Determination of human plasma bile acid metabolic profiles by liquid chromatography-tandem mass spectrometry

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Determination of human plasma bile acid metabolic profiles by liquid chromatography-tandem mass spectrometry

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Determination of human plasma bile acid metabolic profiles by liquid chromatography-tandem mass spectrometry', contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Candidate contributions to co-authored papers

Chapter 2 (in preparation) Chen, Y., Nemati, R., and Lu, J. Adaptation and development of an LC-MS method to measure bile acids in human plasma samples. *Journal of Chromatography B*	Chen 95%, Nemati provided previous method Lu 5%
Chapter 3 (submitted) Chen, Y., Nemati, R., Murphy, R. and Lu, J. Acute Changes of Bile Acids and FGF19 after Sleeve Gastrectomy and Roux-en-Y Gastric Bypass in a randomized trial. *Obesity Surgery*.	Chen 85%, Nemati 5%, Murphy 5%, Lu 5%
Chapter 4 (in preparation) Chen, Y., Murphy, R. and Lu, J. Potential contribution of probiotic supplementation Lactobacillus rhamnosus HN001 in early pregnancy to improve maternal glucose metabolism associated with alterations in plasma conjugated bile acids: a randomised controlled trial. *Diabetologia.*	Chen 90%, Murphy 5%, Lu 5%

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Abbreviations

AUC: Area under the curve
$AUC_{0\text{-}120min}$: Area under the curve over 120 minutes in an oral glucose tolerance test
BA: Bile acid
BMI: Body mass index
BSR: Basal insulin secretion rate
CA: Cholic acid
CA-d4: Cholic acid-d4
CDCA: Chenodeoxycholic acid
CFU: Colony-forming units
Chol: Total cholesterol
CV: Coefficient of variation
CYP7A1: Cholesterol 7α-hydroxylase
CYP8B1: sterol 12α-hydroxylase
DCA: Deoxycholic acid
ESI: Electrospray ionisation
FFA: Free fatty acids
FGF19: Fibroblast growth factor19
FXR: Farnesoid X receptor
GC: Gas chromatography
GCA: Glycocholic acid
GCDCA: Glycochenodeoxycholic acid
GC-MS: Gas chromatography-tandem mass spectrometry
GDCA: Glycodeoxycholic acid
GDM: Gestational diabetes mellitus
Glu sens: Glucose sensitivity
GUDCA: Glycoursodeoxycholic acid
HDCA: Hyodeoxycholic acid
HDL-c: High density lipoprotein-cholesterol
HN001: *Lactobacillus rhamnosus* HN001
HOMA-IR: Homeostatic model assessment of insulin resistance
HPLC: High-performance liquid chromatography
IQR: Interquartile range
ISI: Insulin sensitivity index
LC: Liquid chromatography
LCA: Lithocholic acid
LC-MS/MS: Liquid chromatography-tandem mass spectrometry
LDL-c: Low-density lipoprotein-cholesterol
LOD: Limit of detection
mL: Milliliter (10^{-3} L)
mM: Millimolar (10^{-3} mol L^{-1})
MRM: Multiple reaction monitoring
MS: Mass spectrometer
NA: not applicable
OGTT: Oral glucose tolerance test
PF: Potentiation factor
r^2: Correlation coefficient

Rate sens: Rate sensitivity
RYGB: Roux-en-Y gastric bypass
S/N: Signal-to-noise ratio
SD: standard deviation
T2DM: Type 2 diabetes mellitus
TCDCA: Taurochenodeoxycholic acid
TDCA: Taurodeoxycholic acid
TGR5: Takeda G protein-coupled receptor5
THDCA: Taurohyodeoxycholic acid
TIS: Total insulin secretion
TLCA: Taurolithocholic acid
TUDCA: Tauroursodeoxycholic acid
TβMCA: Tauro-conjugated-beta-muricholic acid
UDCA: Ursodeoxycholic acid
VSG: Vertical sleeve gastrectomy
μL: Microliter (10^{-6} L)
μM: Micromolar (10^{-6} mol L^{-1})

Abstract

Metabolic diseases, particularly type 2 diabetes mellitus (T2DM), are becoming serious health issues globally. Metabolic diseases are associated with changes in a number of biomarkers, such as glucose, insulin, and adiponectin. Many biomarkers have been used to predict or assess metabolic diseases, which are essential clinical tools for disease diagnosis and management. Recently, bile acids (BAs) have been shown to be linked with metabolic changes and diseases. Hence, this thesis focuses on applying a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure BAs in blood samples collected from a bariatric surgery study and a gestational probiotic intervention study. It intends to find out whether BAs are changing with those interventions and whether BAs are a good indicator of changes in metabolic diseases.

An LC-MS/MS method was successfully developed in the analytical laboratory of the Auckland University of Technology. The separation of BAs was achieved with a reverse-phase C18 column with the gradient elution of aqueous and methanol acetate mobile phases that contained formic acid. BAs were monitored and quantified based on their unique production of ions. Then the method was applied to measure plasma BA concentrations in blood samples collected from 19 patients at three days and three months after bariatric surgeries, including 8 gastric bypasses (GBP) and 11 sleeve gastrectomies (SG). The same method was used to measure BAs in plasma samples from 348 women at gestation, with 172 taking the probiotic supplement and 176 on placebo.

In the bariatric surgery trial, several BA species increased acutely after bariatric operations, along with fibroblast growth factor19 (FGF19). Significant correlations were observed between the post-operation changes of either BA compositions or FGF19 versus selected metabolic indices, such as the area under the curve (AUC) over 120 minutes (AUC0-120min) of glucose or insulin, insulin resistance, insulin sensitivity index (ISI), basal or total insulin secretion rate, and C-peptide at either three days or three months. In the probiotic gestational trial, a higher taurine-conjugated BA level was correlated with the higher concentration of one-hr glucose after an oral glucose test ($r=0.10$, $P=0.05$).

It is apparent from this study that plasma BAs are linked to metabolic diseases. Some BAs showed significant acute and short-term modifications induced by different types of bariatric surgery in obese individuals with T2DM. The alterations of BAs might contribute to improvements in glycemic control after surgery. In the probiotic intervention in women at gestation, changes in BAs indicate that taking probiotic supplements improves maternal glycemic control, particularly among obese women with gestational diabetes. The alteration of fasting plasma BAs by probiotic supplementation, which was notably recognised as a decrease in taurine-conjugated BAs, might play a role in the improvement of glucose metabolism. In conclusion, BAs are likely to link to metabolic diseases and have the potential to be developed into a biomarker for disease monitoring and management.

Chapter 1 Introduction

Bile acids (BAs) are a group of end products of cholesterol catabolism characterised by a steroid scaffolding with a carboxyl group at the side chain. The classic bile acid synthesis pathway is initiated by cholesterol 7α-hydroxylase (CYP7A1) for the synthesis of two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), from cholesterol. BAs are conjugated to the amino acids taurine or glycine before biliary secretion. Secondary BAs are derived from primary BAs in the intestine by bacterial enzymes (Schaap, Trauner, & Jansen, 2014).

BAs are synthesised exclusively in the liver, secreted across the canalicular membrane of the hepatocytes into the bile, and stored in the gallbladder. After a meal, gallbladder BAs are released into the intestinal tract, efficiently reabsorbed in the ileum, and transported back to the liver via portal blood for re-excretion into the bile. This process is referred to as enterohepatic circulation of BAs (Dawson, 2018). The enterohepatic circulation of BAs provides a negative feedback mechanism to maintain BA homeostasis (Chiang, 2015). The alteration of BA synthesis, secretion, and transport in the enterohepatic circulation may cause liver diseases (Kullak-Ublick, Stieger, & Meier, 2004), gallstone diseases (Carey, 1993), diabetes, and obesity (Li & Chiang, 2015).

BAs also serve as signalling molecules that activate several intracellular signalling pathways. BAs are known to activate the farnesoid X receptor (FXR) and the Takeda G protein-coupled receptor5. Primary-unconjugated BAs are potent endogenous ligands of FXR, whereas secondary BAs are more potent agonists of TGR5 (Li & Chiang, 2014). FXR was shown to induce the intestine fibroblast growth factor19 (FGF19), which may act as an endocrine hormone to repress CYP7A1 gene transcription and thereby regulate the synthesis and secretion of BAs, influences lipid and glucose metabolism in the liver (Kliewer & Mangelsdorf, 2015). TGR5 can activate cAMP signalling in many cells and plays a role in energy metabolism (Donepudi, Boehme, Li, & Chiang, 2017). It has been suggested that BAs play a critical role in maintaining lipid, glucose, and energy homeostasis through activation of FXR (Chiang, 2009) as well as TGR5 (Thomas et al., 2009).

Given all above, BAs are regarded as useful biomarkers for the prediction or assessment of metabolic diseases. It has been extensively reported that aberrant BA metabolism is associated with obesity, gestational diabetes mellitus (GDM), and type 2 diabetes mellitus (T2DM). For instance, in diabetic patients, plasma 12α-OH BAs and their conjugates are increased and correlated with insulin resistance (Haeusler, Astiarraga, Camastra, Accili, & Ferrannini, 2013). Individuals with GDM were reported to have significant increases in several BA species, including GHDCA and THDCA, in comparison to healthy controls (Gao et al., 2016). Therefore, studies of the BA metabolic profile in human bodily fluids are essential for disease diagnosis as well as management.

To date, a number of findings have shown that BAs are recognised as essential mediators that are related to the regulation of lipid, glucose, insulin, and drug metabolism. For example, bariatric surgeries are effective in reducing weight and improving insulin resistance and are increasingly introduced in T2DM. Several studies reported that plasma BAs were increased after Roux-en-Y gastric bypass (RYGB) surgery in obese and diabetic patients (Jahansouz et al., 2016; Schauer et al., 2014). The changes of BAs were shown to be correlated with the improvement of glycemic control, lipid oxidation, and energy expenditure (Gerhard et al., 2013; Patti et al., 2009; Simonen et al., 2012). Ryan et al. reported that an increase in circulating BAs, associated changes in gut microbial communities, reduction of body weight, and improved glucose tolerance were observed after vertical sleeve gastrectomy (VSG) in mice (Ryan et al., 2014). Gerhard et al. reported higher increases in serum BA and FGF19 concentrations among T2DM patients who went into remission compared with those without diabetes and those who did not show remission after RYGB (Gerhard et al., 2013). The above finding indicated that the regulation of the FXR signalling pathway of BAs might play a role in the remission of T2DM following bariatric surgery. Moreover, previous studies showed that probiotic supplementation has the ability to induce BA deconjugation as well as BA hydrolysis (Travers et al., 2016), which might be related to its therapeutic effects to improve glycemic control in pregnant women (Luoto, Laitinen, Nermes, & Isolauri, 2010). To sum up, clarifying the modifications of human plasma BA metabolic profiles after medical interventions may facilitate an understanding of any underlying therapeutic mechanisms of particular treatments and can contribute to clinical management exploitation of various human diseases.

In the presented thesis, we optimised and applied a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure BAs in human plasma. The BAs were collected from a bariatric surgery study and a gestational probiotic intervention study. The thesis consists of five chapters.

Chapter 1 offers an introduction to the overall studies.

In Chapter 2, an LC-MS/MS method is validated and optimised to analyse BAs in human plasma. The chromatographic resolution of 15 BAs, instrumental repeatability, and reliability, along with the intra-day and the inter-day precisions of the assay, were determined in this study. The validated method was then applied to the studies described in the following chapters.

Chapter 3 investigates the acute (three-day) and short-term (three-month) changes of human plasma BAs, FGF19, along with other metabolic profiles after bariatric surgeries. T2DM patients scheduled for either gastric bypass (GBP, n=8) or sleeve gastrectomy (SG, n=11) from three hospitals in the Auckland region (Auckland City Hospital, North Shore Hospital, and Middlemore Hospital) were recruited for the study. We hypothesised that the acute influences of two types of surgeries on fasting and postprandial total BAs, as well as fasting FGF19 levels and metabolic parameters, might be different. We hypothesised that the changes in BAs and FGF19 would be common to both types of bariatric surgery. The modifications of BAs and FGF19 at both early time points after operations would be significantly correlated with body weight and also glucose and lipid metabolisms.

Chapter 4 studies the effects of probiotic supplementation *L. rhamnosus* HN001 (HN001) on glucose, lipid, and BA metabolism associated with GDM in pregnant women during early pregnancy. 423 women in Auckland and Wellington, New Zealand, were recruited for the study and randomised to daily consume HN001 (n=212) or placebo (n=211) from 14-16 weeks' to 24-30 weeks' gestation. We hypothesise that improved glycemic control, as well as lipid metabolism would be observed in the HN001 intervention group. The BA pool would be modified by the probiotic treatment, in which an increase of primary- or secondary- unconjugated BAs would be seen, whereas a decrease of conjugated BAs would be seen.

Chapter 5 is an overall conclusion, which summarises the main findings obtained from the investigations. Proposed researches for further study are also pointed out in this chapter.

Chapter 2

Validation and optimisation of a liquid chromatography-tandem mass spectrometry method for determination of human plasma bile acid metabolic profiles

Abstract

Aims

To validate and optimise an established liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the analysis of bile acids (BAs).

Materials and methods

The separations of standard and human plasma-derived BAs were achieved using a reverse-phase C18 column (2.1 × 150 mm, 2.7 μm) with gradient elution of mobile phase A (5 mM ammonium acetate, 0.012 % formic acid) and mobile phase B (methanol, 5 mM ammonium acetate, 0.012 % formic acid). The column was kept at 45℃, and the flow rate was 0.3 mL min^{-1}. Quantitative data were gained in the multiple reaction monitoring (MRM) mode based upon the product ion parameters for each BA. The injection volume was 10 μL, and the elution was quantified by internal standard curves.

Results

We optimised the LC condition to achieve baseline separation of 15 BA standards. The analysis achieved high stability, with retention-time and area coefficients of variation (CV) of 0.7 ± 0.6 % and 6.3 ± 3.4 %, respectively. The correlation coefficients for the internal standard curves ranged from 0.9924 to 0.9999. The precisions of BAs were represented by CV areas of 9.5 ± 6.4 % for intra-day and 12.8 ± 7.4 % for inter-day analyses.

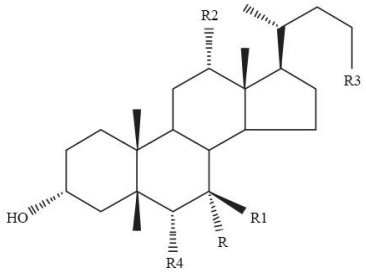
Conclusions

Based on encouraging experimental outcomes, the established separation method was validated for determining and quantifying profiles of human plasma BAs.

Keywords: Bile acids; Mass spectrometry; HPLC; LC-MS/MS.

2.1 Introduction

Bile acids are a group of acid sterols synthesised from cholesterol in the liver (Heubi, Setchell, & Bove, 2007). Two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) are primarily synthesised in hepatocytes. Enterohepatic circulation of BAs covers the processes including secretion of primary BAs into bile mainly as their glycine and taurine conjugates, and subsequently a release into the intestine in which primary BAs can be further modified by intestinal bacteria and converted into secondary BAs (shown in Figure 4.1) (Humbert et al., 2012). The alteration of enterohepatic circulation, and subsequent potential accumulation of unusual BAs, is thought to induce severe diseases (Sundaram, Bove, Lovell, & Sokol, 2008). Therefore, BAs serve as useful biomarkers for the diagnosis of many diseases given that either the level or the ratio of the different classes of BAs can yield important information about the nature of the disease under investigation (Brouwers et al., 2015; Walters, 2014). Studies of the BA metabolic profile in human body fluids is essential not only for disease diagnoses but also for therapy monitoring (Sergi et al., 2012). For example, maternal total BA levels were suggested as an appropriate indicator of the risk of various adverse perinatal outcomes (Cui, Zhong, Zhang, & Du, 2017).



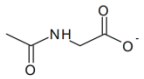
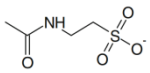
BA species	R	R1	R2	R3	R4
CA	-OH	-H	-OH		-H
CDCA	-OH	-H	-H		-H
DCA	-H	-H	-OH		-H
LCA	-H	-H	-H		-H
UDCA	-H	-OH	-H		-H
HDCA	-H	-H	-H		-OH
Glycine-conjugated					
Taurine-conjugated					

Figure 2.1. Chemical structure of bile acids and their conjugates.

The analysis of BA metabolism requires methods which not only account for the complexity of this structurally diverse group of compounds but also possess a high detection sensitivity and reliability due to the relatively small amounts found in body fluids. Methods to identify BAs include gas chromatography (GC) (Bonazzi, Calaresu, & Galeazzi, 1984), gas chromatography coupled to tandem mass spectrometry (GC-MS) (Kumar et al., 2011) and high-performance liquid chromatography (HPLC) (Kakiyama et al., 2014). GC requires gasification of BAs, which is difficult to achieve due to their high boiling points (A. Zhang, Sun, Wang, Han, & Wang, 2012). HPLC is not

applicable for the detection of BAs present at micromolar concentrations such as in biological samples because BAs have a relatively weak UV absorption (A. Zhang et al., 2012). Among all of the proposed methods being reported, the coupling of liquid chromatography (LC) with a mass spectrometer (MS) is desirable (Becker, Kortz, Helmschrodt, Thiery, & Ceglarek, 2012). To enhance the detection sensitivity and improve the signal-to-noise ratio, methods were proposed using a combination of two mass analysers dedicated to processing both pseudo-molecular ions of each BA and their product ions generated by the spectrometer fragmentation (Tagliacozzi et al., 2003). To date, a series of methods have been established using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in order to analyse BAs in human plasma without inducing derivatisation (Alnouti, Csanaky, & Klaassen, 2008; Han et al., 2014; Scherer, Gnewuch, Schmitz, & Liebisch, 2009). For routine quantification of clinical samples, LC-MS/MS appears to be a suitable method for screening BA profiles. Validation of the previously established methods will help to study BA functions in detail and may lead to a newly optimised method with an improved detection sensitivity, reproducibility and reliability.

A proposed method for analysis of BAs (Tagliacozzi et al., 2003) was validated and optimised. The presented method was then applied to analysis of human plasma BA metabolic profiles originating from different studies (see Chapter 3 and Chapter 4).

2.2 Materials and methods

2.2.1 Bile acid standards used in this study

Table 2.1 lists all 16 BA standards used in the study's analyses. Upon receipt, all chemical compounds were stored at -20℃.

Table 2.1. Bile acid standards used in this study. Abbreviations and applicable information are provided.

Number	Bile acids	Abbreviations	Catalogue ID	Suppliers	Molecular Weight	Amounts
1	Chenodeoxycholic acid	CDCA	C0940-000	Steraloids, USA	392.57	5mg
2	Cholic acid	CA	C1900-000	Steraloids, USA	408.57	5mg
3	Cholic acid-d4	CA-d4	C1900-015	Steraloids, USA	412.6	10mg
4	Deoxycholic acid	DCA	C1070-000	Steraloids, USA	392.57	5mg
5	Glycocholic acid	GCA	C1925-000	Steraloids, USA	465.62	5mg
6	Glycochenodeoxycholic acid	GCDCA	C0960-000	Steraloids, USA	449.62	5mg
7	Glycodeoxycholic acid	GDCA	C1085-000	Steraloids, USA	449.62	5mg
8	Glycoursodeoxycholic acid	GUDCA	C1025-000	Steraloids, USA	449.62	5mg
9	Hyodeoxycholic acid	HDCA	C0860-000	Steraloids, USA	392.57	5mg
10	Lithocholic acid	LCA	C1420-000	Steraloids, USA	376.57	5mg
11	Taurochenodeoxycholic acid	TCDCA	C0990-000	Steraloids, USA	499.7	5mg
12	Tauroursodeoxycholic acid	TUDCA	C1052-000	Steraloids, USA	515.7	5mg
13	Taurodeoxycholic acid	TDCA	C1160-000	Steraloids, USA	499.7	5mg
14	Taurohyodeoxycholic acid	THDCA	C0890-000	Steraloids, USA	499.7	5mg
15	Taurolithocholic acid	TLCA	C1470-000	Steraloids, USA	483.71	5mg
16	Ursodeoxycholic acid	UDCA	C1020-000	Steraloids, USA	392.57	5mg

2.2.2 Materials required for the analysis of bile acids

The materials needed for extracting human plasma samples or performing LC-MS/MS analysis are provided in Table 2.2.

Table 2.2. Materials required for the study. Suppliers and relevant information are provided.

Number	Materials	Suppliers
1	InfinityLab Poroshell 120 C18 column (2.1×150 mm, 2.7 μm)	Agilent Technologies, USA
2	Methanol, Optima™ LC/MS Grade	Fisher scientific, USA
3	Acetonitrile, Optima™ LC/MS Grade	Fisher scientific, USA
4	Formic acid 98% - 100%for LC-MS LiChropur®	Sigma-aldrich, NZ
5	Ammonium acetate, eluent additive for LC-MS	Sigma-aldrich, NZ
6	2ml Amber Screw Cap Vial with Write-on Spot & Fill Lines, Wide Opening	Interlab, NZ
7	9mm Blue Cap, Short Screw Thread, Polypropylene	Interlab, NZ
8	Insert 250 μL glass tapered for 2 mL vials	Thermo Fisher, NZ

2.2.3 Preparation of bile acid stock standards

BAs were dissolved in 50 % methanol in 1.5 mL Eppendorf tubes to reach a final concentration of 10 g L^{-1} for each BA. Stock standards were aliquot into 2 mL amber screw-cap vials and stored at -20℃.

2.2.4 Preparation of bile acid working solutions

BA working solutions of 200 mg L^{-1} were prepared in 50% methanol for individual BA standards. A mixture containing all BA analytes (excluding internal standard) was also prepared in similar fashion.

