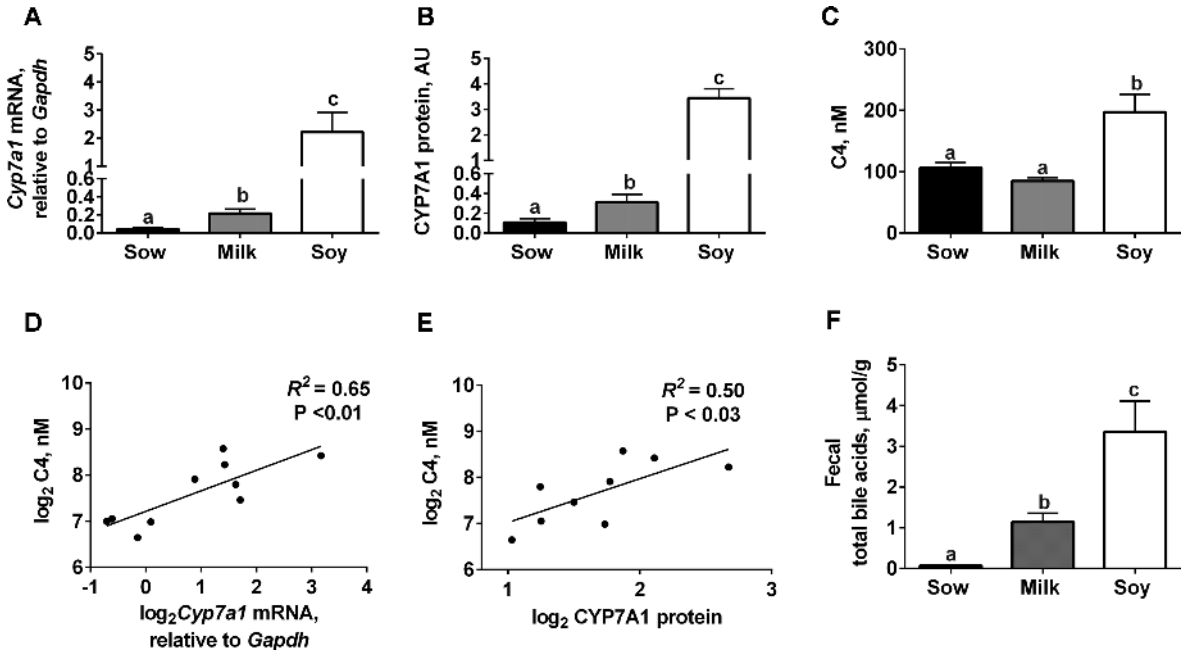

FIGURE 1 *Cyp7a1* mRNA (A) and CYP7A1 protein (B) expression in the liver and fasting serum C4 concentrations (C) in Sow, Milk, and Soy group piglets. In the Soy group, $\log_2$-transformed data were used in Pearson's coefficient correlations between C4 and *Cyp7a1* mRNA (D) and C4 and CYP7A1 protein (E). Fecal total bile acid concentrations (F) were determined for each diet group by the summing of all individual bile acid forms quantified by LC-MS. Values are means ± SEMs, $n = 12$. Labeled means without a common letter differ, $P < 0.05$. AU, arbitrary units; CYP7A1, cholesterol 7α hydroxylase; Milk, dairy-based formula; Sow, sow milk; Soy, soy-based formula.

much higher in the Soy group compared with the Milk group. CYP7A1 enzymatic activity was assessed indirectly by measuring C4, an intermediary metabolite in bile acid synthesis in serum (32). Interestingly, C4 concentrations significantly increased only in the Soy group when compared with the Milk and Sow groups (Figure 1C); we did observe a positive correlation between C4 and *Cyp7a1* mRNA and protein expression ($P < 0.05$), suggesting increased CYP7A1 activity in the Soy group (Figure 1D, E). Next, we measured fecal conjugated and unconjugated bile acid concentrations in all diet groups with the use of LC-MS; only unconjugated bile acids were detected in each sample. As seen in Figure 1F, total fecal file bile acid excretion was 14- and 47-fold higher in the Milk and Soy groups, respectively, compared with that in the Sow group ($P < 0.05$). Hyocholic acid (HCA) is the major circulating bile acid in domestic pigs (*Sus scrofa*) (33), which represented 36% of the fecal bile acids in the Sow group (**Table 2**). In the Milk and Soy groups, the secondary bile acid hyodeoxycholic acid (HDCA) comprised the majority of the excreted bile acids (~50%), followed by lithocholic acid (LCA; 32%) in the Milk group and HCA (32%) in the Soy group. In both Milk and Soy groups, HDCA, chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and LCA concentrations were significantly higher compared with those in the Sow group (Table 2). HCA and ursodeoxycholic acid concentrations were higher in the Soy group than in the Milk and Sow groups ($P < 0.05$).