2.2.5 Preparation of bile acid concentration series

Working solutions for the BA mixed standard were diluted in 50% methanol to reach the final concentration series as 10000, 5000, 2500, 1250, 625, 312.5, 156.25, and 78.125 μg L^{-1}. CA-d4 served as an internal standard and was added into all calibrations at a final concentration of 10 mg L^{-1}. BA dilution series were stored at -20℃ after preparation and were placed at 4℃ one day prior to their analysis. The ratio of area under the curve (AUC) between the internal standard and each individual BA standard was utilised for quantification of BAs. Analytical sensitivity of each BA was identified from the slope of the calibration curve, in which lower sloped curve represented lower detection sensitivity. The determination of the limit of detections (LOD) was based on a signal-to-noise ratio (S/N) approach. The measured signals of samples with known concentrations of analyte were compared with those of blank samples, by which the minimum concentration at which the analyte can be reliably detected was established. LOD in the LC-MS/MS assays was estimated according to a signal-to-noise ratio of 3:1 (S/N=3).

2.2.6 Extraction of human plasma samples

Human plasma samples were kept at -80℃ prior to analysis. After thawing at 4 ℃, the samples were vortexed for at least 1 minute in order to dissolve most materials in the matrix. 800 μL of acetonitrile was added to 250 μL of each plasma sample in Eppendorf tubes, followed by vortex-mixing for 1 minute. All proteins in the samples were likely precipitated in this step. Samples were then centrifuged at 13000 g for 15 minutes. 900 μL of the clear supernatant was transferred to a new Eppendorf tube after centrifugation and was evaporated to complete dryness with applied airflow. After evaporation, 250 μL of 50 % methanol was added to the tube. The samples were centrifuged again at the same settings to ensure clearance of any undissolved materials. Samples were aliquotted into 2 mL amber screw-cap vials and stored at -20℃ before measurement.

2.2.7 LC-MS/MS phase compositions

The compositions of the mobile and stationary phases are listed in Table 2.3. MilliQ water (or methanol), ammonium acetate, and formic acid are combined to define solvents A and B.

Table 2.3. Mobile phase and stationary phase compositions.

Solvent	Description	Contents
A	Mobile phase	MilliQ water, 5mM ammonium acetate, 0.012% formic acid
B	Stationary phase	Methanol, 5mM ammonium acetate, 0.012% formic acid

2.2.8 Preparation of LC-MS/MS worklist

The worklist required for conducting LC-MS/MS analysis was compiled in the Agilent MassHunter Acquisition Worklist Editor (Agilent, Canada). Three no injection blanks were placed at the start of the worklist with the purpose of equilibrating the column to the initial experimental conditions as well as flushing out any accumulated contaminants from the previous run. A shutdown script was added at the end of the worklist to put the instrument on standby.

2.2.9 Methodology of LC-MS/MS analysis

The parameters included in the LC-MS/MS analysis for this study are described in Table 2.4. BAs were analysed using an Agilent triple quadrupole mass spectrometer (6420 Triple Quadrupole LC/MS, Agilent, Canada) operated in the ion evaporation mode with a gas temperature of 325℃, a gas flow of 6.00 L min^{-1} and a sprayer voltage of -4500 V. The column was kept at 45℃ and the flow was set at 0.3 mL min^{-1}. The injection volume for each analyte was set at 10 µL.

Table 2.4. Parameters included in the liquid chromatography–tandem mass spectrometry method.

Parameters	Details
Flow	
Flow speed (mL min^{-1})	0.300
Low pressure limit (bar)	2.00
High pressure limit (bar)	590
Maximum flow gradient (mL min^{-2})	3.00
Left temperature (℃)	45.0±2.00
Right temperature (℃)	45.0±2.00
Injection	
Injection mode	Standard injection
Injection volume (μL)	10.0
Ion source parameters	
Sprayer voltage (V)	-4500
Gas temperature (℃)	325
Gas flow (L min^{-1})	6.00

The analysis included two segments and the electrospray ionisation (ESI) was operated in the negative mode (Table 2.5). Chromatographic and spectral interpretation of the analytes were acquired by MassHunter Workstation QQQ quantitative Analysis (Agilent, Canada).

Table 2.5. Setting of the analytical time segments.

Time segment	Start time (min)	Scan type	Ion mode	Div value	Delta EMV
1	3.50	MRM	ESI	To MS	200
2	14.7	MRM	ESI	To MS	200

Quantitative data were obtained from the multiple reaction monitoring (MRM) mode. The establishment of the acquisition method (Table 2.6), which comprises determining the precursor ion (Prec ion) and product ion (Prod ion) according to the most intense fragment identified for each BA, determining the fragmentation energy (Frag.), and the energy of collision cell (CE), was based on the suggestions from the previous study (Tagliacozzi et al., 2003). Data points were processed using MassHunter Workstation Qualitative Analysis Software B.06.00 (Agilent, Canada). The second time-segment was explicitly set for collecting the signal of LCA with a dwell time (Dwell) of 600 ms in consideration of its low detection sensitivity (Tagliacozzi et al., 2003).

Table 2.6. Details for bile acid mass spectrometry

BAs	Time segment	Prec ion (m/z)	Prod ion (m/z)	Dwell (ms)	Frag. (V)	CE (V)	Polarity
THDCA & TUDCA & TCDCA & TDCA	1	498.29	498.29	60	95	0	Negative
THDCA & TUDCA & TCDCA & TDCA	1	498.29	79.90	60	95	74	Negative
TLCA	1	482.29	482.29	60	95	0	Negative
TLCA	1	482.29	79.90	60	95	86	Negative
GCA	1	464.30	464.30	60	90	0	Negative
GCA	1	464.30	74.00	60	90	38	Negative
GCDCA & GDCA & GUDCA	1	448.31	448.31	60	105	0	Negative
GCDCA & GDCA & GUDCA	1	448.31	74.00	60	105	36	Negative
CA	1	407.28	407.28	60	240	0	Negative
DCA & CDCA & UDCA & HDCA	1	391.28	391.28	60	200	0	Negative
LCA	2	375.29	375.29	600	260	0	Negative

Instrument optimisation was performed automatically using the "AutoTune" functionality included in the instrument's protocol. Elutions were quantified by internal standard curves after a gradient procedure of 24 minutes in total (Table 2.7).

Table 2.7. The gradient procedure of separation of bile acids

Time (min)	**Solvent A (%)**	**Solvent B (%)**
0.0	50.0	50.0
7.0	20.0	80.0
14.0	5.0	95.0
16.0	50.0	50.0
24.0	50.0	50.0

2.3 Results

2.3.1 Chromatograms of bile acids

Figure 2.2 showed the separation of a mixture of BA standards at a level of 10 mg L^{-1} per component. A good chromatographic resolution of all the 15 BAs analysed was achieved, even for co-eluting BAs.

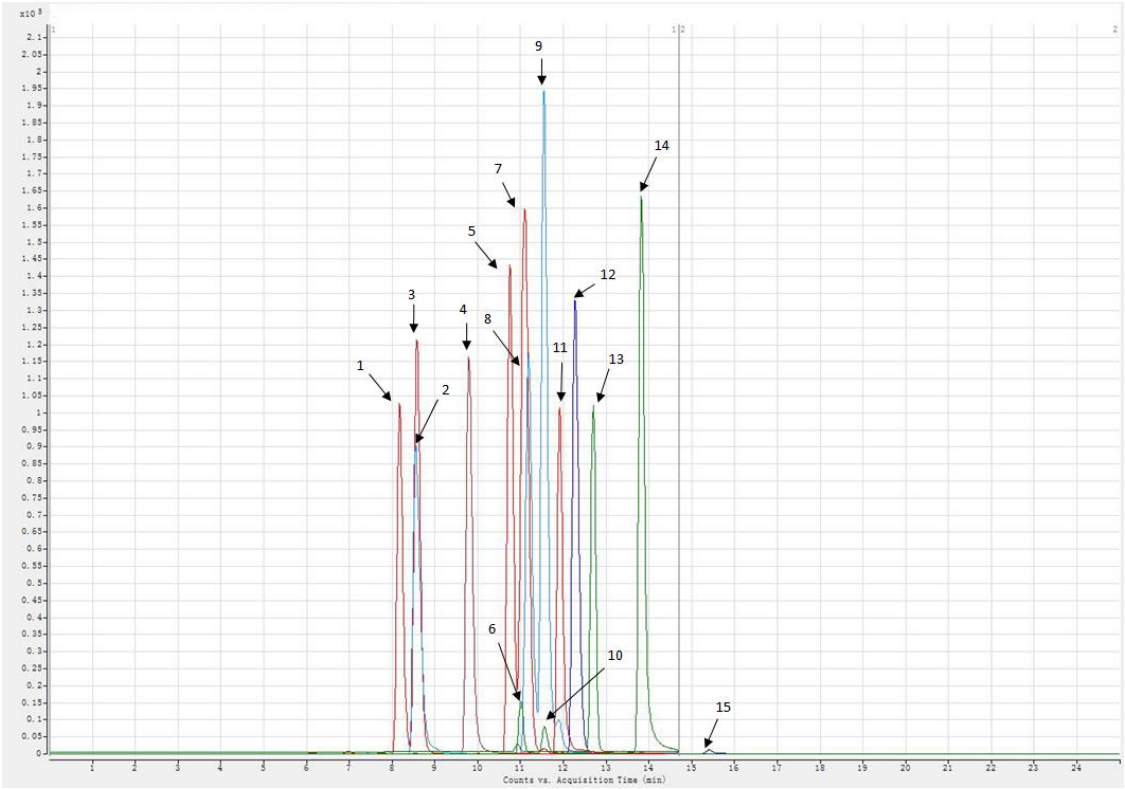


Figure 2.2. The chromatogram of bile acid standards. (1, TUDCA; 2, GUDCA; 3, THDCA; 4, GCA; 5, TCDCA; 6, UDCA; 7, TDCA; 8, GCDCA; 9, GDCA; 10, HDCA; 11, CA; 12, TLCA; 13, CDCA; 14, DCA; 15, LCA)

2.3.2 Instrumental repeatability and reliability

A mixed BA standard at a concentration of 10 mg L^{-1} for each component acid was injected and analysed three times to investigate the reproducibility of the retention time as well as peak area of each individual BA. As listed in Table 2.8, the analysis achieved

good repeatability and reliability, with the retention-time and area coefficients of variation (CV) of 0.7 ± 0.6 % and 6.3 ± 3.4 %, respectively.

Table 2.8. Instrumental retention times of bile acids (n = 3). AUC values are also presented. Both retention time and AUC data yielded mean, SD, and CV information for assessment.

Bile acids		1	2	3	Mean	SD	CV (%)
THDCA	t (min)	8.45	8.33	8.36	8.38	0.06	0.75
	Area ($\times 10^3$)	1.43	1.42	1.66	1.50	0.13	8.76
TUDCA	t (min)	7.86	7.89	7.94	7.90	0.04	0.51
	Area ($\times 10^6$)	1.08	1.22	1.17	1.16	0.07	6.10
TCDCA	t (min)	10.55	10.57	10.61	10.58	0.03	0.31
	Area ($\times 10^4$)	6.09	5.90	5.73	5.91	0.18	2.98
TDCA	t (min)	10.92	10.95	10.98	10.95	0.03	0.27
	Area ($\times 10^4$)	1.92	1.54	1.77	1.74	0.19	11.03
TLCA	t (min)	12.17	12.13	12.12	12.14	0.03	0.22
	Area ($\times 10^4$)	2.07	2.06	2.06	2.06	0.01	0.27
GCA	t (min)	9.50	9.60	9.64	9.58	0.07	0.75
	Area ($\times 10^4$)	2.10	2.05	2.00	2.05	0.05	2.45
GCDCA	t (min)	11.08	11.02	11.34	11.15	0.17	1.53
	Area ($\times 10^4$)	2.83	3.01	2.59	2.81	0.21	7.55
GDCA	t (min)	11.34	11.77	11.81	11.64	0.26	2.24
	Area ($\times 10^4$)	2.14	2.34	2.54	2.34	0.20	8.71
GUDCA	t (min)	8.37	8.33	8.22	8.31	0.08	0.94
	Area ($\times 10^6$)	2.24	2.25	1.89	2.13	0.21	9.66
CA	t (min)	11.73	11.77	11.81	11.77	0.04	0.34
	Area ($\times 10^4$)	3.14	3.42	3.40	3.32	0.16	4.72
DCA	t (min)	13.80	13.74	13.70	13.75	0.05	0.37
	Area ($\times 10^5$)	5.52	5.62	5.41	5.52	0.11	1.93
CDCA	t (min)	12.67	12.53	12.79	12.66	0.13	1.03
	Area ($\times 10^5$)	4.47	4.99	4.83	4.77	0.27	5.59
UDCA	t (min)	10.79	10.85	10.89	10.84	0.05	0.46
	Area ($\times 10^4$)	1.04	1.23	1.24	1.17	0.11	9.74
HDCA	t (min)	11.44	11.43	11.37	11.41	0.04	0.33
	Area ($\times 10^2$)	6.70	8.35	7.67	7.57	0.83	10.95
LCA	t (min)	15.30	15.35	15.41	15.35	0.06	0.36
	Area ($\times 10^4$)	1.21	1.15	1.27	1.21	0.06	4.63

2.3.3 Determination of bile acid standard curves

The linearity of response was tested by analysing BA standard solutions prepared at a dilution series ranging from 78.125 to 10000 μg L^{-1}. The concentration unit of BAs was converted to μmol L^{-1} (μM) when performing the quantitative assay. As shown in Table 2.9, the correlation coefficients (r^2) for the internal standard curves ranged from 0.9924 to 0.9999. HDCA and LCA had the lowest analytical sensitivities among all of the explored BAs.

Table 2.9. Linearity of bile acids response to method of analysis.

Bile acids	Linearity range (μmol L^{-1})	Calibration curves	r^2	LOD (μmol L^{-1})
THDCA	0.043~22	y=0.872x-0.014	0.9934	0.002
TUDCA	0.037~9.6	y=0.720x+0.012	0.9978	0.002
TCDCA	0.043~11	y=1.137x+0.007	0.9985	0.005
TDCA	0.043~22	y=1.259x+0.013	0.9974	0.008
TLCA	0.040~21	y=0.919x+0.012	0.9953	0.008
GCA	0.042~21	y=0.841x+0.009	0.9959	0.007
GCDCA	0.043~22	y=1.103x+0.019	0.9924	0.005
GDCA	0.043~5.6	y=1.121x+0.001	0.9949	0.005
GUDCA	0.043~11	y=1.101x+0.006	0.9983	0.005
CA	0.050~24	y=0.706x+0.000	0.9999	0.006
DCA	0.050~25	y=1.311x+0.012	0.9973	0.005
CDCA	0.050~25	y=1.176x-0.001	0.9999	0.004
UDCA	0.050~25	y=0.094x+0.000	0.9999	0.006
HDCA	0.050~25	y=0.056x-0.000	0.9999	0.010
LCA	0.053~27	y=0.006x-0.000	0.9959	0.008

2.3.4 Precision of the LC-MS/MS assay

Analysis of BAs in human plasma extractions was repeated in triplicate on the same day to study the intra-day CV of each BA analyte. For the investigation of inter-day CV, the same plasma extraction was injected on three different days in triplicate. According to Table 2.10, the CV of BAs ranged from 3.2 to 25.5 % for intra-day and 2.8 to 28.9 % for inter-day sample sets. The concentrations of HDCA and LCA in the tested plasma samples were lower than the detection limits of the methodology.

Table 2.10. Intra-day and inter-day precisions of the LC-MS/MS analysis.

Bile acids	**Intra-day values (mean ± SD, µmol L^{-1})**	**CV (%)**	**Inter-day values (mean ± SD, µmol L^{-1})**	**CV (%)**
THDCA	0.130±0.033	25.5	0.157±0.045	28.9
TUDCA	0.039±0.003	6.9	0.044±0.009	19.7
TCDCA	2.330±0.313	13.4	2.245±0.382	17.0
TDCA	1.284±0.121	9.4	1.348±0.038	2.8
TLCA	0.041±0.001	3.2	0.043±0.005	10.9
GCA	3.044±0.115	3.9	2.915±0.299	10.3
GCDCA	3.245±0.209	6.4	3.240±0.205	6.3
GDCA	3.041±0.244	8.0	3.085±0.317	10.3
GUDCA	0.436±0.025	5.8	0.450±0.032	7.2
CA	1.725±0.337	19.6	1.942±0.099	5.1
DCA	0.820±0.048	5.9	0.871±0.130	15.0
CDCA	1.871±0.122	6.6	2.195±0.474	21.6
UDCA	0.728±0.063	8.6	0.760±0.082	10.8
HDCA	<LOD	-	<LOD	-
LCA	<LOD	-	<LOD	-

2.4 Discussion

This study has presented the validated chromatography and MS spectra of BAs taken from human plasma samples. The analytical parameters introduced by an established method were optimised for flow speed, injection volume, column temperature, and mobile phase gradient composition for achieving high level detection and quantification standards under current experimental conditions. A good separation of all of the 15 BAs was achieved by C18-reverse phase chromatography. It can be noted that BAs were eluted sequentially in the order of each tauro-conjugated, glycine-conjugated and unconjugated form as a function of their hydrophilicity/hydrophobicity balance (Humbert et al., 2012). Specific scanning of both pseudo-molecular and product ion pairs of BAs in the MRM mode allowed the determination of overlapping elution peaks. The analysis had a good reproducibility with the retention-time CV < 2.2 % and the peak area CV < 11.0 %. To take any analyte loss throughout the assay into consideration, quantification of BAs was accomplished by the use of an internal standard. Linearity values were in the calibration range, with correlation coefficients ranging from 0.9924 to 0.9999. As presented, we did not achieve a good intra-day or inter-day precision in this study, with CV < 25.5 % and CV < 28.9 % respectively. The accuracy of the assay might be improved by applying a set of each stable-isotope labelled BA as internal standards rather than a single standard when performing the analysis (Scherer et al., 2009).

In conclusion, we validated and optimised a novel LC-MS/MS method to analyse BAs in human plasma. The presented method demonstrated good reproducibility and reliability. The wide range of detection sensitivity allows for assays of both prominent and minor species of BAs which might be potentially relevant as biomarkers of disease.

Chapter 3

Acute changes of bile acids and FGF19 after sleeve gastrectomy and gastric bypass in type 2 diabetes

Abstract

Context

Gastric bypass (GBP) and sleeve gastrectomy (SG) are both effective bariatric treatments that cause sustained weight loss as well as improvement of type 2 diabetes mellitus (T2DM). The change of bile acids (BAs) has been suggested to play a vital role in the remission of T2DM after bariatric surgery, and is associated with multiple metabolic improvements.

Aims

The aim of this study was to investigate the acute and short-term effect of GBP and SG on BA compositions in obese individuals with T2DM. Additionally, assessments regarding the potential contribution of changes in BAs and fibroblast growth factor19 (FGF19) to any metabolic improvements were carried out.

Methods

The levels of plasma BA compositions, FGF19 and various metabolic indices were measured at three days and three months after GBP and SG in 19 obese patients (GBP=8, SG=11) with T2DM.

Results

Body weight loss was observed after both GBP and SG 3-month postoperatively, with no significant difference between the two intervention groups (15.0±3.1% vs 13.9±5.2%, P=0.761). A significant increase in both fasting and postprandial BAs, as well as fasting FGF19 levels was observed three days after GBP and SG, followed by a reduction in levels by the three month time point. The concentrations of most of the BA compositions in both surgery groups at three months (total, primary, secondary, conjugated, glycine-conjugated, secondary-conjugated and 12α-OH BAs for both surgeries; unconjugated, primary-conjugated, secondary-unconjugated, 12α-OH and

non-12α-OH BAs for GBP; taurine-conjugated, primary-unconjugated BAs for SG) were significantly higher than the pre-surgery values. At three days, fasting and prandial increases in secondary (r=0.57, P=0.02; r=0.58, P=0.01), conjugated (r=0.50, P=0.01; r=0.48, P=0.04), glycine-conjugated (r=0.52, P=0.05; r=0.46, P=0.05) and secondary-conjugated (r=0.53, P=0.02; r=0.60, P=0.01) BAs were correlated with decreases in the postprandial states of glucose (defined by area under the curve (AUC) over 120 minutes ($AUC_{0\text{-}120min}$)). The increases in fasting and prandial taurine-conjugated BA were correlated with the decreases of both basal insulin secretion rate (r=0.47, P=0.04; r=0.48, P=0.04) and C-peptide level (r=0.45, P=0.05; r=0.47, P=0.04). At three months, the increases in both fasting and prandial 12α-OH BA were correlated with the decreases of glucose AUC (r=0.46, P=0.05; r=-0.41, P=0.04). Fasting and prandial increases in secondary (r=0.51, P=0.03; r=0.48, P=0.04), secondary-conjugated (r=0.52, P=0.02; r=0.51, P=0.03) and non-12α-OH (r=0.51, P=0.02; r=0.58, P=0.01) BA were also found to correlate with the increases in Stumvoll insulin sensitivity index. The increases in fasting FGF19 level was only found to have a weak correlation with the increases in free fatty acids (r=0.06, P=0.02) at three days.

Conclusions

This study provides evidence regarding the acute and short-term modification of BAs induced by different types of bariatric surgery in obese individuals with T2DM. Both GBP and SG resulted in similar increases in many BA species as early as 3 days and sustained at 3 months. Rises in secondary BA and conjugated forms were correlated with early improvements in glucose metabolism at 3 days, while these and 12α-OH BA were correlated with improvements in glucose metabolism at 3 months, suggesting they may contribute to the T2DM remission observed.

Keywords: Bile acids; Type 2 diabetes mellitus; FGF19; Gastric bypass; Sleeve gastrectomy.