In the liver, total bile acid concentrations were 23% ($P = 0.08$) and 33% ($P < 0.05$) greater in the Milk and Soy groups, respectively, compared with the Sow group (**Figure 2**A). In the Sow group, HCA conjugates represented 77% of the bile acids, followed by HDCA (14%) and CDCA (7%) conjugates, respectively (Figure 2B). Conversely, HDCA conjugates were the most abundant bile acids (55%) in the Milk and Soy groups, followed by CDCA (22%) and HCA (16%) conjugates. In the Milk and Soy groups, HDCA, CDCA, and LCA conjugates were significantly higher compared with those in the Sow group. Ursodeoxycholic acid conjugates were also significantly higher in the Soy group, and DCA conjugates higher in the Milk group relative to the Sow group ($P < 0.05$). Although we did not measure biliary secretion directly, hepatic mRNA expressions of farnesoid X receptor (*Fxr*) transcription factor, and its target bile salt export pump (*Bsep*), were significantly upregulated in the Milk and Soy groups, with the highest expression for *Bsep* observed in the Soy group (Figure 2C, D). Hepatic mRNA expression of *Shp*, which negatively regulates *Cyp7a1* transcription, was reduced in the Milk and Soy groups when compared with that in the Sow group ($P < 0.05$) (Figure 2E). Correspondingly, SHP protein was 30% lower in the Soy group ($P < 0.05$) and tended to be lower in the Milk group ($P = 0.06$) compared with the Sow group (Figure 2F). In addition, serum fibroblast growth factor 19 (FGF19) concentrations were reduced in the

TABLE 2 Fecal bile acid concentrations in Sow, Milk, or Soy group piglets at postnatal day 21[1]

	Group		
Bile acid	Sow	Milk	Soy
HCA, µmol/g	0.025 ± 0.01^a	0.10 ± 0.04^a	1.0 ± 0.5^b
CA, µmol/g	<LOD	<LOD	0.0013 ± 0.0004
CDCA, µmol/g	0.00054 ± 0.0001^a	0.29 ± 0.01^b	0.24 ± 0.014^b
HDCA, µmol/g	0.015 ± 0.004^a	0.60 ± 0.1^b	1.6 ± 0.31^c
DCA, µmol/g	0.00012 ± 0.0001^a	0.0043 ± 0.001^b	0.0039 ± 0.001^b
LCA, µmol/g	0.022 ± 0.006^a	0.35 ± 0.05^b	0.38 ± 0.05^b
UDCA, µmol/g	0.0011 ± 0.001^a	0.0027 ± 0.001^a	0.023 ± 0.01^b

[1]Values are means ± SEMs, $n = 12$. Labeled means in a row without a common superscript letter differ, $P < 0.05$. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; LOD, limit of detection; Milk; dairy-based formula; Sow; sow milk; Soy; soy-based formula; UDCA, ursodeoxycholic acid.

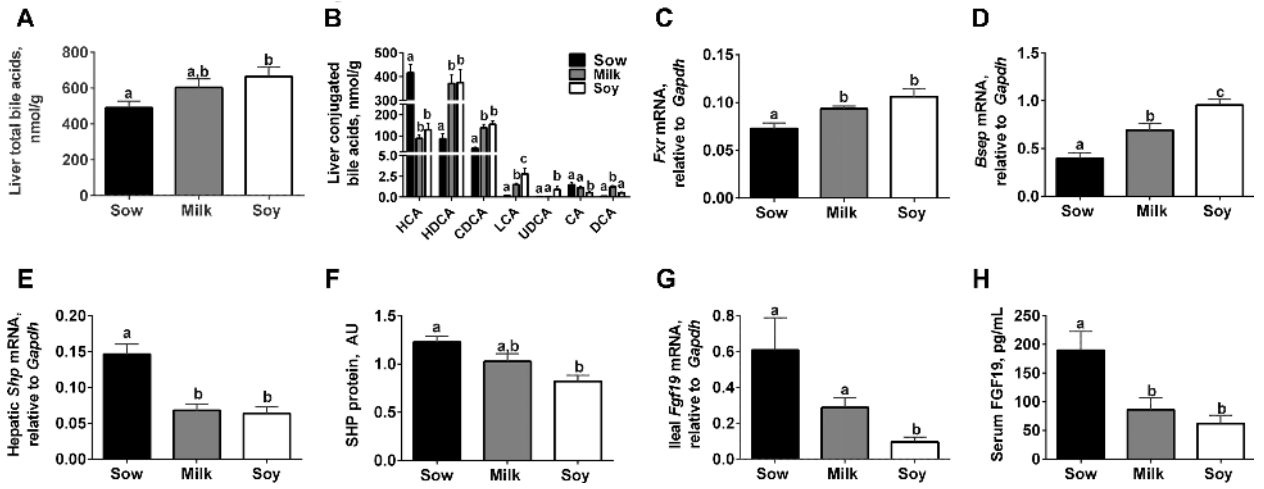

FIGURE 2 Liver total (A) and conjugated (B) bile acids; *Fxr* (C), *Bsep* (D), and *Shp* mRNA (E); and SHP protein (F) in Sow, Milk, and Soy group piglets and *Fgf19* mRNA in ileum (G) and serum FGF19 concentrations (H). Total bile acids are the sum of all individual bile acids; conjugated bile acids are the sum of glycine- and taurine-conjugated forms of each bile acid. Values are means ± SEMs, $n = 12$. Labeled means without a common letter differ, $P < 0.05$. *Bsep*, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FGF19, fibroblast factor 19; *Fxr*, farnesoid X receptor; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; Milk, dairy-based formula; SHP, short heterodimer protein; Sow, sow milk; Soy, soy-based formula; UDCA, ursodeoxycholic acid.

Milk and Soy groups relative to the Sow group ($P < 0.05$). In the ileum, *Fgf19* mRNA expression was also significantly reduced in the Soy group when compared with the Milk and Sow groups (Figure 2H).

Formula-fed piglets have lower serum total bile acid concentrations relative to the sow-milk control. In serum, total conjugated bile acids were 56% and 72% lower in the Milk and Soy groups, respectively, in comparison to the Sow group ($P < 0.05$) (**Figure 3**A). Recirculating unconjugated bile acid pools were lower in the Milk group than in the Soy and Sow groups ($P < 0.05$) (Figure 3A). In the Sow group, serum bile acid profiles were similar to those identified in the liver, with a greater abundance of HCA conjugates when compared with the Milk and Soy groups ($P < 0.05$) (Figure 3B). Conversely, circulating HDCA and LCA conjugates were significantly higher in the Milk and Soy groups compared with the Sow group. In the ileum, we observed no differences in mRNA expression of sodium-bile acid transporter (*Abst)* or organic solute transporter α (*Osta*) between diet groups to explain the loss in serum conjugated bile acid concentrations in the Milk or Soy groups (Figure 3C). Interestingly, hepatic gene expression Na^+-taurocholate co-transporting polypeptide (*Ntcp*), which is primarily responsible for uptake of conjugated bile acids in serum, was upregulated in the Milk and Soy groups, with the higher expression in the Soy group compared with the Sow group ($P < 0.05$) (Figure 3D).

Formula feeding increases expression of intestinal genes involved in cholesterol synthesis and transport. In comparison to the Sow group, fecal cholesterol concentrations in the Milk and Soy groups were 33% and 56% lower, respectively ($P < 0.05$) (**Figure 4**A). Cholesterol concentrations were significantly lower in the Soy group when compared with the Milk group. In the ileum, mRNA expression of transporters involved in intestinal cholesterol uptake [Niemann-Pick C1-like 1 (*Npc1l1*)] and efflux [ATP binding cassette subfamily G, members 5 and 8 (*Abcg5/8*)] were significantly upregulated in the Milk and Soy groups when compared with the Sow group (Figure 4B, C). Likewise, we observed increased gene expression of the sterol-transcription factor, *Srebp2*, and its target, *Hmgr*, in the Milk and Soy groups relative to the Sow group (Figure 4D). Interestingly, mRNA expression of the basolateral cholesterol transporter, ATP cassette subfamily A, member 1 (*Abca1*), was increased in the Soy group relative to the Milk and Sow groups ($P < 0.05$) (Figure 4B).

Formula-specific differences in the duodenal microbiome influence serum conjugated bile acid profiles. In **Figure 5**, Pearson's correlations between duodenal bacterial and serum conjugated bile acids are presented. Interestingly, glycohyocholic acid, taurohyocholic acid, and taurocholic acid positively correlated with 15 genera that were higher in abundance in the Sow group. These genera include *Clostridium*, *Arcanobacterium*, *Rothia*, *Turicibacter*, *Porphyromonas*, *Actinomyces*, *Veillonella*, and *Bifidobacterium*. In contrast, LCA conjugates, more specifically taurolithocholic acid, were negatively associated with many of these same genera that were more abundant in the Sow group (Figure 5). In addition, glycohyocholic acid and taurohyocholic acid were also negatively associated with genera in the Firmicutes phylum that were in higher abundance in the Milk and Soy groups, including *Caloramator* and *Geobacillus*. DCA conjugates were positively associated with several genera that were in greater abundance in the Milk and Soy groups, including *Acinetobacter*, *Lactobacillus*, *Citrobacter*, and *Lactococcus*. In addition, taurochenodeoxycholic acid and taurocholic acid were also positively associated with Firmicutes genera *Lactobacillus* and *Streptococcus*, respectively.