3.1 Introduction

Bariatric surgery is currently the most effective treatment for obesity, and it is thought to relate with considerable improvement in metabolic co-morbidities, such as type 2 diabetes mellitus (T2DM) (Schauer et al., 2014). While there are a number of distinct types of bariatric surgery that have been developed, the two most popular bariatric procedures are gastric bypass (GBP) and sleeve gastrectomy (SG) (Chang et al., 2014). Both operations have been reported to have effectiveness in inducing long-term reduction in body weight and blood glucose levels, which are thought to associate with the remission of obesity-related T2DM (Mingrone et al., 2015; Schauer et al., 2017). Interestingly, amelioration of glycemic control often occurs before significant weight loss after intervention (Madsbad, Dirksen, & Holst, 2014), indicating that weight loss-independent mechanisms underpinning changes in glucose metabolism might be related to a direct effect of bariatric surgery contributing to the resolution of T2DM. However, the underlying mechanisms implicated in the metabolic improvements associated with bariatric surgery remain poorly understood.

Bile acids (BAs) serve as signalling molecules that modulate numerous metabolic processes, including glucose and lipid metabolism and energy expenditure (Fiorucci, Mencarelli, Palladino, & Cipriani, 2009). Many of these metabolic effects are modulated through activating dedicated BA receptors such as the nuclear farnesoid X receptor (FXR) on mucosal cells of the terminal ileum (Penney, Kinross, Newton, & Purkayastha, 2015). After a meal, BAs release into the intestine, bind to and activate FXR, and thereby stimulate synthesis of fibroblast growth factor 19 (FGF19) (Kir, Kliewer, & Mangelsdorf, 2011). Interestingly, FGF19 is also recognised as a secreted factor that mediates the inhibition of Cholesterol 7α-hydroxylase (CYP7A1), an enzyme that catalyses the first and the rate-limiting step in the synthesis of primary BAs, and it is the key target of the feedback regulation. BAs inhibit the transcription of the CYP7A1 gene, accordingly down-regulate their own synthesis (Kir et al., 2011). Gerhard et al. reported higher increases in serum BA and FGF19 concentrations in those T2DM patients who went into remission compared with those without diabetes and those who did not show remission after Roux-en-Y gastric bypass (RYGB), implicating the FGF19-CYP7A1-BA pathway in the remission of T2DM following bariatric surgery (Gerhard et al., 2013). However, the timing of the changes in mechanism BAs and FGF19 corresponding with the resolution of T2DM has not been fully elucidated.

Whether changes of FGF19 levels correlate with any metabolic improvements after the operations are also unclear.

Several studies have reported that fasting and postprandial circulating BA levels are increased after GBP or SG in cohorts without T2DM or with mixed T2DM status (Nakatani et al., 2009; Nemati et al., 2018a; Patti et al., 2009; Ryan et al., 2014). Additionally, there is some evidence that FGF19 levels increase following RYGB (Gerhard et al., 2013; Sachdev et al., 2016) as well as after SG (Shimizu, Hatao, Imamura, Takanishi, & Tsujino, 2017). A number of research findings show a general increase in BAs long term after GBP or SG, but rare researches have clarified how early after surgery the increases in the efficacy of the operation for early improvement of T2DM. Dutia et al. (2015) observed a reduction or no change at one month after RYGB surgery for most BA composite variables among obese women with T2DM. Escalona et al. (2016) reported a decrease in BA synthesis after laparoscopic sleeve gastrectomy at one month among obese patient without diabetes. In contrast, Pournaras et al. (2012) reported that fasting BAs were increased as early as four days after gastric bypass. Nakatani et al. (2009) found that total BAs had significantly increased with GBP and SG groups at both one and three months after surgery among obese individuals (with or without diabetes). Furthermore, the relationship between BAs versus body weight, glucose-related (as well as lipid-related) parameters is debated. Thus, gaps exist in understanding the influence of bariatric surgery on BA metabolism and its association with obesity and T2DM. A clear understanding of the short-term change of bile acid levels corresponding with any metabolic improvements after surgery may help to better elucidate whether BA are important in the mechanism of action of bariatric surgery, and may lead to novel treatments for T2DM.

The objective of this study is to investigate the acute effect of GBP and SG on circulating BA levels and composition in the fasting and postprandial states, and to assess the fasting FGF19 levels measured at 3 days and 3 months, relative to pre-operative values, in obese individuals with T2DM. We also investigated relationships between BAs and three criteria (body weight, and glucose and lipid metabolism) in the above population. This study makes a unique contribution to the existing literature as it focuses particularly on the subjects with T2DM, includes two different types of operations (GBP and SG), assessed in the acute period (after 3 days and 3 months), and carefully characterises changes in both fasting and postprandial BA compositions and

FGF19, in relation to alterations in glucose and lipid related profiles after bariatric surgery. Considering that GBP and SG have distinct effects on gastric size and nutrient flow, we hypothesised that the acute influences of two types of surgeries on fasting and postprandial total BAs, as well as fasting FGF19 levels and metabolic parameters, might be different to some extend. We hypothesised that if certain BA and FGF19 are essential to achieving metabolic improvements seen after bariatric surgery, then these changes would be common to both types of bariatric surgery, and a significant correlation between BAs and FGF19 versus body weight, and glucose and lipid metabolisms, would emerge.

3.2 Materials and methods

3.2.1 Patients

Patients with T2DM scheduled for either GBP (n = 8) or SG (n = 11) in three different hospitals in the Auckland region (Auckland City Hospital, North Shore Hospital and Middlemore Hospital) between August 2010 and March 2012 were recruited for the study. Patients with T2DM, between 25 and 55 years of age, were eligible for surgery if BMI was at least 35 kg/m^2. Patients receiving insulin therapy, incretin hormone-based therapy or diet-controlled T2DM were excluded. All subjects consented to a 75-g oral glucose tolerance test (OGTT) one day before, three days and three months after surgery. Plasma samples were collected from antecubital veins (after inserting a cannula) during fasting and at 30, 60, 90 and 120 minutes after the oral glucose load. Samples were immediately centrifuged at 4 °C, and subsequently stored as aliquots at -80 ºC until analysis.

3.2.2 Surgical Protocol

All subjects scheduled for either GBP or SG were prescribed a hypocaloric diet with three servings of Optifast® (152 cals) plus vegetables daily for three weeks before surgery. Surgery was conducted after an 8-h overnight fast. Eight patients underwent GBP with a 100-cm antecolic Roux limb with hand-sewn pouch jejunostomy, or a 60-cm biliopancreatic limb with a hand-sewn small bowel anastomosis. Eleven patients underwent SG, involving a longitudinal resection of the stomach against a 32-French bougie from just lateral to the angle of His to 2 cm proximal to the pylorus. After surgery, patients were administered PlasmaLyte® intravenous fluids 1 L every 24 h, until oral fluid intake began at approximately 48 h post-surgery.

3.2.3 Biochemical analysis

Samples from fasting and all postprandial time points were used to measure BAs, glucose, insulin, and C-peptide levels. Area under the curve (AUC) analyses of the indices over 120 minutes ($AUC_{0\text{-}120min}$), the definite integral in the plot of the indices in

plasma versus time, are the basis of the study's data collections. Plasma lipids and FGF19 were only measured in the fasting state.

All 13 individual BAs were measured by a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method described previously (Tagliacozzi et al., 2003) with slight modifications to achieve optimal detection. The LC-MS/MS system consists of an HPLC Agilent 1200 series apparatus and the Agilent 6420 Triple Quadrupole MS/MS (Agilent Technologies, Santa Clara, CA, USA). BAs analysed include chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and each of their glycine (G-) and taurine (T-) conjugates.

FGF19 levels were measured by a commercially available human FGF19 sandwich ELISA kit (RayBiotech, Inc. GA, USA).

Lipid profile, plasma glucose, insulin and C-peptide amounts were determined by an autoanalyser (Roche Diagnostics, Basel, Switzerland) based on Roche's manufacturer protocols.

3.2.4 Calculation

BA compositions were classified based on their site of synthesis (primary vs secondary), conjugation state (conjugated vs unconjugated), or 12α-hydroxylation (12α-OH vs non-12α-OH). The molar sum of BA concentrations in each category was used to determine the levels of each BA composition. Compositions included: (1) Total BAs=all 13 BAs; (2) Primary BAs=CA, GCA, CDCA, GCDCA and TCDCA; (3) Secondary BAs=DCA, GDCA, TDCA, UDCA, GUDCA, TUDCA, LCA and TLCA; (4) 12α-OH BAs=CA, GCA, DCA, GDCA and TDCA; (5) non 12α-OH BAs=CDCA, GCDCA, TCDCA, LCA, TLCA, UDCA, GUDCA, TUDCA; (6) Conjugated BAs=all glycine- and taurine-conjugated BAs; (7) Unconjugated BAs=all unconjugated BAs; (8) Glycine-conjugated; (9) Taurine-conjugated; (10) Primary-conjugated; (11) Primary-unconjugated; (12) Secondary-conjugated; and (13) Secondary-unconjugated.

Diabetes indices for this study are the homeostatic model assessment of insulin resistance (HOMA-IR) and the Stumvoll insulin sensitivity index (ISI).

Established mathematical modelling which describes the relationship between insulin secretion and glucose concentration coupled with a model of C-peptide kinetics (Mari, Schmitz, et al., 2002; Mari, Tura, Gastaldelli, & Ferrannini, 2002), was used to obtain more sophisticated variables describing the beta cell (β cell) insulin secretory process. Based on the model, our study evaluated the β cell sensitivity to glucose (glucose sensitivity), β cell sensitivity to the rate of change of glucose (rate sensitivity) and a potentiation factor (representing a relevant potentiation of insulin secretion throughout the OGTT), basal insulin secretion rate (BSR) and the total insulin secretion (TIS) during the OGTT before and at different time points post-surgery. All indices included in the model were estimated by regularised least squares and implemented using MATLAB (The MathWorks, Natick, MA, USA) based on the measured glucose and C-peptide concentrations (Tura, Mari, Winzer, Kautzky‐Willer, & Pacini, 2006).

3.2.5 Statistical analysis

R Studio Version 1.1.414 was used for statistical analysis. Normal distribution of model residuals was tested with the Shapiro-Wilks test. For normally distributed data, the results were presented as the mean ± standard deviation (SD) unless stated otherwise. Comparisons within and between surgical groups were examined using paired and unpaired Student's t-tests for normally distributed data. For non-normally distributed data, Wilcoxon signed-ranks testing and the Mann–Whitney U test were conducted, respectively. The correlation analyses were performed using Pearson rank test. Areas under the curve were calculated according to the trapezoidal rule. Statistical significance was set at $P<0.05$ (two-tailed).

3.3 Results

3.3.1 Subject clinical characteristics before and after each intervention

Subject characteristics and postoperative changes of those characteristics are outlined in Table 3.1 and Table 3.2, respectively. There were no significant changes in either weight or BMI three days after any type of studied intervention. However, three months after surgery, body weight loss reached 18.2 ± 4.7 kg (15.0 ± 3.1%) in the GBP group and 17.3 ± 6.7 kg (13.9 ± 5.2%) in the SG group. BMI decreased significantly three months after both GBP (43.0 ± 4.7 kg m^{-2} (pre-op) vs. 36.6 ± 4.3 kg m^{-2} (3 months post-op); $P<0.001$) and SG (43.4 ± 6.1 kg m^{-2} (pre-op) vs. 37.4 ± 5.9 kg m^{-2} (3 months post-op); $P<0.001$). No significant differences were observed between GBP and SG interventions relative to reducing body weight or BMI three months postoperatively.

Table 3.1. Clinical characteristics, diabetes indices, lipid profile and fasting FGF19 before and after bariatric surgeries.

	Gastric Bypass (n=8) Metformin=5, MF=3			**Sleeve Gastrectomy (n=11)** Metformin=5, Metformin+Glipizide=3, MF=1, Ibuprofen=1, Glipizide=1		
Clinical characteristics	**Pre GBP**	**3 Days post GBP**	**3 Months post GBP**	**Pre SG**	**3 Days post SG**	**3 Months post SG**
n	8	8	8	11	11	11
Gender (men/women)	1/7	1/7	1/7	2/9	2/9	2/9
Age (years)	42.9±9.6			43.9±6.7		
Weight (kg)	120.5±14.9	120.5±14.9	102.3±12.8*†	126.6±17.0	126.6±17.0	109.2±18.5*†
Weight loss (%)	-	0.0	15.0±3.1	-	0.0	13.9±5.2
BMI (kg m^{-2})	43.0±4.7	43.0±4.7	36.6±4.3*†	43.4±6.1	43.4±6.1	37.4±5.9*†
Diabetes indices						
Fasting glucose (mmol L^{-1})	6.1±2.1	6.7±1.4	5.6±1.1	6.4±1.5	6.1±1.5	5.6±1.7
Glucose $AUC_{0\text{-}120min}$ (mol L^{-1} min^{-1})	1.3±0.5	1.07±0.4*	1.18±0.4	1.3±0.4	1.2±0.4*	1.2±0.5*

Fasting insulin (pmol L^{-1})[a]	1.8±0.5	1.8±0.4	1.9±0.4	2.1±0.3	2.0±0.2	1.2±0.3
Insulin $AUC_{0\text{-}120min}$ (nmol L^{-1} min^{-1})	31.2±16.2	32.2±21.3	52.8±18.5	45.9±20.9	36.2±16.6	62.93±50.0
C-peptide (pg mL-1)[a]	3.2±0.2	3.1±0.2	3.2±0.1[†]	3.4±0.2	3.4±0.2	3.3±0.2[*]
C-peptide $AUC_{0\text{-}120min}$ (ng mL^{-1} min^{-1})	356.4±58.2	363.3±164.3	557.8±122.8[*†]	638.4±252.3	598.2±261.3	756.2±452.3
HOMA-IR[a]	0.4±0.6	0.5±0.5	0.4±0.4	0.7±0.4	0.6±0.3	0.5±0.4
ISI composite (Stumvoll)	2.2±1.3	3.5±1.8[*]	5.1±2.4[*†]	1.1±1.3	3.0±1.1[*]	4.6±1.7[*†]
Total ISR (nmol m^{-2})	16.9±2.7	16.6±7.2	27.5±8.2[*†]	28.7±12.0	26.9±13.5	35.5±21.3
Basal ISR (pmol min^{-1} m^{-2})	59.3±24.5	47.7±19.8	65.5±14.9[†]	110.0±43.2	99.7±44.5	83.8±32.3[*]
Glucose sensitivity (pmol min^{-1} m^{-2} mM^{-1})	11.3 (9.2-19.0)	19.1 (7.7-33.7)	24.4 (14.5-32.2)[*]	18.8 (14.1-32.9)	29.1 (16.6-47.6)	33.0 (28.4-71.8)[*]
Rate sensitivity (pmol m^{-2} mM^{-1})	119.2 (2.7-193.0)	111.0 (11.0-732.1)	313.1 (174.7-447.3)	179.2 (0.0-659.9)	245.4 (1.1-786.1)	571.1 (145.0-1460.3)[*†]
Potentiation factor	1.2±0.4	1.3±0.6	1.3±0.6	0.9±0.3	0.9±0.6	1.2±0.8
Lipid profile						
FFA (mmol L^{-1})	2.4±0.9	2.3±1.2	-	2.0±1.0	2.4±0.8	-
LDL-c (mmol L^{-1})	1.8±0.5	1.6±0.6	-	2.3±0.8	2.2±0.6	-
HDL-c (mmol L^{-1})[a]	1.3±0.7	1.1±0.4[*]	-	1.0±0.3	0.9±0.2	-
Triglycerides (mmol L^{-1})	1.0 ±0.3	1.4±0.3[*]	-	2.0±1.2	1.6±0.7	-
Total cholesterol (mmol L^{-1})	3.8±0.7	3.5±0.5	-	4.3±0.9	4.0±0.8	-
Fibroblast growth factor						
Fasting FGF19 (pg mL^{-1})	118.3 ±57.3	363.6 ±131.0[*]	116.2 ±110.2[†]	173.2 ±127.8	422.0 ±243.6[*]	151.6 ±185.2[†]

Data are mean ± s.d. or median (IQR). [*] P<0.05 vs Pre-GBP or Pre-SG. [†] P<0.05 vs 3 Days post GBP or 3 Days post SG. [a] Transformed variables.

Table 3.2. The changes of clinical characteristics, diabetes indices, lipid profile and fasting FGF19 after bariatric surgeries.

Differences (Δ) vs. baseline	3 Days post Surgery		3 Months post Surgery		p-value GBP vs. SG	
	3 Days post GBP	3 Days post SG	3 Months post GBP	3 Months post SG	3 Days	3 Months
Clinical characteristics						
Δ Weight (kg)	0.0	0.0	-18.2±4.7	-17.3±6.7	NA	0.761
Δ BMI ($kg\ m^{-2}$)	0.0	0.0	-6.5±1.6	-6.0±2.5	NA	0.647
Diabetes characteristics						
Δ Fasting glucose ($mmol\ L^{-1}$)	0.6±2.3	-0.3±1.8	-0.5±1.5	-0.8±1.5	0.384	0.692
Δ Glucose $AUC_{0\text{-}120min}$ ($mmol\ L^{-1}\ min^{-1}$)	-250.7 ±215.3	-149.6 ±204.3	-143.3 ±286.6	-188.9 ±255.0	0.313	0.719
Δ Fasting insulin ($pmol\ L^{-1}$)	6.5 (-7.2-28.9)	-12.4 (-103.4-15.4)	1.2 (-6.3-23.9)	-67.2 (-97.2-1.8)	0.442	0.206
Δ Insulin $AUC_{0\text{-}120min}$ ($nmol\ L^{-1}\ min^{-1}$)	0.9±17.7	-9.7±15.2	21.6±19.2	17.0±32.7	0.176	0.729
Δ C-peptide ($pg\ mL^{-1}$)	-305.9 ±405.3	-261.8 ±1629.9	78.3±383.7	-775.5 ±713.1	0.933	**0.007**
Δ C-peptide $AUC_{0\text{-}120min}$ ($ng\ mL^{-1}\ min^{-1}$)	6.9±147.9	-40.2±223.1	201.5±130.9	117.8±266.8	0.611	0.427
Δ HOMA-IR	1.1 (-0.3-2.8)	-2.1 (-5.9-1.2)	0.1 (-0.2-1.1)	-3.9 (-5.4-0.0)	0.272	0.206
Δ ISI composite (Stumvoll)	1.3±0.7	1.9±0.8	2.8±1.9	3.5±1.6	0.100	0.415
Δ Total ISR ($nmol\ m^{-2}$)	-0.3±5.5	-1.8±9.0	10.6±7.5	6.8±12.2	0.689	0.455
Δ Basal ISR ($pmol\ min^{-1}\ m^{-2}$)	-11.7±15.2	-10.2±63.3	6.1±13.6	-26.1±28.5	0.943	**0.009**
Δ Glucose sensitivity ($pmol\ min^{-1}\ m^{-2}\ mM^{-1}$)	13.3±26.5	15.2±40.3	11.3±11.6	26.8±33.4	0.913	0.177
Δ Rate sensitivity ($pmol\ m^{-2}\ mM^{-1}$)	65.1(-132.7-601.6)	0.0(-184.0-99.2)	170.6(32.7-332.8)	571.1(71.6-1243.0)	0.544	0.395
Δ Potentiation factor	0.1±0.4	0.0±0.6	0.1±0.6	0.3±0.9	0.772	0.487
Lipid profile						
Δ FFA ($mmol\ L^{-1}$)	-0.2±1.0	0.4±1.4			0.291	NA
Δ LDL-c ($mmol\ L^{-1}$)	-0.3±0.4	-0.1±0.4			0.558	NA
Δ HDL-c	-0.3±0.3	-0.1±0.2			0.082	NA

(mmol L^{-1})						
Δ Triglycerides (mmol L^{-1})	0.4±0.2	-0.4±1.0			**0.039**	NA
Δ Total cholesterol (mmol L^{-1})	-0.3±0.5	-0.2±0.5			0.905	NA
Fibroblast growth factor						
Δ Fasting FGF19 (pg mL^{-1})	245.4±105.0	248.8±176.1	-2.1±117.7	-21.6±73.4	0.962	0.661

Data are mean ± SD.

3.3.2 Effect of each intervention on diabetes indices postoperatively

As shown in Table 3.1, fasting levels of glucose, insulin or C-peptide did not change acutely three days after both of the interventions (P>0.05). Three months after surgery, improvements of fasting glucose, fasting insulin and insulin resistance were seen in both intervention groups, but none of the changes was significant. A significant decrease in the fasting C-peptide concentration was observed in the SG group three months postoperatively (P=0.005). Decrease in fasting C-peptide without increase in fasting glucose indicated improvement in insulin sensitivity, as observed in the present study. Insulin sensitivity significantly improved both three days and three months postoperatively in each group (GBP, 3 days post-op, P=0.002; 3 months post-op, P=0.004; SG, 3 days post-op, P<0.000; 3 months post-op, P<0.001). Although the pancreatic glucose sensitivity index tended to be higher progressively with time, only the 3-month changes reached a significant level of difference (GBP, P=0.029; SG, P=0.024). The insulin and C-peptide AUC values during OGTT tended to increase in both interventions over time postoperatively, while only the 3-month change in the GBP group was significant (insulin AUC, P=0.016; C-peptide AUC, P=0.003). In the SG group, the AUC of glucose significantly decreased after surgery (3 days post-op, P=0.036; 3 months post-op, P=0.034), and no significant differences were found between the 3-day changes and 3-month changes. Correspondingly, in the GBP group, significant decreases were observed in glucose AUC levels three days after the bypass (P=0.013), but the 3-month reductions were not significant.

Generally, as presented in Table 3.2, the effect of both GBP and SG on all diabetes indices under investigation were similar (P>0.05). Exceptions to this are noted for the 3-

month changes of C-peptide (P=0.007) and basal insulin secretion rates (P=0.009), in which SG induced a decrease while GBP induced an increase.

3.3.3 Effect of each intervention on lipids postoperatively

Table 3.1 presented the preoperative and 3-day postoperative levels of lipid profiles during the fasting state. Significant differences were found longitudinally in high density lipoprotein-cholesterol (HDL-c) levels (1.3 ± 0.7 mmol L^{-1} (pre-op) vs. 1.1 ± 0.4 mmol L^{-1} (3 days post-op); P=0.039) as well as triglycerides levels (1.0 ±0.3 mmol L^{-1} (pre-op) vs. 1.4±0.3 mmol L^{-1} (3 days post-op); P=0.002) 3-day post GBP. Alternatively, in the SG group, all lipid compositions being investigated did not differ before and after the surgery (see Table 3.1). The 3-day changes of triglycerides levels were also found to be different between GBP and SG groups (GBP, 0.4 ± 0.2 mmol L^{-1}; SG, -0.4±1.0 mmol L^{-1}; P=0.039). However, interestingly, the 3-day changes of HDL-c levels did not differ between both intervention groups (GBP, -0.3 ± 0.3 mmol L^{-1}; SG, -0.1 ± 0.2 mmol L^{-1}; P=0.082).

3.3.4 Effect of each intervention on fasting levels of BA compositions and FGF19 postoperatively

During the fasting state, most composite BA levels, as well as FGF19 concentrations, followed the same trend. Levels presented an acute increase at three days, followed by a decrease three months postoperatively, although the level was still higher than the baseline (Figure 3.1 a-f2; Table 3.3). All fasting levels of BA compositions significantly increased three days after both surgeries, but to a greater extent after GBP for the composite variables of primary (P=0.003), secondary (P=0.041), primary-conjugated (P=0.006) and 12α-OH (P=0.002) BAs. Most fasting BA fractions were significantly increased at three months except for taurine-conjugated and primary-unconjugated BAs (Figure 3.1 d1, e1; Table 3.3). In contrast, unconjugated, primary-conjugated, secondary-unconjugated and non-12α-OH BA fractions did not increase significantly three months after SG (Figure 3.1 c2, e2, f2; Table 3.3).

Table 3.3. The concentrations of fasting bile acid fractions preoperatively and postoperatively in given bariatric surgeries.

	Gastric Bypass (n=8) Metformin=5, MF=3			**Sleeve Gastrectomy (n=11)** Metformin=5, Metformin+Glipizide=3, MF=1, Ibuprofen=1, Glipizide=1			**Δ vs. baseline p-value GBP vs. SG**	
	Pre GBP	**3 Days post GBP**	**3 Months post GBP**	**Pre SG**	**3 Days post SG**	**3 Months post SG**	**3 Days**	**3 Months**
Total BAs	6.27(5.63-6.91)	12.06(11.56-13.04)*	8.10(7.63-8.61)*†	6.43(5.87-7.29)	10.93(9.90-11.79)*	6.77(6.48-7.75)*†	0.080	0.064
Primary BAs	4.00(3.60-4.40)	6.72(6.46-7.84)*	5.11(4.66-5.45)*†	4.21(3.79-4.49)	6.05(5.48-6.81)*	4.38(4.09-4.82)*†	**0.003**	**0.004**
Secondary BAs	2.27(1.99-2.46)	4.86(4.55-5.08)*	3.07(2.84-3.25)*†	2.16(2.07-2.65)	4.67(4.19-5.14)*	2.42(2.31-2.54)*†	**0.041**	**0.000**
Conjugated BAs	4.80(4.41-5.48)	9.06(8.53-9.98)*	6.16(5.84-6.75)*†	5.14(4.61-5.39)	8.46(7.37-9.04)*	5.40(4.97-6.06)*†	0.086	0.098
Unconjugated BAs	1.29(1.13-1.38)	3.03(2.50-3.41)*	1.70(1.53-1.94)*†	1.23(1.10-1.76)	2.86(2.42-3.09)*	1.46(1.37-1.63)†	0.245	**0.017**
Glycine-conjugated BAs	3.52(3.13-3.79)	6.64(6.51-7.31)*	4.77(4.38-5.17)*†	3.80(3.38-4.07)	5.98(5.25-6.11)*	3.90(3.56-4.49)*†	0.091	0.095
Taurine-conjugated BAs	1.27(1.09-1.45)	2.10(2.01-3.60)*	1.34(1.18-1.68)†	1.27(1.03-1.55)	2.29(1.79-2.59)*	1.48(1.04-1.70)*†	0.899	0.555
Primary-conjugated BAs	3.28(2.97-3.52)	5.46(5.21-6.77)*	4.52(3.90-4.65)*†	3.54(2.99-3.74)	4.98(4.50-5.65)*	3.76(3.33-4.06)†	**0.006**	**0.004**
Primary-unconjugated BAs	0.68(0.61-0.84)	1.17(0.99-1.37)*	0.71(0.58-0.79)†	0.67(0.54-0.78)	1.06(0.95-1.11)*	0.74(0.63-0.82)*†	0.202	0.255
Secondary-conjugated BAs	1.48(1.40-1.62)	3.10(2.97-3.38)*	1.93(1.76-2.01)*†	1.53(1.42-1.69)	2.91(2.63-3.25)*	1.70(1.40-1.90)*†	0.271	0.230
Secondary-unconjugated BAs	0.67(0.54-0.83)	1.85(1.47-2.16)*	1.13(0.87-1.26)*†	0.69(0.39-1.01)	1.82(1.29-1.96)*	0.74(0.66-1.01)†	0.367	**0.001**

12α-OH BAs	2.51(2.35-2.70)	5.55(5.30-5.87)*	3.29(3.10-3.80)*†	2.85(2.40-2.98)	5.36(4.31-5.90)*	3.10(2.56-3.49)*	**0.002**	**0.006**
Non-12α-OH BAs	3.53(3.19-4.33)	6.16(5.94-6.96)*	4.40(4.24-4.93)*†	3.98(3.28-4.21)	5.88(5.04-6.20)*	4.11(3.55-4.22)†	0.185	0.140
Primary/Secondary	1.83(1.54-2.11)	1.42(1.32-1.69)*	1.71(1.52-1.73)*†	1.71(1.49-1.94)	1.21(1.14-1.41)*	1.82(1.49-1.95)†	0.589	**0.022**
Conjugated/unconjugated	3.56(2.71-4.80)	3.57(2.50-4.20)*	3.90(3.01-4.15)	4.04(2.91-4.39)	3.21(2.32-3.41)*	3.56(3.07-4.74)†	0.169	0.492
12α-OH/non-12α-OH	0.68(0.64-0.77)	0.89(0.78-1.00)*	0.78(0.70-0.85)†	0.70(0.63-0.82)	0.86(0.82-0.99)	0.77(0.60-0.97)	0.717	0.901

All data are median(IQR) in μm L^{-1}.

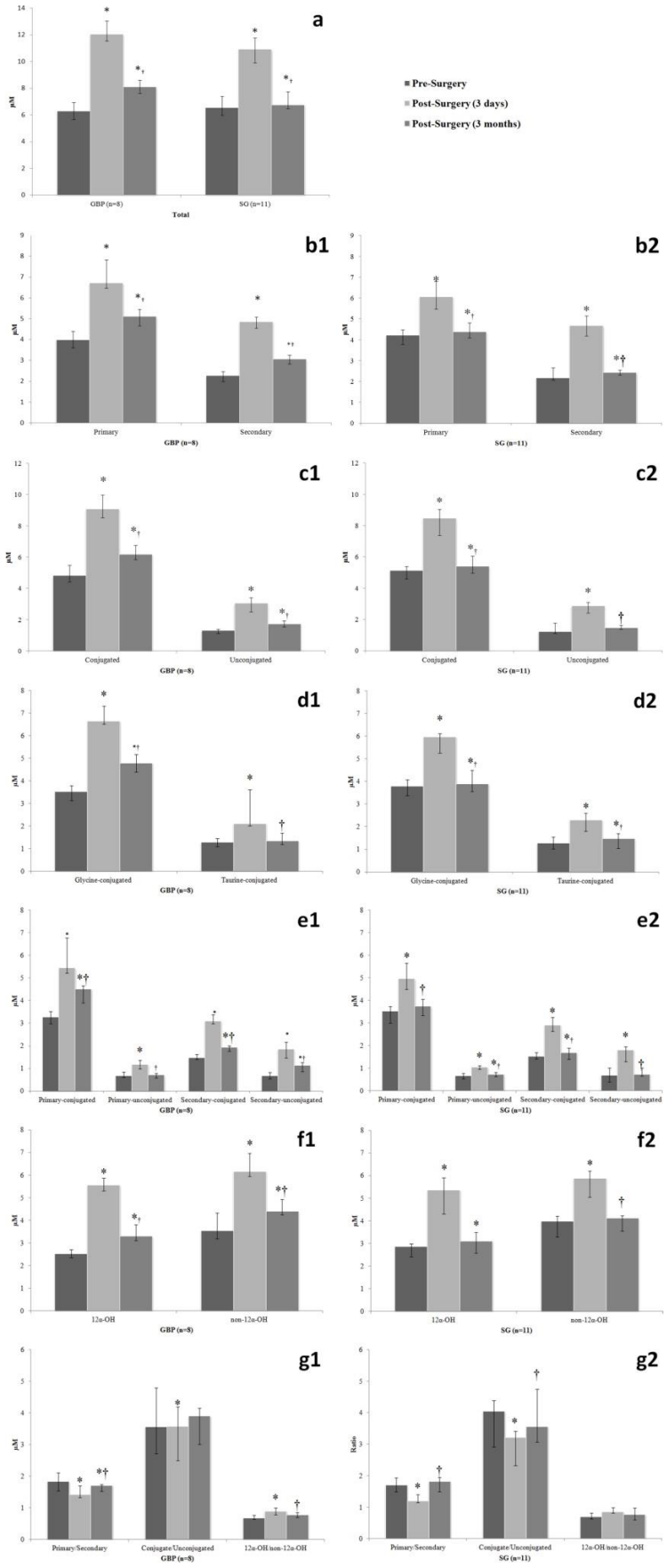


Figure 3.1. Bile acid levels and compositions before surgery and 3-day, 3-month post surgery after gastric bypass and sleeve gastrectomy. Data are median (IQR) *P<0.05 vs. Pre-GBP or Pre-SG. † P<0.05 vs. 3 Days post GBP or 3 Days post SG.

As shown in Table 3.3, compared with SG, GBP induced a greater increase for the composite variables of primary (3 days post-op, P=0.003; 3 months post-op, P=0.004), secondary (3 days post-op, P=0.041; 3 months post-op, P<0.001), primary-conjugated (3 days post-op, P=0.006; 3 months post-op, P=0.004) and 12α-OH BAs (3 days post-op, P=0.002; 3 months post-op, P=0.006). The differences of changes in unconjugated (P=0.017) and secondary-unconjugated BAs (P=0.001) between two intervention groups were only observed at three months after the surgeries, in which both compositions increased to a greater extent after GBP.

In both surgery groups, FGF19 levels increased significantly three days postoperatively, but the levels did not differ from the baseline at three months (Table 3.1). The influences of GBP compared to SG on FGF19 levels showed no significant difference at either three days (P=0.962) or three months (P=0.661) between these two types of surgery.

3.3.5 Effect of each intervention on postprandial levels of BA compositions

A significant increase in BA levels in response to glucose at 30 min for all composite variables was observed from the postprandial BA curves (Figure 3.2 a-m, Figure 3.3 a-m), regardless of longitudinal time relative to surgery or types of operation. For both interventions, the rise in peak BA levels 30 min after ingestion at the three-days post surgery timepoint was more exaggerated compared to the baseline and three months post-surgery levels.

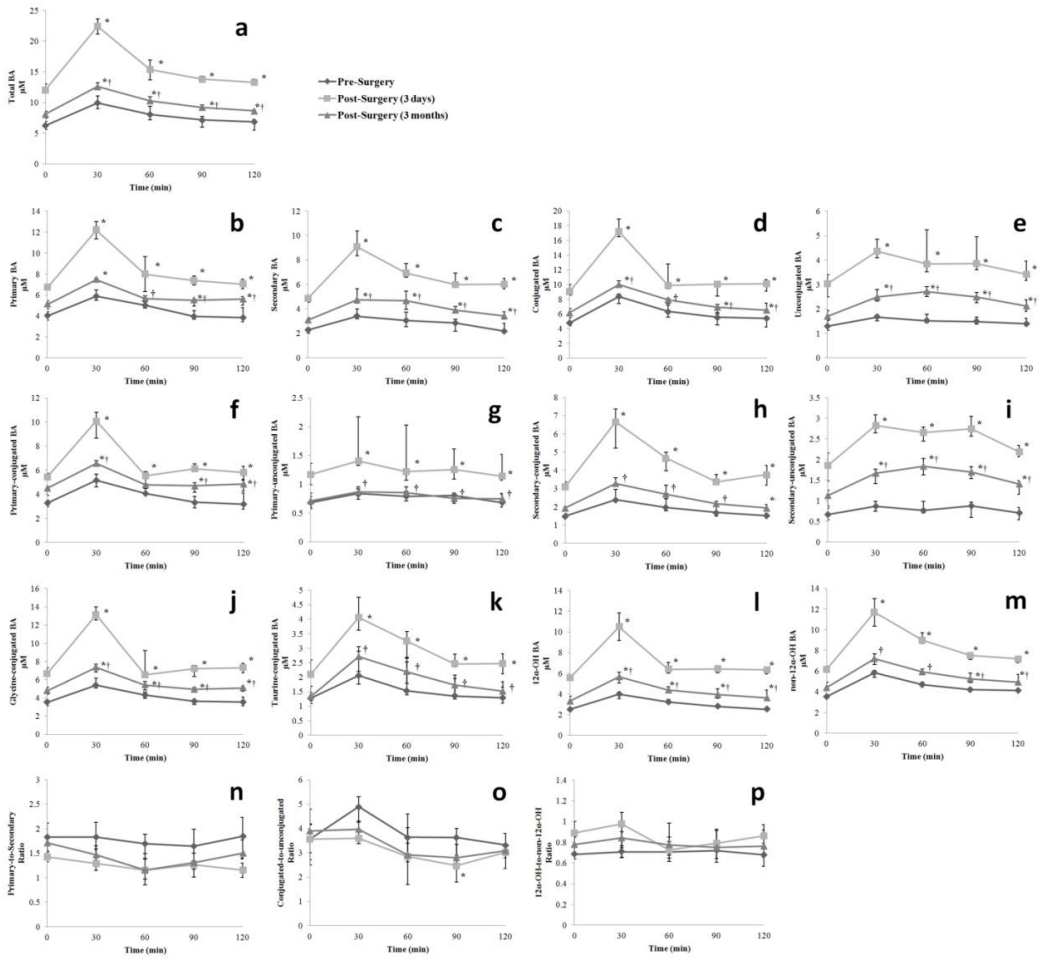


Figure 3.2. Postprandial changes of bile acid levels and compositions before surgery and 3-day, 3-month post-surgery after gastric bypass. Data are median (IQR). *P<0.05 vs. Pre-GBP; † P<0.05 vs. 3 Days post-GBP.

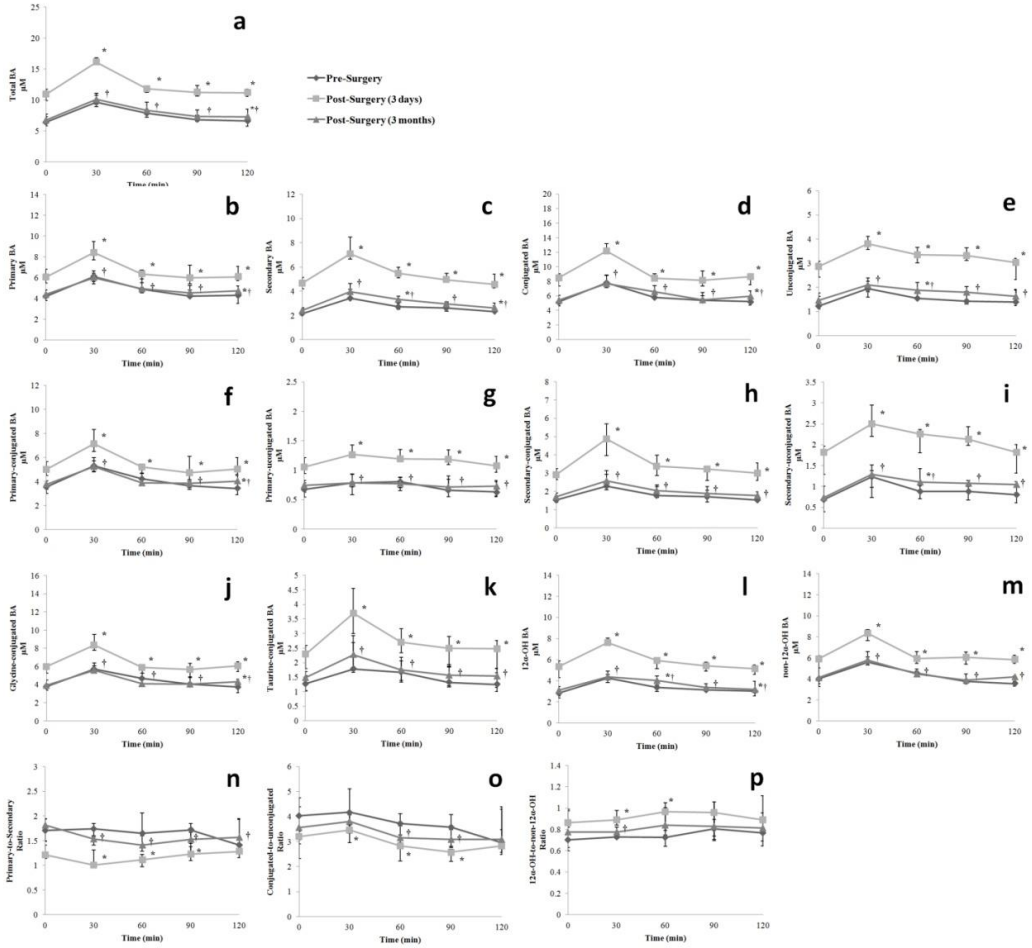


Figure 3.3. Postprandial changes of bile acid levels and compositions before surgery and 3-day, 3-month post-surgery after sleeve gastrectomy. Data are median (IQR). $^{*}P<0.05$ vs. Pre-SG; $^{\dagger}P<0.05$ vs. 3 Days post-SG.

Indeed, the AUC of all BA compositions was significantly higher than the baseline regardless of operation types (Table 3.4). At three months, most BA compositions were higher than the baseline except for glycine-conjugated, primary-unconjugated, secondary-conjugated and non-12α-OH BAs for the GBP group, and primary BAs, glycine-conjugated, primary-conjugated, primary-unconjugated and non-12α-OH for the SG group of BAs ($P>0.05$). The distinctions regarding the postoperative effects on AUC of BA compositions between GBP and SG were more evident at three days compared to those observed at three months, in which significant differences were measured in 10 out of 13 BA compositions between the intervention groups. By comparison, the changes of AUC in only 5 out of 13 BA compositions were found to be different between groups.

Table 3.4. The AUC0-120min of bile acid fractions preoperatively and postoperatively in given bariatric surgeries.

	Gastric Bypass (n=8) Metformin=5, MF=3			**Sleeve Gastrectomy (n=11)** Metformin=5, Metformin+Glipizide=3, MF=1, Ibuprofen=1, Glipizide=1			**Δ vs. baseline p-value GBP vs. SG**	
	Pre GBP	**3 Days post GBP**	**3 Months post GBP**	**Pre SG**	**3 Days post SG**	**3 Months post SG**	**3 Days**	**3 Months**
Total BAs	976.1(824.5-1025.8)	1913.0(1853.0-2015.0)*	1204.0(1169.0-1327.0)*†	923.0(852.5-1031.6)	1535.0(1445.0-1547.0)*	967.5(948.3-1106.0)*†	**0.000**	**0.037**
Primary BAs	550.4(538.8-644.5)	1024.7(912.3-1139.2)*	710.1(690.1-735.9)*†	583.2(534.0-634.0)	817.5(755.2-891.2)*	592.6(561.7-666.8)†	**0.000**	0.558
Secondary BAs	347.0(294.9-399.9)	841.1(770.0-881.4)*	486.1(455.2-558.3)*†	339.5(316.8-373.3)	655.7(635.4-768.0)*	379.6(365.5-418.8)*†	**0.028**	0.086
Conjugated BAs	771.5(655.2-812.9)	1366.0(1312.0-1516.0)*	934.8(884.6-993.1)*†	704.3(673.2-808.3)	1136.0(1033.0-1196.0)*	755.7(709.2-848.7)*†	**0.002**	0.080
Unconjugated BAs	181.1(165.2-200.4)	446.2(428.1-591.6)*	285.5(269.9-310.7)*†	190.5(165.4-224.9)	411.6(355.7-423.0)*	219.6(203.7-252.8)*†	**0.007**	**0.004**
Glycine-conjugated BAs	505.5(479.1-551.2)	987.2(956.7-1103.5)*	675.7(639.1-708.2)†	539.6(499.9-569.6)	783.4(714.7-830.3)*	524.2(492.1-633.3)†	**0.002**	0.100
Taurine-conjugated BAs	184.7(165.2-280.4)	363.7(339.9-396.5)*	242.2(193.0-284.7)*†	176.3(153.1-244.4)	340.6(269.3-398.4)*	209.4(161.8-263.0)*†	0.451	0.290
Primary-conjugated BAs	466.4(442.6-556.4)	782.5(767.8-962.1)*	610.1(589.6-657.3)*†	476.6(467.4-540.5)	660.2(618.5-749.5)*	496.1(462.4-579.8)†	**0.004**	**0.005**

Primary-unconjugated BAs	94.3(82.1-104.8)	148.4(138.0-234.8)*	94.6(88.15-104.9)†	85.0(70.8-102.6)	142.1(135.4-151.0)*	89.4(81.2-106.5)†	0.117	0.886
Secondary-conjugated BAs	225.3(211.6-279.3)	556.3(475.9-573.0)*	302.6(285.8-330.7)†	212.3(199.0-276.1)	436.2(367.5-488.1)*	237.3(222.8-282.2)*†	0.066	0.207
Secondary-unconjugated BAs	97.9(79.17-107.3)	294.4(290.5-313.4)*	194.8(168.5-199.7)*†	110.9(80.2-139.7)	243.0(225.4-285.7)*	132.5(112.6-156.5)*†	**0.021**	**0.002**
12α-OH BAs	367.5(356.5-389.1)	849.0(840.9-917.8)*	515.5(490.5-587.1)*†	410.3(376.5-440.0)	708.5(685.7-762.1)*	458.9(407.9-504.6)*†	**0.000**	**0.002**
Non-12α-OH BAs	578.9(462.5-632.0)	1059.4(936.0-1111.3)*	696.8(617.1-731.9)†	512.7(503.7-582.3)	778.6(733.4-831.0)*	546.2(511.5-613.7)†	**0.004**	0.105

All data are median (IQR) in μm L^{-1} min^{-1}. *P<0.05 vs. Pre-GBP or Pre-SG. † P<0.05 vs. 3 Days post GBP or 3 Days post SG.