Discussion

Breast milk is the "gold standard" for early infant nutrition and is well recognized for its positive influences on a child's developing immune system and digestive tract. Current observational studies have suggested that breastfeeding can influence lipid metabolism in adulthood (14, 15). In a systematic review, Owen et al. (14) observed higher serum cholesterol concentrations in breastfed infants (<12 mo) in comparison to formula-fed

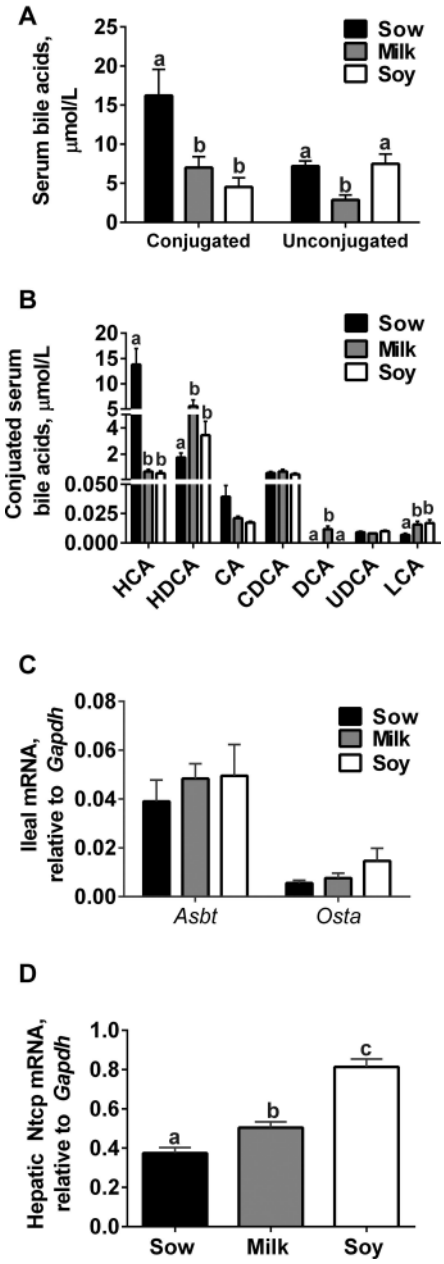

FIGURE 3 Serum total (A) and individual conjugated (B) bile acids; *Abst* and *Osta* mRNA in the ileum (C); and *Ntcp* mRNA in the liver (D) of Sow, Milk, and Soy group piglets. Total bile acids are the sum of all individual bile acids; conjugated bile acids are the sum of glycine- and taurine-conjugated forms of each bile acid. Values are means ± SEMs, $n = 12$. Labeled means without a common letter differ, $P < 0.05$. *Abst*, apical sodium-bile acid transporter; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; *Fgf19*, fibroblast factor 19; *Fxr*, farnesoid X receptor; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; Milk, dairy-based formula; *Osta*, organic solute transporter α; *Ntcp*, Na+-taurocholate cotransporting polypeptide; *Shp*, short heterodimer protein; Sow, sow milk; Soy, soy-based formula; UDCA, ursodeoxycholic acid.