3.3.6 Effect of each intervention on ratios of BA levels during fasting and postprandial

Computed ratios of the composite BA variables revealed a predominance of primary and conjugated BAs, compared with secondary and unconjugated BAs, respectively (Figure 3.1 g1-g2; Table 3.3). During fasting states, there was a trend that primary/secondary BA ratios declined three days after surgery, then rose at three months. A difference between surgery groups was observed at three months postoperative for the primary/secondary BA ratio (P=0.022), where the ratio was significantly lower than the baseline in the GBP group (1.83(1.54-2.11) (pre-op) vs. 1.71(1.52-1.73) (3 months post-op); P=0.033). The ratio did not differ between baseline and three months values (1.71(1.49-1.94) (pre-op) vs. 1.82(1.49-1.95) (3 months post-op); P=0.766). A trend that the primary/secondary BA ratio progressively declined over time in response to glucose was seen in both groups.

During fasting, the conjugated/unconjugated ratio declined at three days post-operative, but appeared relatively unchanged when compared with the baseline at three months in both surgery groups. Conjugated/unconjugated ratios tended to increase at 30 min after oral glucose was taken, and then ratios gradually reduced as the timepoints increased. A significant increase of 12α-OH/non-12α-OH ratios at the 3-day time point post surgery compared with pre-surgery values was observed in the GBP group (0.68(0.64-0.77) (pre-op) vs. 0.89(0.78-1.00) (3 days post-op); P=0.011), but not in the SG group (0.70(0.63-0.82) (pre-op) vs. 0.86(0.82-0.99) (3 days post-op); P=0.054). The 12α-OH/non-12α-OH ratio showed a slight increase at three months relative to baseline values, but the changes were not significant in each group.

3.3.7 Associations between changes in metabolic characteristics and changes in BA compositions and FGF19 postoperatively

The 3-day time point post surgery is shown in Table 3.5.

Table 3.5. Correlations of 3-day changes in fasting and AUC0-120min of bile acid fractions and fasting FGF19 with changes in metabolic characteristics.

	r with Δ Fasting glucose	*r* with Δ AUC Glucose	*r* with Δ Fasting insulin	*r* with Δ AUC Insulin	*r* with Δ HOMA-IR	*r* with Δ Stumvol1	*r* with Δ Total ISR	*r* with Δ Basal ISR	*r* with Δ glu sens	*r* with Δ rate sens	*r* with Δ PFR	*r* with Δ C-peptide	*r* with Δ C-peptide AUC	*r* with Δ FFA	*r* with Δ LDLC	*r* with Δ HDLC	*r* with Δ Triglycerides	*r* with Δ Chol
Δ Total	-0.21	-0.54	0.08	0.21	0.04	-0.33	0.1	0.06	0.04	-0.08	-0.19	0.07	0.09	-0.02	-0.27	-0.17	0.21	-0.06
Δ Primary	0.1	-0.01	0.01	0.32	-0.08	-0.38	0.26	0.32	0.15	-0.18	-0.17	0.31	0.28	-0.27	-0.13	-0.07	0.24	0
Δ Secondary	-0.25	**-0.57***	0.08	0.13	0.06	-0.24	0.03	-0.03	0	-0.03	-0.15	-0.02	0.01	0.06	-0.25	-0.16	0.15	-0.07
Δ Conjugated	-0.2	**-0.5***	0.09	0.19	0.03	-0.34	0.08	0.09	0.03	-0.11	-0.22	0.1	0.09	-0.05	-0.25	-0.17	0.22	-0.06
Δ Unconjugated	-0.16	**-0.46***	0.02	0.23	0.03	-0.11	0.16	-0.19	0.12	0.2	0.08	-0.18	0.09	0.13	-0.2	-0.03	-0.01	-0.08
Δ Glycine-conjugated	-0.23	**-0.52***	0.08	0.17	0.03	-0.32	0.05	0.05	0.03	-0.09	-0.22	0.06	0.06	-0.03	-0.28	-0.17	0.21	-0.08
Δ Taurine-conjugated	0.34	**0.31***	0.06	0.22	0.02	-0.18	0.33	**0.47***	-0.07	-0.28	0.01	**0.45***	0.3	-0.24	0.36	-0.03	0.08	0.35
Δ Primary-conjugated	0.12	0	-0.01	0.3	-0.11	-0.32	0.25	0.31	0.11	-0.16	-0.13	0.3	0.27	-0.29	-0.15	-0.12	0.25	-0.03
Δ Primary-unconjugated	-0.12	-0.1	0.2	0.16	0.23	**-0.44***	0.08	0.08	0.32	-0.17	-0.28	0.1	0.09	0.08	0.16	0.35	0.01	0.2
Δ Secondary-conjugated	-0.25	**-0.53***	0.1	0.11	0.07	-0.26	0	0	0	-0.07	-0.19	0.01	0.01	0.04	-0.22	-0.14	0.16	-0.05
Δ Secondary-unconjugated	-0.14	**-0.48***	-0.04	0.21	-0.03	0.02	0.16	-0.24	0.03	0.28	0.18	-0.23	0.07	0.12	-0.27	-0.14	-0.01	-0.15
Δ 12α-OH	-0.04	-0.37	-0.1	**0.42***	-0.18	-0.3	0.27	0.14	0.24	0.05	0	0.14	0.25	-0.05	-0.24	-0.31	0.22	-0.11
Δ Non-12α-OH	-0.22	**-0.5***	0.12	0.14	0.08	-0.3	0.04	0.03	-0.01	-0.1	-0.21	0.04	0.04	-0.01	-0.24	-0.11	0.18	-0.05
Δ Primary/Secondary	-0.26	0.25	-0.27	−0.42	-0.31	0.02	-0.2	0.05	-0.18	-0.78	-0.4	0.05	-0.22	-0.26	-0.08	0.2	-0.23	-0.19
Δ Conjugate/unconjugated	-0.26	-0.21	-0.01	0.04	-0.11	-0.08	0.05	0.17	0	**-0.3***	-0.32	0.17	0.13	-0.32	-0.29	-0.22	0.39	-0.09
Δ 12α-OH/non-12α-OH	-0.16	-0.11	-0.18	0.06	-0.17	0.29	0.07	-0.15	0.1	0.21	0.22	-0.16	0.02	0.52	-0.16	-0.21	-0.17	-0.32
Δ FGF19	-0.12	-0.1	0.04	−0.22	0.07	0.14	-0.44	-0.15	0.04	-0.32	-0.28	-0.15	-0.38	**0.06***	-0.16	0.21	-0.21	-0.22

Δ AUC Total	-0.03	**-0.47***	-0.01	0.26	-0.06	**-0.49***	0.88	0.01	-0.01	0.15	0.06	0	0.07	-0.23	-0.16	-0.35	0.35	-0.01
Δ AUC Primary	-0.06	-0.12	-0.29	0.03	-0.35	**-0.46***	-0.1	-0.11	-0.06	0.09	0.03	-0.12	-0.08	-0.34	-0.19	-0.17	0.3	-0.04
Δ AUC Secondary	0	**-0.58***	0.21	0.34	0.18	-0.35	0.18	0.09	0.03	0.14	0.05	0.09	0.16	-0.07	-0.08	-0.38	0.27	0.01
Δ AUC Conjugated	-0.15	**-0.48***	-0.11	0.16	-0.17	**-0.46***	-0.04	-0.03	-0.02	0.07	-0.01	-0.03	-0.03	-0.21	-0.23	-0.37	0.33	-0.07
Δ AUC Unconjugated	0.3	-0.19	0.27	0.4	0.28	-0.3	0.35	0.09	0.02	0.27	0.22	0.09	0.31	-0.17	0.12	-0.12	0.21	0.18
Δ AUC Glycine-conjugated	-0.2	**-0.46***	-0.15	0.08	-0.2	**-0.46***	-0.11	-0.12	-0.05	0.04	-0.08	-0.11	-0.1	-0.22	-0.3	-0.29	0.34	-0.1
Δ AUC Taurine-conjugated	0.27	-0.23	0.2	**0.45***	0.13	-0.1	0.41	**0.48***	0.14	0.16	0.36	**0.47***	0.35	0.02	0.34	**-0.51***	0.01	0.12
Δ AUC Primary-conjugated	-0.09	-0.12	-0.39	-0.05	**-0.45***	**-0.44***	-0.17	-0.15	-0.09	0.07	0.03	-0.16	-0.17	-0.26	-0.25	-0.25	0.29	-0.11
Δ AUC Primary-unconjugated	0.09	-0.01	0.23	0.25	0.21	-0.17	0.2	0.1	0.08	0.08	0.02	0.11	0.23	-0.29	0.13	0.21	0.09	0.2
Δ AUC Secondary-conjugated	-0.14	**-0.6***	0.17	0.27	0.13	-0.31	0.09	0.09	0.04	0.04	-0.05	0.1	0.1	-0.08	-0.12	-0.33	0.24	-0.02
Δ AUC Secondary-unconjugated	0.38	-0.27	0.22	0.39	0.25	-0.31	0.36	0.05	-0.04	0.35	0.32	0.05	0.27	-0.01	0.08	-0.36	0.24	0.09
Δ AUC 12α-OH	-0.07	**-0.42***	-0.13	0.27	-0.2	**-0.44***	0.03	-0.05	0.1	0.14	0.12	-0.05	0.03	-0.27	-0.22	-0.36	0.37	-0.1
Δ AUC non-12α-OH	0	-0.43	0.07	0.21	0.03	**-0.45***	0.09	0.04	-0.08	0.13	0.01	0.04	0.08	-0.17	-0.09	-0.3	0.28	0.05

*P<0.05

The change in glucose AUC was negatively correlated with fasting secondary BAs, conjugated BAs, unconjugated BAs, both glycine- and taurine- conjugated BAs, secondary-conjugated BAs, secondary-unconjugated BAs and non-12α-OH BAs, but it was positively correlated with fasting taurine-conjugated BAs. The change in Glucose AUC was also negatively correlated with the AUC of the total, secondary, conjugated, glycine-conjugated, secondary-conjugated and 12α-OH BA compositions. The 3-day change of fasting insulin did not correlate with any of the BA groups, either in fasting or prandial states, but the change of insulin AUC was positively associated with fasting 12α-OH BAs and the AUC of the taurine-conjugated BAs. The HOMA-IR was only negatively correlated with primary-conjugated BA AUC data. The change of ISI values was negatively associated with fasting levels of primary-unconjugated BAs and AUC values of the total, primary, conjugated, glycine-conjugated, primary-conjugated, 12α-OH and non-12α-OH BA compositions. The change of total insulin secretion was only positively correlated with postprandial taurine-conjugated BAs, while the change of basal insulin secretion was positively correlated with both fasting and postprandial taurine-conjugated BAs. The change of rate sensitivity was negatively correlated with fasting conjugate/unconjugated ratios. The change of fasting C-peptide values were positively associated with both fasting and postprandial taurine-conjugated BAs.

The 3-day change of free fatty acids (FFA) had a weak, positive correlation with FGF19, and the change of HDL-c was negatively associated with the AUC of taurine-conjugated BAs.

The associations between 3-month changes in metabolic characteristics and changes in BA compositions and FGF19 are listed in Table 3.6.

Table 3.6. Correlations of 3-month changes in fasting and AUC0-120min of bile acid fractions and fasting FGF19 with changes in clinical and metabolic characteristics.

	r with Δ weight	*r* with ΔBMI	*r* with Δ Fasting glucose	*r* with Δ Glucose AUC	*r* with Δ Fasting insulin	*r* with Δ Insulin AUC	*r* with ΔHOMA-IR	*r* with ΔStum-voll	*r* with Δ total isr	*r* with Δ basal isr	*r* with Δ glu sens	*r* with Δ rate sens	*r* with ΔPF	*r* with ΔC-peptide	*r* with ΔC-peptide AUC
ΔTotal	-0.15	-0.09	-0.07	-0.12	0.04	0.09	0.04	-0.41	0.35	0.34	-0.05	-0.12	-0.13	0.35	0.26
ΔPrimary	-0.02	-0.12	-0.2	-0.41	-0.1	-0.07	-0.18	0.13	-0.04	0.3	-0.15	-0.1	-0.11	0.29	-0.05
ΔSecondary	0.18	0.14	-0.03	-0.03	0.07	0.12	0.1	**-0.51***	0.41	0.31	-0.01	-0.12	-0.12	0.32	0.32
ΔConjugated	0.15	0.1	-0.1	-0.14	0.05	0.1	0.04	-0.41	0.36	0.32	-0.02	-0.07	-0.1	0.32	0.28
ΔUnconjugated	0.05	-0.07	0.15	0.06	-0.04	-0.04	0.01	-0.18	0.09	0.32	-0.19	-0.39	-0.12	0.31	0.01
ΔGlycine-conjugated	0.14	0.08	-0.1	-0.13	0.05	0.12	0.04	-0.4	0.37	0.31	-0.01	-0.07	-0.12	0.31	0.3
ΔTaurine-conjugated	0.16	0.25	0.08	-0.18	-0.07	-0.22	0	0.01	-0.28	0.08	-0.18	-0.01	-0.03	0.07	-0.3
ΔPrimary-conjugated	-0.12	-0.19	-0.24	-0.4	-0.11	-0.1	-0.18	0.13	-0.06	0.3	-0.16	-0.1	-0.13	0.29	-0.07
ΔPrimary-unconjugated	-0.5	-0.4	0.26	0.1	0.09	0.22	0.1	-0.02	0.1	-0.11	0.09	0.07	0.14	-0.1	0.11
ΔSecondary-conjugated	0.22	0.19	-0.04	-0.03	0.09	0.16	0.11	**-0.52***	**0.44***	0.27	0.03	-0.05	-0.1	0.28	0.35
ΔSecondary-	-0.12	-0.22	0.06	0.03	-0.08	-0.12	-0.02	-0.18	0.06	0.38	-	-0.44	-0.16	0.37	-0.03

unconjugated											0.24				
Δ12α-OH	-0.26	-0.33	-0.28	**-0.46***	0.02	0.04	-0.06	0.25	0.01	0.22	0.14	-0.22	-0.12	0.19	0.01
ΔNon-12α-OH	0.23	0.18	-0.01	-0.02	0.04	0.09	0.06	**-0.52***	0.38	0.33	-0.09	-0.08	-0.11	0.34	0.29
ΔPrimary/ Secondary	0.04	0.05	-0.19	-0.33	-0.11	-0.05	-0.19	**0.53***	-0.29	**-0.46***	0.19	0.27	0	**-0.47***	-0.21
ΔConjugate/ unconjugated	0.13	0.17	-0.23	-0.19	0.11	0.23	0.05	-0.25	0.37	0.08	0.15	0.25	-0.06	0.08	0.35
Δ12α-OH /non-12α-OH	-0.21	-0.17	-0.36	-0.3	0.11	0.13	0.02	**0.45***	-0.13	-0.31	0.41	-0.05	-0.26	-0.34	-0.08
ΔFGF-19	-0.01	-0.03	-0.38	-0.56	-0.32	-0.15	-0.41	0.18	-0.09	0.12	-0.1	-0.04	0.05	0.09	-0.13
ΔAUC Total	0.19	0.15	-0.07	-0.07	0.02	0.14	0.01	**-0.47***	0.41	0.41	-0.15	0.01	-0.1	0.42	0.34
ΔAUC Primary	0.1	0.03	-0.15	-0.07	-0.15	0	-0.2	-0.27	0.14	0.36	-0.37	0.06	-0.06	0.35	0.09
ΔAUC Secondary	0.19	0.17	-0.03	-0.06	0.08	0.16	0.08	**-0.48***	**0.44***	0.37	-0.06	-0.01	-0.1	0.38	0.37
ΔAUC Conjugated	0.19	0.15	-0.05	-0.03	0.07	0.14	0.08	**-0.52***	0.42	0.36	-0.1	-0.01	-0.13	0.37	0.33
ΔAUC Unconjugated	0.07	0.05	-0.14	-0.25	-0.24	0.04	-0.35	-0.01	0.18	0.43	-0.33	0.09	0.1	0.44	0.2
ΔAUC Glycine-conjugated	0.2	0.16	-0.08	-0.05	0.04	0.13	0.04	**-0.51***	0.41	0.33	-0.08	-0.02	-0.12	0.33	0.31
ΔAUC Taurine-conjugated	-0.08	-0.02	0.38	0.21	0.34	0.17	0.44	-0.12	0.1	0.36	-0.15	0.05	-0.04	0.39	0.16
ΔAUC Primary-conjugated	0.05	0	-0.15	-0.04	-0.15	-0.02	-0.18	-0.29	0.12	0.37	-0.39	0.04	-0.1	0.36	0.07
ΔAUC Primary-unconjugated	0.31	0.2	0.03	-0.21	-0.04	0.14	-0.11	0.19	0.1	-0.08	0.13	0.12	0.26	-0.08	0.15
ΔAUC Secondary-	0.21	0.18	0	-0.02	0.14	0.18	0.16	**-0.51***	**0.45***	0.29	0.03	-0.03	-0.12	0.31	0.37

conjugated															
ΔAUC Secondary-unconjugated	0	0	-0.15	-0.22	-0.25	0.01	-0.35	-0.06	0.17	**0.49***	-0.39	0.07	0.05	**0.49***	0.18
ΔAUC 12α-OH	-0.19	-0.23	-0.31	**-0.41***	-0.22	-0.07	-0.33	0.16	0.02	0.28	-0.14	-0.07	-0.04	0.26	0
ΔAUC non-12α-OH	0.27	0.24	0.01	0.04	0.09	0.17	0.11	**-0.58***	**0.46***	0.38	-0.13	0.03	-0.1	0.39	0.38

*P<0.05

The change of Glucose AUC was negatively correlated with fasting and postprandial 12α-OH BAs. The change of Stumvoll ISI was negatively correlated with fasting secondary, secondary-conjugated, and non-12α-OH BAs, but was positively correlated with fasting primary/secondary and 12α-OH/non-12α-OH ratio. The change of Stumvoll ISI was also negatively correlated with the AUC of total, secondary, glycine conjugated, secondary-conjugated and non-12α-OH BA compositions. The change of total insulin secretion positively associated with fasting and postprandial secondary-conjugated BAs, postprandial secondary BAs and non-12α-OH BAs. The change of basal insulin secretion, as well as fasting C-peptide levels, had negative associations with the fasting primary/secondary ratio, and was also negatively correlated with postprandial secondary-unconjugated BAs. There were no significant associations observed between changes in fasting FGF19 levels and changes in any clinical and metabolic characteristics at three months post-surgery.

3.4 Discussion

In our study, we observed that physiological values of several BAs and FGF19 were increases acutely after bariatric surgeries, of which total, primary, secondary, conjugated, glycine-conjugated, secondary-conjugated and 12α-OH BAs were common to both types of surgery and at both early time points, suggesting that these signalling molecules may play a vital role in the acute response to bariatric surgery in humans. We found a significant increase at three days post-surgery, for all fasting BA composite variables, as well as primary/secondary and conjugated/unconjugated BA ratios. Our findings are in accordance with several studies showing that most BA compositions increase acutely within one week after different types of bariatric surgery (Ahmad, Pfalzer, & Kaplan, 2013; Jahansouz et al., 2016; Pournaras et al., 2012). We also found that fasting 12α-OH /non-12α-OH BA ratios significantly increase three days after GBP. This finding was somewhat unexpected, considering that a higher 12α-OH /non-12α-OH BA ratio was thought to be associated with lower insulin sensitivity (Haeusler et al., 2013). However, the Stumvoll ISI composites in the GBP group at three days were significantly increased. We did not find any correlation between the 3-day changes of 12α-OH /non-12α-OH BA ratios and insulin resistance.

We found that glucose AUC significantly decreased after both surgical interventions, associating the outcome with higher fasting unconjugated, secondary-conjugated as well as unconjugated, glycine-conjugated and non-12α-OH BAs. Our findings were inconsistent with secondary BAs being the predominant activators of the Takeda G protein-coupled receptor5 (TGR5) pathway, which has been reported to affect glucose metabolism (Chiang, 2009; Fiorucci & Distrutti, 2015).

At three days, we observed a significant reduction of high-density lipoprotein-cholesterol (HDL-c) after GBP. This is in line with the fact that an acute increase of triglycerides was found in our study, indicating that the lipid profile parameters had not improved or even worsened early after the intervention. These observed results might be due to the consistent increase in inflammation and stress resulting from the bariatric surgery (Murri et al., 2010). Benaiges et al. also observed a reduction of HDL-c up to three months after both GBP and SG, but then the progressive increases were also detected over time up to twelve months (Benaiges et al., 2012). Thus, it is tempting to

speculate that the lipid profiles being investigated in this study will progressively improve over a longer duration than the measurements taken three days post-surgery.

In our study, the most striking change in FGF19 and BA fractions was that a sharp rise was observed for fasting FGF19 levels, where both fasting and postprandial BA compositions three days after surgeries were followed by a reduction for each of the measured fractions. The reason for such dramatic changes is unclear, but it might be partly explained by the acute alterations of gut microbiota caused by bariatric surgery (Sweeney & Morton, 2013). Gut microbiota are thought to have mutual interactions with BAs (Wahlström, Kovatcheva-Datchary, Ståhlman, Bäckhed, & Marschall, 2017), including liberating the glycine/taurine conjugation (Ridlon, Kang, & Hylemon, 2006), oxidation, sulfation, and dehydroxylation of BAs (Staels & Fonseca, 2009). The diversity of secondary BA species was directly affected (Kübeck et al., 2016). However, this can not explain the acute increase of primary BAs after the interventions in our study, as the enzyme sterol 12α-hydroxylase (CYP8B1), which is required for CA synthesis (Li-Hawkins et al., 2002) has been reported as not being regulated by microbiota (Sayin et al., 2013). Thus, we inferred from these findings that altered anatomy after bariatric surgeries might not be the only mechanism that is related to elevating plasma BA concentrations acutely. The modifications in hepatic insulin sensitivity, the synthesis or excretion of BAs, enterohepatic cycling and gut permeability -- all of the above acting as possible contributors -- could be attributed as causing changes in BA compositions after bariatric surgery (Dutia et al., 2015). Further investigations are needed to clarify these points.

The acute increase of FGF19 at three days post-surgery could reflect a systemic response to increased circulating BA levels, considering that BAs can bind to the nuclear receptor FXR, which is responsible for stimulating FGF19 synthesis (Chiang, 2009; Wahlström, Sayin, Marschall, & Bäckhed, 2016). Afterwards, the targeted FGF receptor-4 might be activated with the increasing level of FGF19, which in turn negatively feeds back to inhibit hepatic BA synthesis (Chiang, 2009). Bile acid-FXR activation might thereby become inhibited over time, leading to a reduction of FGF19 synthesis. As a result, fasting FGF19 levels, both as fasting and postprandial BA compositions, measured at three months were lower than the 3-day time points post surgeries. It is also worth mentioning that most BA compositions were higher at three months than their initial baseline readings. Such findings might be explained by the

negative correlation between body weight and most BA compositions over long term tracking (Dutia et al., 2015; Gerhard et al., 2013; Pournaras et al., 2012; Risstad et al., 2017). However, we did not find any significant correlation between body weight loss and any changes in BA fractions at three months.

We found that pancreatic glucose sensitivity improved significantly at three months after both interventions, and fasting C-peptide levels, insulin secretion rates, rate sensitivity and the potentiation factor improved after either GBP or SG. Our observations are in line with previous reports, suggesting that bariatric surgery is accompanied by improvement of glycemic control as well as β-cell functions (Guidone et al., 2006; Nakatani et al., 2009). Studies have shown that colesevelam hydrochloride, a precisely engineered BA sequestrant, had a remarkable improvement in glycemic control among patients with T2DM receiving sulfonylurea (Brunetti & DeSantis, 2015) or insulin therapy (Sandhu, Moosavi, Golmohammadi, & Francis, 2016). Additionally, positive correlations were found between the above diabetes indices and specific BA compositions in our study. Thus, it is likely that therapies that modulate downstream pathways of BAs might be effective in improving glycemic control.

Overall, differences between two types of surgery and their effects on BA levels, as either fasting or postprandial readings, were observed for various compositions, in which GBP induced higher increases on their scales. The differences were more exaggerated at three days compared with those taken at three months post-surgery. The reason attributed to such differences is unclear. These differences between the timepoint measurements might be related to malabsorption caused by GBP, or other mechanisms associated with a patient's nutritional status after either of the operations, or possibly alterations in liver metabolism between the two interventions (Coupaye et al., 2014).

Most previous reports have emphasised associations between the achieved levels of fasting BA compositions with the long term clinical state after either GBP or SG among patients without T2DM (Khan et al., 2016; Simonen et al., 2012; Steinert et al., 2013). Therefore, the present study makes a unique contribution to the existing literature as we focused on both acute (three days) and relatively short-term (three months) changes of the plasma BA compositions that occur with two different types of bariatric surgery. We also measured the values in both fasting and postprandial states.

Our study has several limitations. Firstly, the sample size was relatively small, and the sex ratio and glucose tolerance status was different between intervention groups. We only measure the plasma BAs without evaluating portal blood BAs, which have been shown to correlate with peripheral BA levels in both fasting and postprandial states (Angelin, Björkhem, Einarsson, & Ewerth, 1982). Additionally, we did not measure the lipid profile at three months. Thus, the short-term impact of bariatric surgery on lipid metabolism was not fully expatiated in our study.

Overall, our study showed an acute increase in both fasting and postprandial BAs, as well as fasting FGF19 levels after GBP and SG, which was seen as early as three days and sustained till three months. The increases in fasting and postprandial total, secondary-conjugated and 12α-OH BAs were common to both types of surgery and at both early time points. Rises in secondary BA and conjugated forms were correlated with early improvements in glucose metabolism at three days, while these along with 12α-OH BA were correlated with improved glucose metabolism at three months, suggesting they may contribute to improvements in glycemic control after bariatric surgery.

Chapter 4

Potential contribution of probiotic supplementation *Lactobacillus rhamnosus* HN001 in early pregnancy to improve maternal glucose metabolism associated with alterations in plasma conjugated bile acids: a randomised controlled trial

Abstract

Aims:

To investigate the effects of probiotic *Lactobacillus rhamnosus* HN001 (HN001) on glucose, lipid and bile acids (BAs) metabolism associated with gestational diabetes mellitus (GDM) in pregnant women during early pregnancy.

Materials and Methods:

In a double-blind trial, we randomised 423 participants at 14-16 weeks' gestation to consume daily HN001 (6×10^9 colony-forming units) (n=212) or placebo (n=211). The levels of plasma BAs and various metabolic indices were measured at 24-30 weeks.

Results:

The clinical and metabolic parameters of 172 (81%) women taking HN001 and 176 (83%) women taking placebo were assessed. Supplementation with HN001 significantly lowered the levels of fasting glucose compared with placebo intervention (HN001 vs. Placebo, 4.3 (4.1-4.5) vs. 4.4 (4.1-4.6) mmol L^{-1}, P=0.040). Particularly among the obese participants with GDM, a decrease in the levels of two-hr glucose after glucose loaded (HN001 vs. Placebo, 5.8 (5.6-8.0) vs. 6.9 (5.7-8.8) mmol L^{-1}, P=0.036), as well as fasting taurine-conjugated BA (HN001 vs. Placebo, 2.22 (1.41-2.73) vs. 3.85 (2.25-5.39) (μM), P=0.049) was observed by HN001 allocation. No significant differences were found in fasting insulin, HOMA-IR, LDL-c, HDL-c, total cholesterol and triglycerides between the HN001 and the Placebo group. Fasting glucose was positively correlated with GCA (r=0.12, P=0.03) and TCDCA (r=0.12, P=0.03) in individual BAs. The level of one-hr glucose was positively associated with TCDCA (r=0.11, P=0.04), GUDCA (r=0.11, P=0.04), and regarding BA composition, taurine-conjugated BAs

(r=0.10, P=0.05). Higher levels in CA (r=-0.11, P=0.03) were associated with lower two-hr glucose. Both GCDCA and THDCA were positively correlated with fasting insulin (r=0.12, P=0.03; r=0.11, P=0.04) and insulin resistance (r=0.12, P=0.02; r=0.13, P=0.02). Significant correlations were found between HDL-c and GCA (r=-0.10, P=0.05), triglycerides and CDCA (r=0.14, P<0.01) along with GCDCA (r=0.14, P=0.01).

Conclusions:

Probiotic HN001 intervention in early pregnancy improved maternal glycemic control, particularly among obese women with GDM. The alteration of fasting plasma BAs by probiotic supplementation, which was notably recognised as a decrease in taurine-conjugated BAs might play a role in the improvement of glucose metabolism. Elucidating the effect of probiotic supplementation in pregnancy on taurine-conjugated BAs may generate novel therapeutic approaches to GDM.

Keywords: Bile acids; Gestational diabetes mellitus; Glucose; Insulin; *Lactobacillus rhamnosus* HN001; Lipid.

4.1 Introduction

During pregnancy, various dramatic maternal metabolic changes will take place that facilitate ensuring fetal growth and development, as well as preparing for breastfeeding (Koren et al., 2012). However, the disorders of such metabolic changes are also associated with adverse pregnancy outcomes (Dunlop et al., 2015). Given the regulation of glucose metabolism in pregnancy as an example, early pregnancy is characterised by normal tolerance to glucose and insulin, while in late pregnancy, an increase is observed in the plasma insulin concentration accompanied by insulin resistance, which plays an important role in promoting fetal growth by shunting metabolic fuels from the mother to the fetus (Di Cianni, Miccoli, Volpe, Lencioni, & Del Prato, 2003; Lain & Catalano, 2007). However, in some pregnant women, these adaptive processes become abnormal and lead to impaired glucose tolerance. Such individuals are predisposed to gestational diabetes mellitus (GDM). Recent studies have reported that an abnormal maternal lipid profile, including total cholesterol, triglyceride, low-density lipoprotein and high-density lipoprotein increase the health risks associated with pregnancy (Gohil, Patel, & Gupta, 2011; Zeb, 2012). Arterial wall, plasma, as well as intracellular membranes are mainly made up of lipids. Hence, abnormalities of lipid metabolism may result in vascular damage, which plays a vital role in the pathogenesis of GDM (Guariguata et al., 2014). Pregnant women with GDM along with their offspring are at increased risk of developing type 2 diabetes mellitus (T2DM) in the future (Malcolm, 2012). To sum up, during pregnancy, a balanced metabolism regulation is indispensable to reduce the risk of GDM, contributing to a long-term health benefit for mother as well as the infant.

Previous demonstrations suggest that alterations in the gut and vaginal microbiome populations during pregnancy may influence the maternal metabolic profile. A method to achieve such alteration is by implemented probiotics, the lactic acid-producing bacteria such as lactobacilli and bifidobacteria as supplementation during pregnancy, which can regulate gut and vaginal microflora and thereby promote favourable metabolic activity and produce beneficial metabolites (Flach et al., 2018). It has been pointed out that supplementing with probiotics can be served as an alternative strategy to influence multiple aspects of pregnancy including metabolism regulation (De Vrese & Schrezenmeir, 2008). Through a randomised controlled trial on 256 pregnant women, Laitinen et al. reported that dietary counselling with probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12; diet/probiotic group) at the first

trimester of pregnancy lowered the blood glucose and insulin concentrations, improved the glucose tolerance, providing the first evidence of consistently improved glucose metabolism in humans through probiotics intervention (Laitinen et al., 2008). Furthermore, based on the same cohort and applying the same design and interventions, Louto et al. pointed out that the incidence of GDM was significantly reduced in the diet/probiotic group from 34 to 13 % (P=0.003) (Luoto et al., 2010). Recent research carried out by Wicken et al. towards a larger sample size (n=423) further demonstrated that supplementation with the probiotic (*Lactobacillus rhamnosus* HN001) might lower fasting mean blood glucose levels (Wickens et al., 2017). Correspondingly, the probiotic intervention reduces GDM prevalence (P=0.080), particularly among older women (P=0.009) and those with previous GDM (P=0.004).

Among other beneficial effects of probiotics, the reduction of blood lipids is also of particular interest. Evidence has shown that probiotic interventions via probiotic containing capsules (Pereira & Gibson, 2002) or foods (Mikelsaar et al., 2015) may reduce the concentration of plasma lipid in human studies. However, there are still quite a few studies which claim that no significant changes in plasma lipid are detected after intervening with probiotic supplementation (Ivey et al., 2015; Lewis & Burmeister, 2005). Therefore, further clinical research is needed to investigate the potential effects as well as mechanisms of probiotics involved in the regulation of maternal metabolic profiles during pregnancy. Studies have suggested that the modification of bile acids (BAs) by probiotic intervention may correlate with improvements in glycemic control as well as lipid homeostasis.

BAs are cholesterol-derived detergents that play a central role in the absorption of fat and cholesterol. Besides, BAs also act as metabolically active signalling molecules. For example, primary-unconjugated and secondary-unconjugated BAs can activate the farnesoid X receptor (FXR), and the Takeda G protein-coupled receptor5 (TGR5), respectively. The activation of the above receptors may increase the production of various metabolic regulatory mediators that play a critically important role in lipid and glucose metabolism via regulation of different downstream molecules (Chávez-Talavera, Tailleux, Lefebvre, & Staels, 2017; Takebayashi, Aso, & Inukai, 2010). Evidence has shown that aberrant BA homeostasis is associating with GDM or T2DM. Wewalka et al. reported that fasting taurine-conjugated BA concentrations are higher in T2DM and intermediate in impaired compared with normal glucose-tolerant persons and are

positively associated with fasting and prandial glucose, as well as insulin resistance (Wewalka et al., 2014). Gao et al. recently reported that GDM individuals demonstrate significant increases in several BA species, including GHDCA and THDCA, compared to the healthy controls (Gao et al., 2016). Collectively, BAs play a vital role in metabolic homeostasis, and alteration of body BA pool by probiotics may help to improve the glucose as well as lipid metabolism, thereby contribute to the remission of GDM. Moboni et al. recently reported that the intake of probiotic *Lactobacillus reuteri* DSM 17938 among people of different genders with T2DM improved insulin sensitivity, and was associated with increases in DCA levels (Mobini et al., 2017). However, studies have so far not been able to answer the question of whether there is a relationship between the influence of probiotics on metabolic profiles and BAs, particularly in the pregnant woman with or without GDM. Thus, it is vital to analyse the probable modification in plasma BA levels induced by probiotic supplementation during pregnancy corresponding with any metabolic improvements, which may help to better elucidate the mechanism of action of probiotic intervention, thereby leading to novel treatments for GDM.

The objective of the present study is to investigate whether the intake of probiotic *L. rhamnosus* HN001 (HN001) supplement by pregnant mothers with or without GDM from early pregnancy can lead to any improvements on glucose and lipid metabolism, as well as modifications on circulating BA levels and compositions. We also analyse the relationships between BAs and different clinical and metabolic criteria in the studied population. Based upon the available evidence, we hypothesise that HN001 intervention may improve maternal glycemic control as well as lipid metabolism, corresponding with a promotion of the entire composition (or particular individual amounts) of primary- or secondary- unconjugated BAs. Some specific individual conjugated BAs may be decreased with an intake of HN001 as well.

4.2 Materials and methods

4.2.1 Participants

A total of 432 pregnant women in Auckland and Wellington, New Zealand, were recruited via advertisements placed in pregnancy packs. Inclusion criteria were: age>16 years; 14-16 weeks' gestation; English-speaking; stated availability throughout the study period; tending to breastfeed; having (or, their unborn child's biological father had) a history of asthma, hay fever or eczema requiring medication. Exclusion criteria were: having a history of immune dysregulation, or cardiac valve disease, required in-vitro fertilisation; having major fetal abnormalities; currently using supplements containing probiotics; participating in other clinical trials; refusing notification of their clinical carers; carrying adrenaline for cows' milk allergy; having a history of a transplant or HIV; administration of antibiotic therapy <3 months before randomisation; miscarrying between screening and enrolment.

4.2.2 Study design

We performed a two-centre, double-blind, randomised, placebo-controlled trial with two parallel groups of participants receiving either 6×10^9 colony-forming units (CFU) daily of *Lactobacillus rhamnosus* HN001 (Fonterra Co-operative Group Ltd, New Zealand) or placebo (Grain Processing Corporation, USA). The design of the study capsules containing HN001 or placebo has been previously described (Wickens et al., 2017). Participants were stratified according to a computer-generated randomisation schedule and an allocation ratio of 1:1, randomised to HN001 or placebo in blocks of twenty by the Fonterra study centre. All researchers, relevant staff and participants were blinded to study treatment allocation. The effects of the HN001 intervention on the plasma glucose, insulin, lipids, and BA concentrations in pregnant women were investigated. An overview of the study design is shown in Figure 4.1.

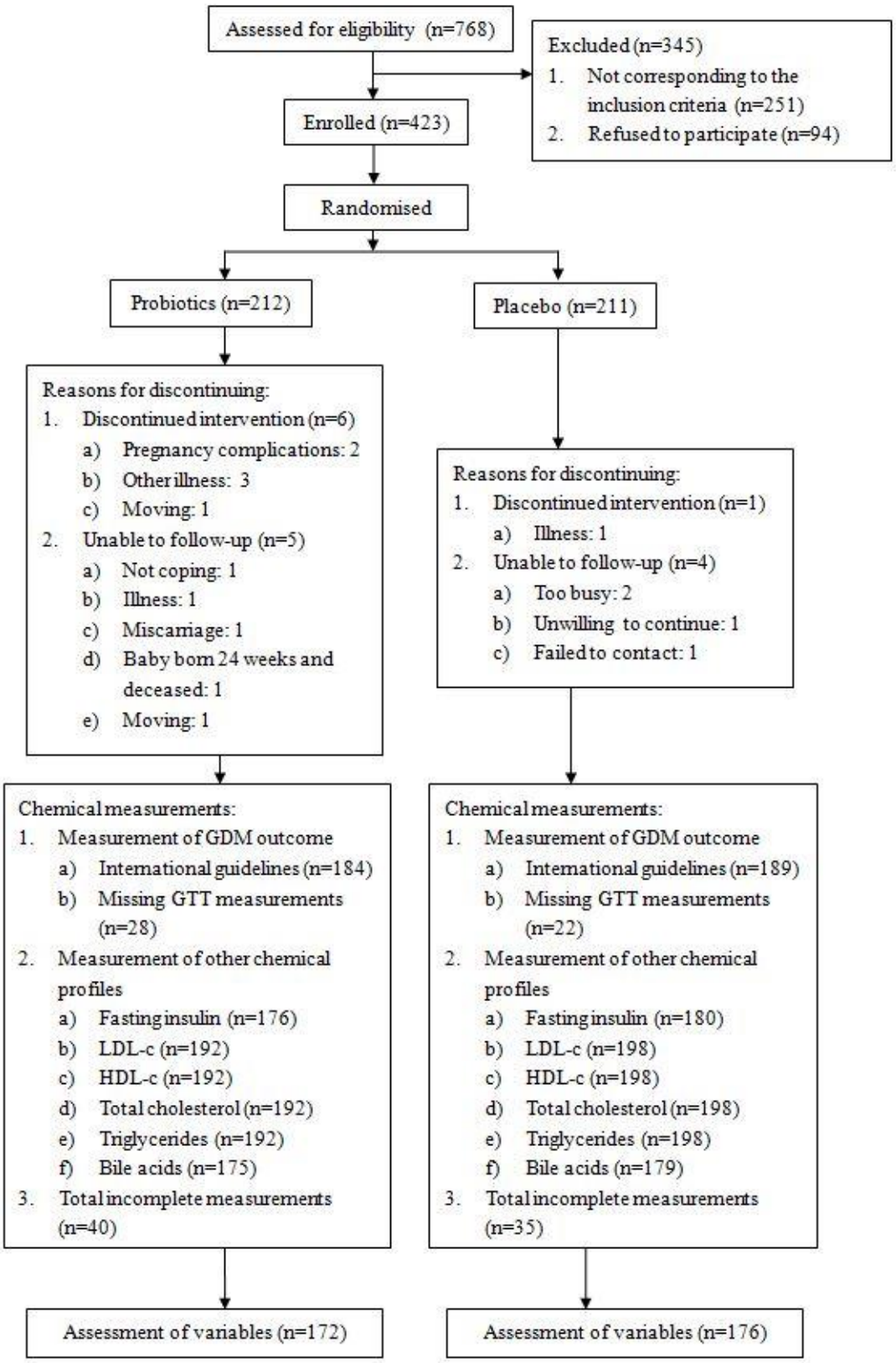


Figure 4.1. Design of the study, status of study participants as well as data collections through the trial.

4.2.3 Laboratory measurements

The maternal age, weight (kg), waist circumference (cm), and body mass index (BMI) (kg m^{-2}) details of the participants were recorded when enrolled. The oral glucose tolerance test (OGTT) and the evaluation of plasma glucose were conducted at a community laboratory among studied participants at 24-30 weeks' gestation. Plasma samples were collected from antecubital veins during fasting and at one and two hours after the 75-g glucose load. Samples were immediately centrifuged at 4 °C, and subsequently stored as aliquots at -80 ºC until analysis.

The concentrations of insulin, low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c), total cholesterol, and triglycerides were measured in the fasting state by an auto-analyser (Roche Diagnostics, Switzerland) according to Roche's manufacturer protocols.

The assessment of BAs was based on an established liquid chromatography-tandem mass spectrometry (LC-MS/MS) method described previously (Tagliacozzi et al., 2003) with slight modifications to optimise the detection sensitivity according to previous publication (Nemati et al., 2018b). The LC-MS/MS system consists of an HPLC Agilent 1200 series apparatus and the Agilent 6420 Triple Quadrupole MS/MS (Agilent Technologies, USA). Fasting BAs analysis includes CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; TCDCA , taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; TLCA, taurolithocholic acid and THDCA, taurohyodeoxycholic acid. Due to undetectable plasma concentrations, the records of LCA and HDCA were removed from all calculations and analyses.

4.2.4 Calculation

The assessment of GDM status was determined based on the IADPSG recommendations (Diabetes & Panel, 2010): fasting plasma glucose ≥5.1 mmol L^{-1}, or one-hr glucose ≥10 mmol L^{-1} or two-hr glucose ≥8.5 mmol L^{-1}.

The definition of obese was based on the National Institutes of Health suggested BMI of 30 kg m^{-2} and above.

Diabetes indices for this study include the homeostatic model assessment of insulin resistance (HOMA-IR).

BA compositions were classified according to their site of synthesis (primary vs. secondary) or conjugation state (conjugated vs. unconjugated). The molar sum of BA concentrations in each category was used to determine the levels of each BA composition. Compositions included: (1) Total BAs=all 13 BAs; (2) Primary BAs=CA, GCA, CDCA, GCDCA and TCDCA; (3) Secondary BAs=DCA, GDCA, TDCA, UDCA, GUDCA, TUDCA, TLCA and THDCA; (4) Conjugated BAs=all glycine and taurine conjugated BAs; (7) Unconjugated BAs=all unconjugated BAs; (8) Glycine-conjugated BAs; (9) Taurine-conjugated BAs; (10) Primary-conjugated BAs; (11) Primary-unconjugated BAs; (12) Secondary-conjugated BAs; and (13) Secondary-unconjugated BAs.