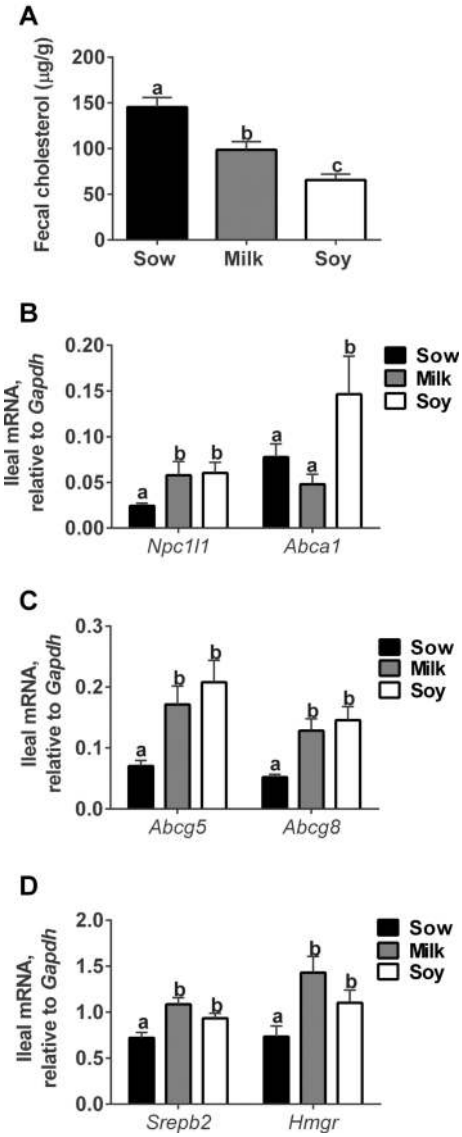

FIGURE 4 Total fecal cholesterol concentrations (A), cholesterol transporters *Npc1l1* and *Abca1* mRNA (B), and *Abcg5* and *Abcg8* mRNA (C) and *Srebp2* and *Hmgr* mRNA (D) in the ileum of Sow, Milk, and Soy group piglets. Values are means ± SEMs, $n = 12$. Labeled means without a common letter differ, $P < 0.05$. *Abca1*, ATP cassette subfamily A, member 1; *Abcg5*, ATP binding cassette subfamily G, member 5; *Abcg8*, ATP binding cassette subfamily G, member 8; *Hmgr*, Hmg-CoA reductase; Milk, dairy-based formula; *Npc1l1*, Niemann-Pick C1-like 1; Sow, sow milk; Soy, soy-based formula; *Srepb2*, sterol regulatory element-binding protein 2.

infants, yet as adults, serum cholesterol concentrations were significantly lower in those who were primarily breastfed as neonates relative to formula-fed individuals. The molecular basis for this apparent nutritional programming effect on cholesterol metabolism is not known, and it is not understood how typical commercial infant formulas, which are uniformly low in dietary cholesterol, alter the nutritional programming effect.

In formula-fed piglets, we observed a marked increase in hepatic CYP7A1 protein expression and bile acid synthesis pathway components in both the Milk and Soy groups relative to the Sow group. We believe this finding explains, at least in part, the link between lower serum cholesterol and higher cholesterol synthesis as observed in both formula-fed infants and piglets (8–11, 34, 35). Increased cholesterol catabolism to bile acids would effectively lower intracellular cholesterol concentrations, which would remove cholesterol's inhibitory effect on *Srebp2* transcriptional activity (36), thus increasing de novo cholesterol synthesis and receptor-mediated cholesterol uptake into