4.2.5 Statistical analysis

Generated data were analysed using RStudio Version 1.1.414. Normal distribution of model residuals was tested with the Kolmogorov-Smirnov test or Shapiro-Wilks test as appropriate. Significant differences between groups were evaluated using unpaired Student's t-tests or Mann–Whitney U test based on the distribution of the data. Data were presented as mean ± SD, number (%) or median (IQR) as required. The correlation assays were performed using Pearson rank tests. Statistical significance was set at $P<0.05$ (two-tailed).

4.3 Results

4.3.1 Characteristics of the study population

Participants (n=423) were randomised to the HN001 (n=212) or placebo group (n=211) between December 2012 and November 2014 at an average rate of 4.2 a week. GDM assessments were completed by February 2015 and biochemical analysis was done by January 2018. As shown in Figure 4.1, the rates for discontinuance were similar between the study group (HN001 vs. Placebo, 5% vs. 3%).

Among randomised participants, 184 (87 %) participants in the HN001 group, and 189 (90 %) in the placebo group completed the 24–30 weeks' OGTT results containing all three time points (fasting, 1 h and 2 h), at 27.7 ± 4.6 and 28.0 ± 8.6 weeks' gestation, respectively. Incomplete biochemical assessments (HN001 vs. Placebo, 40 (19%) vs. 35 (17%)) were either due to discontinued intervention, loss to follow-up, or inadequate records of plasma glucose levels at necessary time points required by IADPSG guideline definitions during OGTT, insufficient aliquoting of the samples, or other unexpected failures during experiments. In total, the data for 172 (81%) and 176 (83%) participants in the HN001 group and the placebo group, respectively, were adopted for the assessment of all clinical variables or biochemical indices (Figure 4.1).

Baseline characteristics of the 348 studied participants are shown in Table 4.1. There were no significant differences between the groups at baseline concerning parameters of age, weight, waist circumference, BMI or ethnicities.

4.3.2 Effect of *L. rhamnosus* HN001 supplementation on glucose and lipid profiles

As shown in Table 4.1, we observed a significant decrease in fasting glucose levels in the HN001 group compared with the placebo group (HN001 vs. Placebo, 4.3 (4.1-4.5) vs. 4.4 (4.1-4.6) mmol L^{-1}, P=0.040). The one-hr glucose was slightly lower in the HN001 group, but did not differ significantly between groups (HN001 vs. Placebo, 6.6 (5.6-7.7) vs. 6.7 (5.7-8.1) mmol L^{-1}, P=0.258). We did not observe any significant effect of HN001 supplementation on two-hr glucose, fasting insulin, HOMA-IR, LDL-c, HDL-c, total cholesterol and triglycerides among all 348 studied participants regardless of their GDM or obesity status.

Table 4.1. Clinical characteristics, ethnicities, diabetes indices and lipid profiles of the participants supplemented with L. rhamnosus HN001 or placebo.

	HN001 (n=172)	**Placebo (n=176)**	**p-value HN001 vs. Placebo**
Baseline characteristics			
Age (years)	33.1±4.2	33.8±4.3	0.144
Weight (kg)	68.4 (63.0-79.1)	71.1 (63.3-81.9)	0.194
Waist circumference (cm)	86.4 (79.8-93.8)	86.8 (80.6-99.1)	0.146
BMI (kg m^{-2})	25.1 (22.9-28.6)	25.8 (23.0-30.0)	0.209
Ethnicities			0.568
European	139 (80.8)	137 (77.8)	
Maori	17 (9.9)	25 (14.2)	
Pacific	4 (2.3)	3 (1.7)	
Asian	12 (7.0)	10 (5.7)	
Other	0 (0.0)	1 (0.6)	
Diabetes indices			
Fasting glucose (mmol L^{-1})	4.3 (4.1-4.5)	4.4 (4.1-4.6)	**0.040**
One-hr glucose (mmol L^{-1})	6.6 (5.6-7.7)	6.7 (5.7-8.1)	0.258
Two-hr glucose (mmol L^{-1})	5.5 (4.9-6.3)	5.5 (4.7-6.5)	0.791
Fasting insulin (pmol L^{-1})	64.4 (48.6-92.4)	60.1 (41.9-86.0)	0.134
HOMA-IR	1.9 (1.3-2.5)	1.7 (1.1-2.5)	0.281
Lipid profiles			
LDL-c (mmol L^{-1})	3.8 (3.2-4.6)	3.7 (3.0-4.6)	0.287
HDL-c (mmol L^{-1})	1.9 (1.6-2.2)	1.9 (1.7-2.2)	0.451
Total cholesterol (mmol L^{-1})	6.3 (5.5-7.1)	6.1 (5.3-7.0)	0.426
Triglycerides (mmol L^{-1})	1.7 (1.3-2.1)	1.6 (1.3-2.0)	0.264

Data are mean ± SD., number (%) or median (IQR).

Table 4.2 lists the results of HN001 intervention associated with different metabolic profiles, where the groups were further stratified by GDM and obesity status of the

studied participants. We determined that HN001 intervention significantly lowered the two-hr glucose level particularly among the obese participant with GDM (HN001 vs. Placebo, 5.8 (5.6-8.0) vs. 6.9 (5.7-8.8) mmol L^{-1}, P=0.036). However, HN001 did not seem to have a similar effect on two-hr glucose in the other stratifications. Notably, in the non-obese & non-GDM group, fasting insulin (HN001 vs. Placebo, 59.8 (43.8-76.6) vs. 52.0 (37.1-68.4) pmol L^{-1}, P=0.006) and HOMA-IR (HN001 vs. Placebo, 1.6 (1.2-2.1) vs. 1.5 (1.0-1.9), P=0.014), were significantly increased by HN001 allocation. No significant differences were observed in lipids between HN001 and Placebo groups in any stratification.

Table 4.2. Diabetes indices and lipid profiles of the participants supplemented with L. rhamnosus HN001 or placebo stratified by different GDM or obesity status.

	Obese & GDM			**Obese & non-GDM**			**non-Obese & GDM**			**non-Obese & non-GDM**		
	HN001 (n=7)	**Placebo (n=10)**	**p-value HN001 vs. Placebo**	**HN001 (n=25)**	**Placebo (n=29)**	**p-value HN001 vs. Placebo**	**HN001 (n=6)**	**Placebo (n=15)**	**p-value HN001 vs. Placebo**	**HN001 (n=134)**	**Placebo (n=122)**	**p-value HN001 vs. Placebo**
Diabetes indices												
Fasting glucose (mmol L^{-1})	5.1 (4.9-5.2)	5.1 (4.6-5.5)	0.557	4.5 (4.2-4.6)	4.6 (4.3-4.7)	0.489	4.3 (4.2-4.8)	4.7 (4.6-5.1)	0.289	4.2 (4.0-4.5)	4.3 (4.1-4.5)	0.386
One-hr glucose (mmol L^{-1})	10 (8.6-10)	9.4 (8.6-10)	0.977	7.8 (6.7-8.5)	7.0 (6.2-8.0)	0.084	11 (10-11)	10 (9.5-11)	0.300	6.2 (5.1-7.1)	6.2 (5.4-7.4)	0.617
Two-hr glucose (mmol L^{-1})	5.8 (5.6-8.0)	6.9 (5.7-8.8)	**0.036**	6.4 (6.7-6.8)	6.0 (5.2-6.3)	0.231	8.2 (6.5-8.8)	7.3 (6.7-8.8)	0.667	5.3 (4.7-5.8)	5.3 (4.6-5.9)	0.829
Fasting insulin (pmol L^{-1})	103 (96.3-144)	137 (90.5-198)	0.475	96.1 (81.1-137)	108 (75.6-140)	0.810	139 (81.6-206)	71.7 (63.1-94.4)	0.132	59.8 (43.8-76.6)	52.0 (37.1-68.4)	**0.006**
HOMA-IR	3.3 (2.7-4.7)	4.7 (2.9-6.2)	0.601	2.8 (2.2-4.4)	3.2 (2.2-4.1)	0.692	4.2 (2.3-6.5)	2.1 (1.8-2.8)	0.235	1.6 (1.2-2.1)	1.5 (1.0-1.9)	**0.014**
Lipid profile												
LDL-c (mmol L^{-1})	3.7 (2.7-4.2)	3.3 (2.4-4.2)	0.689	3.6 (3.1-4.7)	3.7 (3.4-4.5)	0.979	3.8 (3.0-5.3)	3.9 (2.6-4.7)	0.503	3.8 (3.3-4.5)	3.6 (3.0-4.6)	0.328

HDL-c (mmol L^{-1})	1.6 (1.4-1.7)	1.7 (1.5-2.3)	0.265	2.0 (1.5-2.4)	1.9 (1.7-2.0)	0.824	1.9 (1.8-2.5)	2.0 (1.4-2.2)	0.315	1.9 (1.6-2.2)	2.0 (1.7-2.2)	0.209
Total cholesterol (mmol L^{-1})	5.9 (5.5-6.5)	5.3 (5.1-6.7)	0.751	6.4 (5.6-7.8)	6.4 (5.6-7.0)	0.623	6.4 (5.7-7.6)	6.4 (5.1-7.4)	0.369	6.3 (5.5-7.0)	6.1 (5.4-7.0)	0.631
Triglycerides (mmol L^{-1})	2.0 (1.9-2.3)	1.9 (1.4-2.4)	0.653	2.1 (1.6-2.4)	1.9 (1.8-2.4)	0.850	1.8 (1.6-1.9)	1.9 (1.6-2.4)	0.417	1.6 (1.3-2.0)	1.5 (1.3-1.8)	0.066

Data are median (IQR).

4.3.3 Effect of *L. rhamnosus* HN001 supplementation on bile acids

We did not observe a shift of any individual BA population as a consequence of HN001 treatment (Table 4.3, Figure 4.2 b). We have also compared the difference in BAs between the GDM and the non-GDM participants (Figure 4.2 a). Total BAs in the GDM group were slightly higher than the non-GDM group (GDM vs. non-GDM, 26.03 (17.02-42.28) vs. 24.95 (14.90-40.71) (μM), P=0.336), which was mainly due to the alteration of taurine-conjugated BA composition (GDM vs. non-GDM, 3.55 (2.30-5.92) vs. 2.88 (1.44-4.95) (μM), P=0.103), though neither of the differences was significant. We have also investigated the modifications in BA compositions under HN001 exposures among different stratifications of participants based on their GDM and/or obesity status. Combining Figure 4.2 c-1 and c-2, it can be noticed that the total BAs appeared to be lower under HN001 treatment particularly in the GDM group (HN001 vs. Placebo, 19.15 (16.61-32.30) vs. 32.20 (18.90-47.24) (μM), P=0.259). However, BA individuals or compositions were not determined to be different significantly with HN001 intervention, among GDM, non-GDM, obese or non-obese participants (Figure 4.2 c-1 to d-2).

Table 4.3. Fasting levels of bile acid individuals of the participants supplemented with L. rhamnosus HN001 or placebo.

	HN001 (n=172)	**Placebo (n=176)**	**p-value HN001 vs. Placebo**
Primary-unconjugated			
CA (μM)	1.86 (0.77-4.23)	1.61 (0.84-7.04)	0.511
CDCA (μM)	1.10 (0.50-1.91)	1.24 (0.63-1.89)	0.413
Secondary-unconjugated			
DCA (μM)	1.03 (0.52-1.94)	1.00 (0.51-1.78)	0.981
UDCA (μM)	0.93 (0.47-1.71)	0.82 (0.41-1.76)	0.366
Glycine-conjugated			
GCA (μM)	3.41 (1.62-5.63)	3.48 (2.24-6.06)	0.243
GCDCA (μM)	4.24 (2.26-7.44)	4.39 (2.70-7.89)	0.433
GDCA (μM)	3.07 (1.37-6.09)	3.18 (1.74-5.77)	0.488
GUDCA (μM)	0.36 (0.19-0.75)	0.43 (0.22-0.76)	0.480
Taurine-conjugated			
TCDCA (μM)	1.29 (0.69-2.63)	1.64 (0.76-3.10)	0.133
TDCA (μM)	1.13 (0.50-1.95)	1.15 (0.55-1.87)	0.759
TUDCA (μM)	0.06 (0.02-0.12)	0.05 (0.03-0.10)	0.907
TLCA (μM)	0.06 (0.04-0.09)	0.06 (0.04-0.10)	0.459
THDCA (μM)	0.03 (0.01-0.07)	0.03 (0.01-0.06)	0.662

Data are median (IQR).

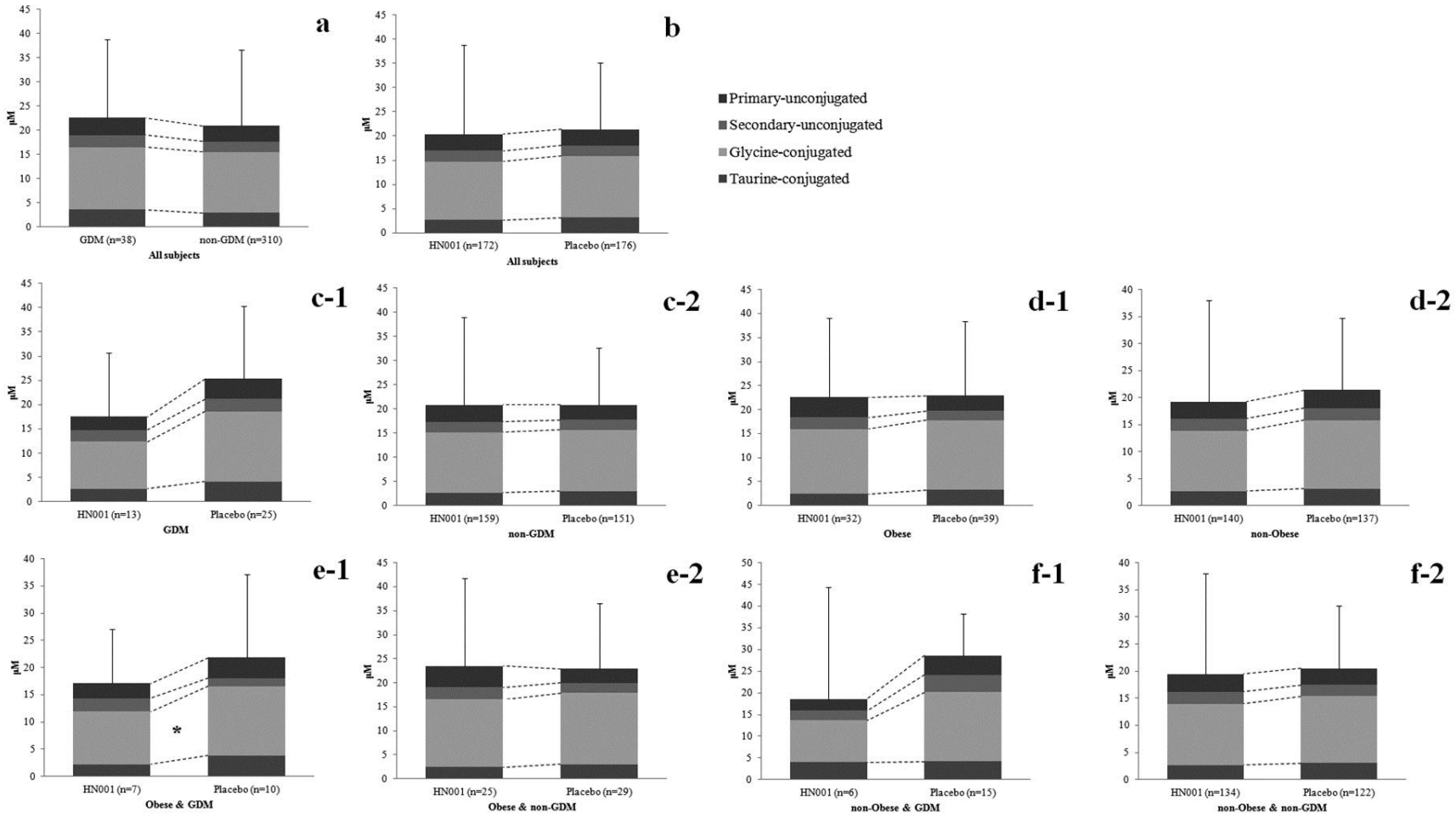


Figure 4.2. Fasting levels of bile acid compositions in the participants supplemented with L. rhamnosus HN001 or placebo stratified by different GDM or obesity status. Data are median (IQR). * P<0.05.

Interestingly, as shown in Figure 4.2 e-1, we observed that taurine-conjugated BA composition was significantly decreased in the HN001 group compared with the placebo group (HN001 vs. Placebo, 2.22 (1.41-2.73) vs. 3.85 (2.25-5.39) (μM), P=0.049), which mainly arose from the alteration of TDCA (HN001 vs. Placebo, 0.76 (0.40-1.17) vs. 1.64 (1.21-2.13) (μM), P=0.028) (Table 4.4). However, we did not find the similar and significant alterations in bile acids among other stratifications.

Table 4.4. Fasting levels of individual bile acids in the participants supplemented with L. rhamnosus HN001 or placebo stratified by different GDM or obesity status.

	Obese & GDM			Obese & non-GDM			non-Obese & GDM			non-Obese & non-GDM		
	HN001 (n=7)	**Placebo (n=10)**	**p-value HN001 vs. Placebo**	**HN001 (n=25)**	**Placebo (n=29)**	**p-value HN001 vs. Placebo**	**HN001 (n=6)**	**Placebo (n=15)**	**p-value HN001 vs. Placebo**	**HN001 (n=134)**	**Placebo (n=122)**	**p-value HN001 vs. Placebo**
Primary-unconjugated												
CA (μM)	0.77 (0.66-3.60)	1.25 (0.71-2.90)	0.813	2.34 (0.96-7.30)	1.67 (0.88-18.4)	0.959	1.96 (1.52-3.11)	3.31 (1.71-7.67)	0.470	1.86 (0.75-3.56)	1.47 (0.82-7.02)	0.759
CDCA (μM)	2.31 (0.54-2.42)	0.97 (0.73-2.39)	0.824	1.12 (0.58-2.86)	1.32 (0.75-2.10)	0.618	0.45 (0.21-0.81)	1.85 (0.79-2.54)	0.085	1.17 (0.52-1.88)	1.24 (0.59-1.77)	0.855
Secondary-unconjugated												
DCA (μM)	1.59 (1.02-1.69)	0.99 (0.55-1.48)	0.475	0.96 (0.48-2.22)	1.19 (0.52-2.00)	0.837	0.86 (0.45-1.58)	1.77 (0.67-2.80)	0.179	1.03 (0.55-1.95)	0.94 (0.51-1.69)	0.688
UDCA (μM)	0.75 (0.57-1.53)	0.41 (0.30-0.85)	0.315	0.93 (0.42-1.81)	0.67 (0.42-1.13)	0.438	1.11 (1.03-1.54)	1.52 (0.62-2.19)	0.519	0.93 (0.48-1.72)	0.88 (0.42-1.82)	0.585
Glycine-conjugated												
GCA (μM)	2.23 (1.64-2.88)	3.43 (2.35-5.61)	0.315	3.99 (3.23-7.53)	3.85 (2.62-8.39)	0.810	4.19 (3.86-5.80)	4.95 (2.93-9.89)	0.791	3.27 (1.48-5.44)	3.26 (1.95-5.39)	0.572
GCDCA (μM)	3.86 (2.62-6.12)	3.88 (2.75-6.99)	0.669	5.43 (2.48-9.33)	4.21 (2.69-12.2)	0.630	4.20 (2.61-5.52)	5.39 (3.25-12.2)	0.470	4.13 (2.27-7.31)	4.37 (2.67-6.91)	0.810

GDCA (μM)	3.66 (2.03-3.95)	4.63 (2.80-5.76)	0.307	2.73 (1.93-4.79)	3.01 (2.18-8.33)	0.594	1.72 (1.45-2.69)	4.05 (3.17-7.67)	0.095	3.21 (1.32-6.76)	2.98 (1.52-5.10)	0.905
GUDCA (μM)	0.31 (0.23-0.86)	0.44 (0.35-0.87)	0.536	0.45 (0.24-0.92)	0.64 (0.27-1.18)	0.480	0.49 (0.32-0.67)	0.38 (0.27-0.62)	0.791	0.35 (0.19-0.69)	0.39 (0.18-0.72)	0.825
Taurine-conjugated												
TCDCA (μM)	1.24 (1.00-1.39)	2.05 (0.65-3.44)	0.093	1.53 (0.81-2.35)	1.63 (1.01-3.10)	0.594	2.26 (1.80-3.72)	2.73 (1.53-5.59)	0.863	1.23 (0.69-2.69)	1.61 (0.73-2.89)	0.426
TDCA (μM)	0.76 (0.40-1.17)	1.64 (1.21-2.13)	**0.028**	0.93 (0.51-2.08)	1.28 (0.45-1.97)	0.986	1.04 (0.63-1.96)	1.63 (1.22-2.19)	0.470	1.17 (0.50-2.02)	1.04 (0.54-1.62)	0.533
TUDCA (μM)	0.06 (0.03-0.10)	0.03 (0.02-0.13)	0.737	0.05 (0.02-0.13)	0.09 (0.04-0.14)	0.119	0.08 (0.06-0.21)	0.08 (0.05-0.14)	0.439	0.06 (0.03-0.10)	0.05 (0.02-0.09)	0.458
TLCA (μM)	0.07 (0.05-0.08)	0.07 (0.06-0.08)	0.887	0.07 (0.02-0.10)	0.05 (0.04-0.09)	0.945	0.04 (0.02-0.05)	0.06 (0.04-0.12)	0.112	0.06 (0.04-0.09)	0.06 (0.03-0.10)	0.654
THDCA (μM)	0.02 (0.01-0.02)	0.03 (0.01-0.04)	0.364	0.04 (0.01-0.08)	0.04 (0.02-0.06)	0.692	0.09 (0.03-0.12)	0.04 (0.02-0.08)	0.622	0.03 (0.01-0.07)	0.02 (0.01-0.06)	0.281

4.3.4 Correlations between clinical and metabolic profiles and bile acids

The associations between the baseline clinical characteristics, glucose as well as lipid profiles, and BA individuals (as well as classified compositions) are shown in Table 4.5. No significant correlation was found between maternal weight or BMI and any BA individual/composition. Fasting glucose was positively correlated with GCA (r=0.12, P=0.03), TCDCA (r=0.12, P=0.03) in individual BAs. In terms of BA compositions, positive correlations had been observed between fasting glucose and total (r=0.14, P=0.01), primary (r=0.13, P=0.01), secondary (r=0.11, P=0.05), conjugated (r=0.13, P=0.02), unconjugated (r=0.11, P<0.01), glycine-conjugated (r=0.11, P=0.04) and primary-conjugated (r=0.13, P=0.01) BAs. The level of one-hr glucose was positively associated with TCDCA (r=0.11, P=0.04), GUDCA (r=0.11, P=0.04) and taurine-conjugated (r=0.10, P=0.05) BAs. We found that higher levels in CA (r=-0.11, P=0.03) associated with lower two-hr glucose. GCDCA was positively correlated with fasting insulin (r=0.12, P=0.03) as well as HOMA-IR (r=0.12, P=0.02). Similarly, a positive association was identified between THDCA and fasting insulin (r=0.11, P=0.04) as well as insulin resistance (r=0.13, P=0.02). Although significant correlations had been found between HDL-c and GCA (r=-0.10, P=0.05), triglycerides and CDCA (r=0.14, P<0.01) as well as GCDCA (r=0.14, P=0.01), no significant association was observed between lipid profiles with any classified BA compositions.

Table 4.5. Correlations of fasting bile acids with clinical and metabolic parameters.

	Weight		BMI		Fasting glucose		One-hr glucose		Two-hr glucose		Fasting insulin		HOMA-IR		LDL-c		HDL-c		Total cholesterol		Triglycerides	
	r	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**
CA	-0.05	0.33	-0.01	0.87	0.09	0.11	-0.02	0.74	-0.11	**0.03**	0.00	0.98	0.01	0.87	-0.01	0.84	-0.02	0.76	-0.01	0.78	0.02	0.73
CDCA	0.09	0.08	0.10	0.07	0.05	0.37	0.06	0.27	0.05	0.35	-0.04	0.41	-0.04	0.44	-0.04	0.46	0.02	0.67	-0.01	0.90	0.15	**0.00***
GCA	0.06	0.28	0.08	0.14	0.12	**0.03***	0.01	0.83	0.03	0.64	0.07	0.18	0.08	0.16	-0.04	0.50	-0.10	**0.05***	-0.06	0.26	0.08	0.13
GCDCA	0.07	0.21	0.09	0.10	0.10	0.06	0.07	0.19	0.01	0.92	0.12	**0.03***	0.12	**0.02***	-0.06	0.28	-0.04	0.42	-0.07	0.23	0.06	0.29
TCDCA	0.00	0.98	0.05	0.38	0.12	**0.03***	0.11	**0.04***	0.06	0.30	0.09	0.10	0.09	0.08	-0.10	0.08	0.03	0.60	-0.07	0.16	0.01	0.91
DCA	0.04	0.45	0.04	0.44	0.08	0.15	0.06	0.23	0.04	0.50	-0.04	0.47	-0.04	0.50	-0.03	0.54	0.01	0.82	-0.02	0.69	0.05	0.37
UDCA	-0.05	0.40	-0.05	0.31	0.07	0.21	0.01	0.86	-0.07	0.21	-0.01	0.84	-0.01	0.87	0.03	0.63	0.10	0.07	0.04	0.42	-0.02	0.71
GDCA	0.04	0.41	0.06	0.30	0.06	0.25	0.05	0.35	0.03	0.59	0.09	0.10	0.09	0.09	0.01	0.82	-0.03	0.52	0.00	0.99	0.07	0.20
GUDCA	0.02	0.66	0.06	0.23	0.06	0.23	0.11	**0.04***	0.03	0.58	0.07	0.22	0.07	0.19	0.00	0.99	-0.04	0.51	0.01	0.86	0.14	**0.01***
TDCA	0.00	0.94	0.05	0.37	0.09	0.11	0.08	0.14	0.05	0.34	0.08	0.12	0.09	0.10	-0.06	0.24	0.05	0.40	-0.05	0.40	0.00	0.96
TUDCA	-0.04	0.41	0.00	0.94	0.01	0.92	0.07	0.17	0.03	0.62	0.08	0.14	0.08	0.14	0.02	0.77	-0.06	0.25	0.01	0.91	0.10	0.07
TLCA	-0.05	0.37	-0.03	0.62	0.01	0.93	0.03	0.58	-0.05	0.35	-0.02	0.77	-0.01	0.78	0.01	0.93	0.06	0.23	0.01	0.88	-0.08	0.16
THDCA	0.01	0.80	0.03	0.60	0.04	0.46	0.06	0.24	0.03	0.62	0.11	**0.04***	0.13	**0.02***	-0.04	0.49	-0.04	0.47	-0.04	0.40	0.01	0.81
Total	0.01	0.79	0.06	0.29	0.14	**0.01***	0.06	0.28	-0.03	0.62	0.08	0.15	0.09	0.11	-0.04	0.43	-0.02	0.71	-0.04	0.41	0.06	0.28
Primary	0.02	0.73	0.06	0.26	0.13	**0.01***	0.04	0.49	-0.03	0.54	0.07	0.20	0.08	0.15	0.33	0.33	-0.05	0.37	-0.06	0.27	0.06	0.25
Secondary	0.00	1.00	0.03	0.62	0.11	**0.05***	0.08	0.12	0.00	0.97	0.07	0.21	0.07	0.18	0.00	0.93	0.05	0.35	0.00	0.94	0.03	0.60
Conjugated	0.05	0.38	0.08	0.12	0.13	**0.02***	0.08	0.14	0.03	0.57	0.11	**0.04***	0.12	**0.03***	-0.05	0.32	-0.04	0.45	-0.06	0.26	0.06	0.25

Unconjugated	-0.05	0.40	-0.01	0.83	0.11	**0.04***	0.00	0.99	-0.11	**0.04***	-0.01	0.81	0.00	0.95	-0.01	0.88	0.02	0.70	0.00	0.98	0.03	0.59
Glycine-conjugated	0.07	0.23	0.09	0.10	0.11	**0.04***	0.05	0.33	0.02	0.66	0.11	**0.05***	0.11	**0.04***	-0.03	0.53	-0.07	0.17	-0.05	0.35	0.08	0.11
Taurine-conjugated	-0.02	0.77	0.04	0.50	0.10	0.06	0.10	**0.05***	0.03	0.53	0.08	0.15	0.08	0.12	-0.08	0.16	0.06	0.31	-0.06	0.30	-0.02	0.68
Primary-conjugated	0.06	0.28	0.09	0.10	0.13	**0.01***	0.06	0.23	0.03	0.59	0.11	**0.04***	0.11	**0.03***	-0.07	0.21	-0.06	0.25	-0.08	0.15	0.07	0.22
Primary-unconjugated	-0.04	0.46	0.00	0.96	0.09	0.09	-0.01	0.85	-0.11	**0.05***	-0.01	0.91	0.00	0.95	-0.02	0.78	-0.01	0.80	-0.02	0.78	0.04	0.51
Secondary-conjugated	0.02	0.75	0.05	0.31	0.08	0.13	0.09	0.11	0.03	0.62	0.09	0.08	0.10	0.07	-0.01	0.79	0.01	0.85	-0.01	0.79	0.04	0.48
Secondary-unconjugated	-0.03	0.57	-0.04	0.48	0.08	0.12	0.03	0.62	-0.05	0.33	-0.02	0.70	-0.02	0.73	0.01	0.78	0.09	0.09	0.03	0.53	-0.01	0.92

* $P<0.05$.

4.4 Discussion

Glycemic control, lipid homeostasis and BA metabolism are mutually linked via an intriguing interaction between BAs and two major BA receptors, FXR and TGR5. The synthesis, metabolism, and distribution of BAs in the body can be regulated by gut microbiota, including deconjugation, dehydrogenation, dehydroxylation and epimerisation of BAs (Molinaro, Wahlström, & Marschall, 2017). The gut microbiota modulation induced by probiotics may modify the metabolic production of BAs, and in turn triggers the development of glucose and lipid metabolism. Thus, the study regarding the effect of probiotics supplementation during pregnancy on BAs corresponding with glucose and lipid metabolism is of great interest for seeking a therapeutic approach to regulating maternal metabolism, and thereby contribute to the prevention and/or treatment of GDM.

In the presented study, we conducted a two-centre, double-blind, randomised, placebo-controlled trial and showed that pregnant women at 14-16 weeks' gestation receiving probiotic *L. rhamnosus* HN001 administrations had a decreased amount of fasting glucose levels compared with the placebo group. A positive correlation was found between fasting glucose and total BAs. We have also compared the BA compositions between the GDM and the non-GDM participants, as well as the HN001 and the placebo groups. Based on our observations, the total BA value was relatively higher in the GDM group vs. the non-GDM group, lower in the HN001 group vs. the placebo group, but neither of the differences was statistically significant. Whether there was a link between the lower total BA concentrations induced by HN001 intervention and the decreased levels of fasting glucose, is unclear. Nevertheless, the above findings might indicate that altering BA metabolism could play a role in the regulation of maternal glycemic control by the HN001 supplement.

One-hr and two-hr post-load glucose, fasting insulin, along with insulin resistance were not altered by HN001 intervention significantly throughout the whole studied populations. Notably, our data also suggested that the probiotic HN001 at a dose of 6×10^9 CFU/d may lower two-hr glucose, taurine-conjugated BAs (mainly TDCA) among the obese participants with GDM. Our study is consistent with a randomised clinical trial revealing that the probiotic supplement containing lactobacilli species appeared to affect glucose metabolism among pregnant women, especially those with GDM

(Dolatkhah et al., 2015). Nevertheless, as far as we are aware, no studies have investigated the influence of probiotic intervention in pregnancy on BAs associated with maternal GDM or obesity status. Therefore, our results from a relatively large cohort of women in early pregnancy make a unique contribution to the existing literature on the composition/individual presence of plasma BA changes that occur after intervention with HN001, especially among obese participants with GDM.

Previous studies have indicated that various strains of lactobacillus have the ability to induce BA deconjugation as well as BA hydrolysis (Allain et al., 2018; Travers et al., 2016). Additionally, emerging evidence has shown that there is an underlying link between the gut microbiome and human obesity (Liu et al., 2017). Comparison of gut microbial compositions between lean and obese individuals revealed significant differences in gene abundances as well as species (Le Chatelier et al., 2013). Moreover, it is likely that GDM can alter the microbiota of pregnant women (Wang et al., 2018). A recent research reported differences across GDM and non-GDM pregnant women in the relative abundance of various compositions of gut microbiota (FESTA et al., 2018). Hence, the impact of probiotic supplement *L. rhamnosus* HN001 implemented in our study on the gut microbiome, and subsequently, BA and glucose metabolism, might be different from the maternal GDM as well as obesity status, as was observed in our study.

We also showed that taurine-conjugated BAs positively associated with one-hr glucose. Our observation is in line with a previous report, suggesting that taurine-conjugated BAs were positively correlated with fasting glucose, post-load glucose, fasting insulin and HOMA-IR (Wewalka et al., 2014). The positive correlations between taurine-conjugated BAs and the above parameters were also observed in our study, but most of the correlations were not significant. Apart from research on the metabolism of total BAs, few studies have been carried out regarding taurine-conjugated BAs, due to their low levels in humans (Hylemon et al., 2009). Accordingly, the molecular mechanisms for how HN001 itself, or altered gut microbiota, suppresses taurine-conjugated BAs, and how this effect relates to maternal glycemic control are currently unknown, but it might be FXR-associated based on currently understood mechanisms of action.

FXR serves as an essential regulator of glucose and lipid homeostasis, and can be primarily activated by primary-unconjugated BAs (Claudel, Staels, & Kuipers, 2005). The activation of FXR can suppress hepatic gluconeogenesis, but also increase hepatic glycogen synthesis as well as glycogen content through a mechanism involving

promoted insulin sensitivity (Y. Zhang et al., 2006). FXR-deficient mice show impaired glucose tolerance, decreased insulin sensitivity, and significantly blunted insulin responsiveness in both skeletal muscle and liver (Ma, Saha, Chan, & Moore, 2006). It has been suggested that tauro-conjugated-beta-muricholic acid (TβMCA) as an FXR antagonist, and taurine conjugation was essential for FXR antagonistic activity in mice (Sayin et al., 2013). However, given there are substantial differences in murine and human bile acid composition, the BA-related metabolic changes observed in mice might not reflect similar changes in human.

Unconjugated BAs, mainly CA as one of the primary-unconjugated BAs, were found to be negatively correlated with two-hr glucose. The above observation might be related with the FXR-dependent mechanism, as primary- and secondary- unconjugated BAs have been known as the FXR and TGR5 agonists, respectively (Molinaro et al., 2017). However, we did not find any significant differences on primary-unconjugated or secondary-unconjugated BAs between the HN001 and the placebo group, which is contrary to our hypothesis. This might imply that neither FXR- nor TGR5- dependent mechanisms play a particularly crucial role in the alteration in the BA pool by HN001 intervention among pregnant women.

According to our observations, fasting insulin, along with HOMA-IR, was significantly increased by the HN001 administration on non-obese participants with no GDM. These findings are somewhat unexpected, and is inconsistent with our hypothesis that HN001 might improve maternal glycemic control, with a lowering effect on insulin resistance. Worth mentioning, we found that THDCA was positively correlated with fasting insulin as well as HOMA-IR. Additionally, as shown in Table 4.4, THDCA value was slightly higher in the HN001 group compared with the placebo group (HN001 vs. Placebo, 0.03 (0.01-0.07) vs. 0.02 (0.01-0.06), (μM), P=0.281), but the differences were not statistically significant. It has also been pointed out that THDCA values were higher in GDM people (Gao et al., 2016), which might be associated with impaired maternal glycemic control. Although it was uncertain that whether HN001 intervention would directly or indirectly affect maternal THDCA level， which led to any changes in insulin metabolism of the stratified participants, the interval time or the dosage of probiotic supplementation administrated to the pregnant women should be more telling depending on the current GDM status and/or maternal weight of the user.

We did not find any significant alteration in LDL-c, HDL-c, total cholesterol or triglycerides by HN001 allocation. Similarly, a single-blinded clinical trial reported that no significant differences were found in plasma LDL-c, HDL-c, total cholesterol or triglycerides concentrations between placebo and treatment groups administrated with probiotic from lactobacilli species. However, we determined a negative correlation between GCA and HDL-c, and positive correlations between triglycerides and CDCA along with GUDCA based on our findings. Further research is required to investigate the altering effects of probiotic intervention in pregnancy on BAs, and whether it would subsequently affect maternal lipid metabolism.

The current study has several limitations. Our measurement was limited to plasma concentrations of BAs without evaluating portal blood BAs. It is also of great importance to assess BAs in portal venous pools which has been shown to correlate with peripheral BA values (Angelin et al., 1982). The parallel changes in glucose, lipid and BAs between the values in the baseline at 14-16 weeks' gestation, and after intervention at 24-30 weeks' gestation were not available to be evaluated in our study. However, our study has the larger sample size and better follow-up rates compared with previous studies.

In conclusion, this is the largest study to evaluate BA alterations associated with metabolic outcomes after HN001 intervention in early pregnancy, to the best of our knowledge. Probiotic HN001 intervention in early pregnancy improved maternal glycemic control particularly among obese women with GDM. Probiotic supplement appears to be associated with a decrease in taurine-conjugated BAs, which may play a role in the improvement of glucose metabolism. The result may present a possible clinical management for GDM.

Chapter 5 Conclusions

In this study, we validated and optimised a reliable method for the quantitative determination of a total of 15 BAs in human plasma. LC-MS/MS provided satisfactory results regarding specificity, reproducibility, and detection sensitivity. The specific scanning of both pseudo-molecular and product ion pairs of BAs in the MRM mode offered a sensitive detection system, allowing the selective detection of both prominent and minor classes of BAs. The optimised method had a relatively short running time, in which 24 minutes were required for a sample-to-sample injection. The short analysing duration could allow for the implementation of this method for mass batches of the assay. To improve the intra-day as well as the inter-day precision of the assay, the presented method could be further optimised by introducing various stable-isotope labelled BAs as internal standards instead of a single standard when performing the analysis.

Based on the validated LC-MS/MS method, we studied the modification of plasma BA compositions together with FGF19 and various metabolic indices, at three days and three months after bariatric surgeries, and we were provided evidence regarding the acute and short-term modification of BAs induced by GBP and SG in obese individuals with T2DM. We also conducted a two-centre, double-blind, randomised, placebo-controlled trial among pregnant women at 14 to 16 weeks' gestation to investigate the influence of probiotic *L. rhamnosus* HN001 administration on plasma BAs, glucose, and lipid metabolism. The differences in BAs, glucose, and lipid indices of the participants stratified by different GDM or obesity status were compared between the probiotic group and the placebo group.

In the bariatric surgery trial, according to our observation, total, primary, secondary, conjugated, glycine-conjugated, secondary-conjugated, and 12α-OH BAs were common to both types of surgery and at both early time points, suggesting that these signalling molecules may play a vital role in the acute response to bariatric surgery in humans. Bariatric surgery can contribute to acute alterations of gut microbiota, hepatic insulin sensitivity, synthesis or excretion of BAs, enterohepatic cycling, and gut permeability, which may result in an acute change of BA compositions (Dutia et al., 2015; Sweeney & Morton, 2013). A sharp rise was observed in fasting FGF19 levels at three days. The

acute increase of FGF19 post-surgery might reflect a systemic response to increased circulating BA levels, considering that BAs can bind to the nuclear receptor FXR, which is responsible for stimulating FGF19 synthesis. Secondary BAs serve as predominant activators of the TGR5 pathway, which has been reported to affect glucose metabolism (Chiang, 2009; Fiorucci & Distrutti, 2015). Consistently, we found that glucose AUC significantly decreased after both surgical interventions, associating the outcome with higher fasting secondary BAs. In addition, early improvements of pancreatic glucose sensitivity, fasting C-peptide levels, insulin secretion rates, rate sensitivity, and the potentiation factor were observed after either GBP or SG. Our findings suggest that bariatric surgery can improve glycemic control as well as β-cell functions, which could contribute to early remission of T2DM. Apart from the encouraging results listed above, at three days, a significant reduction of HDL-c along with an acute increase of triglycerides after GBP was seen in this study. The above observations indicate that the lipid profile parameters had not improved (and even worsened early after the operations), which might be due to the consistent increase in inflammation and stress resulting from bariatric surgery (Murri et al., 2010). Nevertheless, it is tempting to speculate that the lipid parameters may progressively improve over time after post-surgery according to a previous report showing a progressive increase of HDL-c over time and up to twelve months after bariatric operations, while a reduction was also detected at three months (Benaiges et al., 2012).

The most striking finding of the probiotic gestational trial was that the probiotic HN001 at a dose of 6×10^9 CFU/d might lower two-hr glucose, along with taurine-conjugated BAs (mainly TDCA), among the obese participants with GDM. The findings indicated that probiotic HN001 intervention in early pregnancy improved maternal glycemic control, particularly among obese women with GDM. It is worth mentioning that a previous study suggested that taurine-conjugated BAs were positively correlated with fasting glucose, post-load glucose, fasting insulin, and HOMA-IR (Wewalka et al., 2014). Similarly, our data showed a positive correlation between taurine-conjugated BAs and one-hour post-load glucose. Moreover, lactobacillus species have been reported to have an ability to induce BA deconjugation, as well as BA hydrolysis (Allain et al., 2018; Travers et al., 2016), which might contribute to the modification of taurine-conjugated BA under HN001 administration. Accordingly, it can be highlighted from our study that probiotic intervention *L. rhamnosus* HN001 could induce a decrease in taurine-conjugated BA level, which might play a role in the improvement of glucose

metabolism. However, it is still unclear how HN001 itself, or altered gut microbiota, suppresses taurine-conjugated BAs and how this effect relates to maternal glycemic control. Apart from research on the metabolism of total BAs, few studies have been conducted on taurine-conjugated BAs because of their low levels in humans (Hylemon et al., 2009). Therefore, further research is required to investigate the altering effects of probiotic intervention in pregnancy on BAs, particularly taurine-conjugated BAs. Elucidating the impact of probiotic supplementation in pregnancy on taurine-conjugated BAs might generate novel therapeutic approaches to GDM.

The presented studies have several limitations. Our measurements were limited to plasma concentrations of BAs without evaluating portal blood BAs, which were shown to correlate with peripheral BA values and also of great importance to assess (Angelin et al., 1982). In the bariatric surgery trial, the sample size was relatively small, and the sex ratio and glucose tolerance status was different between intervention groups. The assay of the lipid parameters was not available at the three-month time point. In other words, the early impact of bariatric surgery on lipid was not fully expatiated in our study. As for the probiotic gestational trial, we did not evaluate the parallel changes in glucose, lipid, and BAs between the values in the baseline at 14-16 weeks' gestation, and after intervention at 24-30 weeks' gestation. However, the bariatric surgery trial had focused particularly on subjects with T2DM, explored the influences of two different types of bariatric surgery, and characterised changes in both fasting and postprandial BA compositions. Additionally, the probiotic gestational trial had a larger sample size and better follow-up rates than previous studies. To the best of our knowledge, we had conducted the largest study to evaluate BA alterations associated with metabolic outcomes after HN001 intervention in early pregnancy. Therefore, the presented studies made a unique contribution to the existing literature regarding the influence of bariatric operation, as well as probiotic intervention, on human plasma BA metabolic profiles.

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