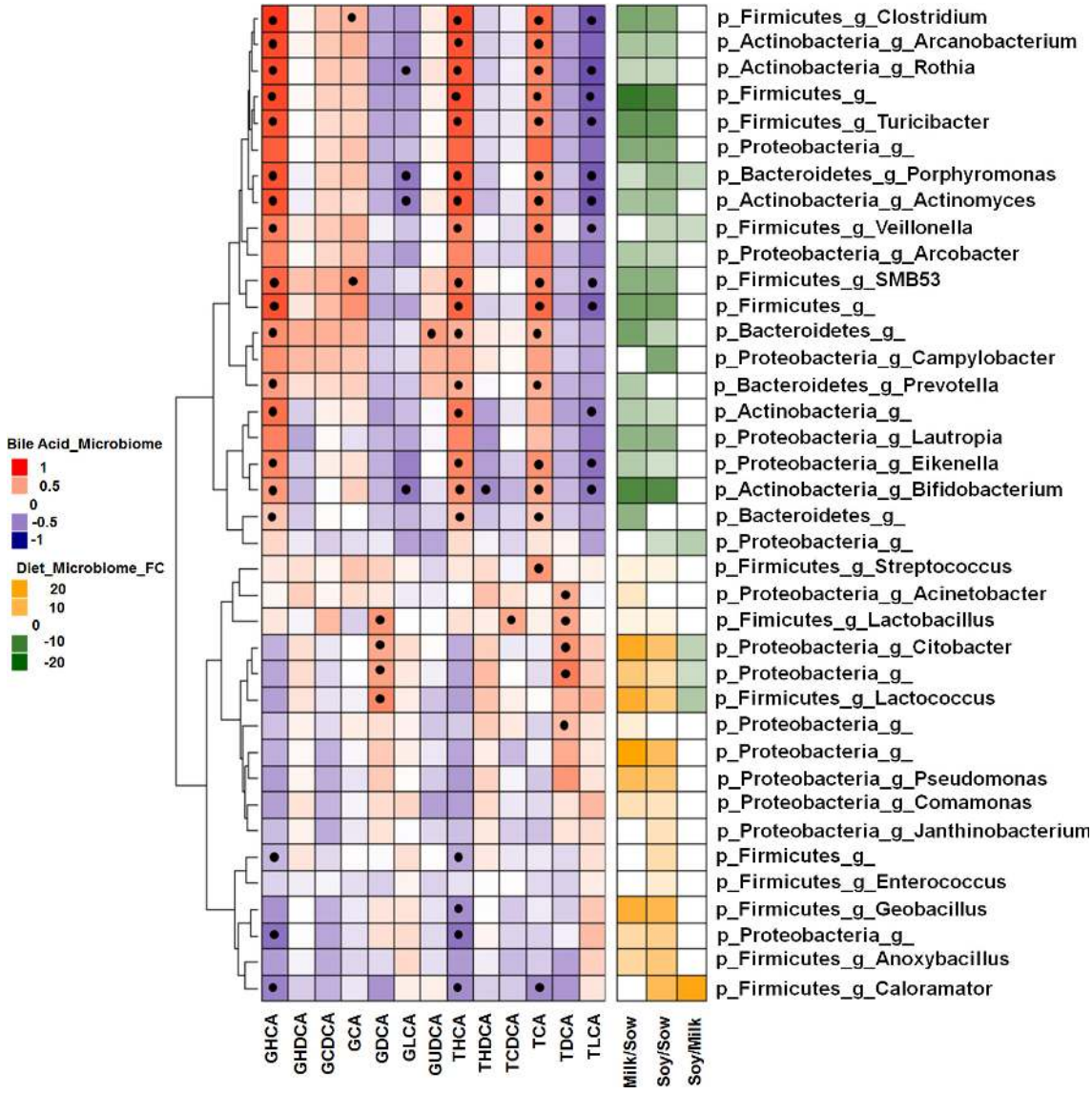

FIGURE 5 The heat map on the left shows Pearson's correlations between duodenum genera that significantly differed (>$\log_2$-fold) between Sow, Milk, and Soy group piglets and serum glycine- and taurine-conjugated primary and secondary bile acids as quantified by LC-MS. The heat map on the right shows $\log_2$-fold change in the microbial abundance in the 3 diet group comparisons as previously described (25). Black dots indicate significant correlation coefficients ≥0.42 at $P < 0.05$. FC, fold change; g_, genus; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycdeoxycholic acid; GHCA, glycohyocholic acid; GHDCA, glycohyodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; Milk, dairy-based formula; p_, phylum; Sow, sow milk; Soy, soy-based formula; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THCA, taurohyocholic acid; THDCA, taurohyodeoxycholic acid; TLCA, taurolithocholic acid.

the liver. Support for this hypothesis comes from the work of Li et al. (37), who showed that transgenic mice overexpressing CYP7A1 not only have elevated bile acid synthesis but also increased hepatic cholesterol synthesis. The authors attribute this increase in cholesterol synthesis to reduced intracellular cholesterol concentrations resulting from increased CYP7A1 activity.

In the ileum, reabsorbed bile acids activate FXR, thus increasing transcriptional expression of FGF19 (21, 38). This secreted hormone suppresses liver *Cyp7a1* transcription through the cell surface receptor FGFR4 and downstream tyrosine kinase signaling. In both milk- and soy-formula–fed piglets, we observed an apparent attenuation of this specific feedback mechanism. Interestingly, it has been reported in human intestinal cells that SREBP2 negatively regulates *Fgf19* mRNA expression (23). Likewise, intestinal SREBP2 also upregulates *Ncp1l1* gene expression under conditions of dietary cholesterol deprivation in mice and mini-pigs (22, 39, 40). In comparison to sow milk, both milk formula and soy formula are cholesterol deficient. As such, ileal *Srebp2*, *Hmgr*, and *Ncp1l1* mRNA expression levels were upregulated in the Milk and Soy groups. Thus, we propose the formula feeding stimulates cholesterol synthesis in both the ileum and the liver. With respect to piglets, Jones et al. (34) reported no increases in intestinal (jejunum, ileum) HMGR activity in response to formula feeding; however, these studies were performed in male Yorkshire piglets only, and the sample size was small (n = 2–4 piglets/diet). More recently, Dimova et al. (41) described a novel mouse model of postnatal feeding in which dietary cholesterol availability from breast milk is reduced through ezetimibe supplementation. The authors observed increased *Hmgr* mRNA in the proximal intestine in response to reduced cholesterol uptake in adult mice

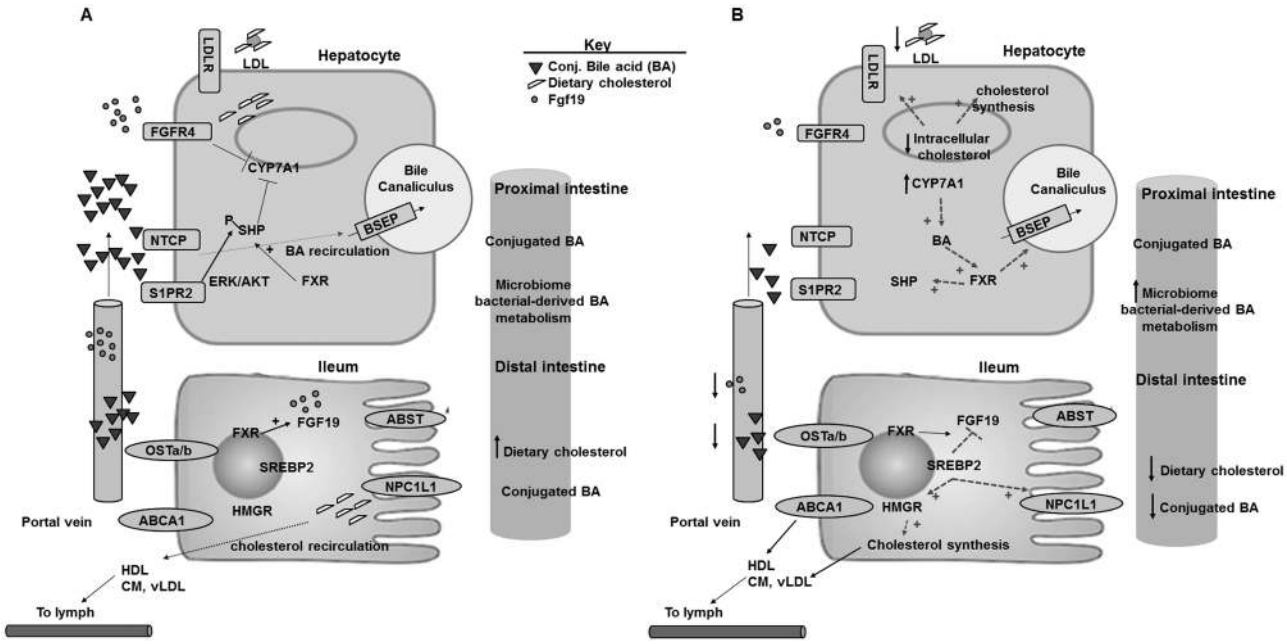

FIGURE 6 Differences in cholesterol and bile acid enterohepatic circulation in Sow, Milk, or Soy group piglets: a working model. In contrast to sow milk (A), formula feeding (B) resulted in lower dietary cholesterol bioavailability, thus stimulating intestinal cholesterol synthesis mediated by SREBP2 activation. In the ileum, Srebp2 inhibits *Fgf19* mRNA expression, reducing serum concentrations. This reduces FGF19-mediated inhibition of CYP7A1 expression and activity in the liver. As formula feeding continues, hepatic bile acid synthesis continues, increasing biliary bile acid output and decreasing intracellular cholesterol concentrations, which stimulates hepatic cholesterol synthesis and receptor-mediated uptake via LDLR. Formula feeding results in low recirculating conjugated BA to the liver, in part driven by diet-specific changes in the duodenal microbiome. Lower serum bile acid conjugates result in reduced liver SHP protein expression through phosphorylation via S1PR2/ERK/AKT signaling pathways, thus reinforcing increased hepatic bile acid synthesis and lower serum cholesterol concentrations. ABCA1, ATP cassette subfamily A, member 1; ABST, apical sodium-bile acid transporter; AKT, PI3 kinase/protein kinase B; BA, conjugated bile acid; BSEP, bile salt export pump; CM, chylomicron; CYP7A1, cholesterol 7α hydroxylase; ERK, extracellular regulated kinase; FGF19, fibroblast growth factor 19; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid X receptor; HMGR, Hmg-CoA reductase; LDLR, LDL receptor; Milk, dairy-based formula; NPC1L1, Niemann-Pick C1-like 1; NTCP, Na+-taurocholate cotransporting polypeptide; OSTa/b, organic solute transporter α/β; SHP, short heterodimer protein; Sow, sow milk; Soy, soy-based formula; SREBP2, sterol regulatory element-binding protein 2; S1PR2, sphingosine 1-P receptor 2.

at 24 wk, indicating a possible programming effect associated with low dietary cholesterol intake during the postnatal feeding period. Additional studies are needed to assess the contribution of intestinal cholesterol synthesis during formula feeding, and to evaluate the potential influence of this process on *Fgf19* mRNA induction and bile acid homeostasis.

Upregulation of several hepatic FXR targets, most notably *Bsep* mRNA, remained intact, consistent with the increased hepatic bile acid pool observed in the Milk and Soy groups. Conversely, *Shp* mRNA and protein expressions were reduced and gene expressions of *Cyp7a1* and *Ntcp* were increased, suggesting loss of SHP-mediated repression. In rodent models, it has been reported that optimal hepatic SHP protein expression results from both FXR-dependent and -independent signaling mechanisms (42). In rat hepatocytes, conjugated bile acids bind to G-protein bile acid receptor sphingosine-1-phosphate receptor 2 (S1PR2) and activate extracellular regulated kinase 1 (ERK1) and PI3 kinase/protein kinase B (AKT) signaling pathways, which in turn, phosphorylate and stabilize SHP, preventing proteasome degradation (24). Therefore, conjugated bile acid–activated S1PR2 signaling may inhibit *Cyp7a1* transcription indirectly through SHP. In our study, serum conjugated bile acid concentrations were significantly lower in the Milk and Soy groups, which is consistent with this concept that SHP expression is dependent on enterohepatic circulation of conjugated bile acids. As for a mechanism, we did not observe any significant differences in *Abst* or *Osta* mRNA expression between diet groups that would explain altered uptake, export, or both of conjugated bile acids in the ileum.

In the intestine, bacteria-derived bile acid metabolism begins with bile salt hydrolase (BSH), which functions to remove the glycine- and taurine-conjugate to produce the unconjugated bile acid (43, 44). Therefore, diet-specific changes to the gut microbiome may have contributed to the lower conjugated bile acids observed in the serum of formula-fed piglets. In this study, we focused on the duodenum microbiome located upstream of the ileum, which is the primary site for conjugated bile acid uptake. In pigs, HCA is the most abundant bile acid (33, 45). Interestingly, HCA conjugates were positively associated with bacterial genera that were found solely in the sow microbiome (25). We also observed a significant increase in the fecal HCA pool and a corresponding loss of HCA conjugates in the serum and liver in the Milk and Soy groups relative to the Sow group. BSH activity is prevalent across all major phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria) within the intestinal microbiome (46, 47). In particular, formula feeding increased the relative abundance of the BSH-expressing genus *Lactobacillus* in the duodenum (25). Unlike other BSH-expressing genera, *Lactobacillus* BSH activity is inducible, with a $\geq$6-fold increase in some species (48). Certain *Lactobacillus* species are also capable of horizontal transfer of the BSH gene to the local bacterial community (43, 48). Taken together, these data suggest that formula feeding leads to a shift in bacteria-derived BSH metabolism within the duodenal microbial community.

In this piglet study, soy milk had the most dramatic effect on whole-body cholesterol homeostasis, CYP7A1 expression, and bile acid synthesis. In adult populations, soy food consumption is linked to hypocholesterolemic effects, including lower serum total- and LDL-cholesterol concentrations (49, 50). More specifically, soy protein isoflavones are potent liver X receptor (LXR) agonists and upregulate intestinal cholesterol uptake and hepatic bile acid synthesis in rodents (35, 51). In the current study, it is possible that isoflavone-mediated LXR activation contributed to the increased *Cyp7a1* expression in the Soy group. However, we did not see increased mRNA expression of *Ncp1l1* or *Abcg5/8* in the Soy group relative to the Milk group, which would suggest isoflavone-mediated activation of LXR targets in the ileum. In one clinical study, Cruz et al. (9) showed a significant increase in cholesterol synthesis rates in infants receiving soy formula, which contains no dietary cholesterol, relative to milk formula–fed infants. Consistent with this finding, our data clearly show diet-specific cholesterol metabolism phenotypes associating with the high, low, or no cholesterol content in the Sow, Milk, and Soy groups, respectively. However, to confirm that the loss of dietary cholesterol is generating these diet-specific phenotypes, additional piglet feeding studies that use cholesterol-supplemented infant formulas are necessary. These studies are currently under investigation in our laboratory. Interestingly, supplementing formulas with cholesterol has only modest effects on cholesterol synthesis rates and serum LDL-cholesterol concentrations in infants (8, 12). Whether or not this observation is a result of low intestinal uptake and bioavailability of formula-based cholesterol, due to the formula matrix or other factors, remains to be determined.

In summary, postnatal infant formula feeding leads to an increased CYP7A1 protein expression and elevated fecal bile acid elimination, suggestive of increased bile acid synthesis. As depicted in a working model (**Figure 6**), it is proposed that low dietary cholesterol bioavailability associated with formula feeding stimulates intestinal cholesterol synthesis, mediated through SREBP2 activation, a transcription factor, which also binds to—and inhibits—*Fgf19* mRNA expression. This unexpected upregulation in *Srebp2* gene expression indicates a potential mechanism through which intestinal cholesterol uptake regulates cholesterol catabolism to bile acids. As formula feeding continues, diet-specific changes in the gut microbiome result in increased bacteria-derived BSH metabolism, driven in part by increased biliary bile acid output. Subsequent lowering of recirculating conjugated bile acids reduces hepatic SHP protein stabilization, thus enhancing liver bile acid synthesis. Our model highlights the central role that dietary cholesterol uptake plays in controlling both cholesterol and bile acid homeostasis. Whether these pathways are affected long term by postnatal feeding through programming mechanisms remains to be evaluated further.

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