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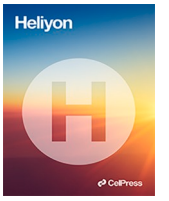

Research article

Gut microbiota-derived metabolites associate with circulating immune cell subsets in unexplained recurrent spontaneous abortion

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ABSTRACT

Currently, the precise causes of over 40 % of recurrent spontaneous abortion (RSA) cases cannot be identified, leading to the term "unexplained RSA" (URSA). Through an exploration of the gut microbiota, metabolites, and immune cell subsets in URSA, this study establishes a link between gut microbiota-derived metabolites and immune cells. The results indicate reduced diversity in the gut microbiota of URSA. Targeted metabolomic analyses reveal decreased levels of gut microbiota-derived deoxycholic acid (DCA), glycolithocholic acid (GLCA), acetate, propionate, and butyrate in URSA. Furthermore, elevated frequencies of Th1, Th17, and plasma B cells, along with decreased frequencies of Tregs and Bregs, are observed in the peripheral blood of URSA. The results demonstrate correlations between the levels of gut microbiota-derived bile acids and short-chain fatty acids and the frequencies of various immune cell subsets in circulation. Collectively, this study uncovers an association between gut microbiota-derived metabolites and circulating immune cell subsets in URSA.

1. Introduction

Recurrent spontaneous abortion (RSA) refers to the occurrence of two or more consecutive natural miscarriages before the 24th week of pregnancy. It is one of the most common conditions in the field of reproductive health, with an incidence rate of 2 %–5 % among women of childbearing age and accounting for 15 %–20 % of all spontaneous abortions [1]. RSA exerts significant physical and psychological impacts on women of childbearing age and places a heavy burden on families [2]. The etiology and pathogenesis of RSA are highly complex, and it lacks specific clinical manifestations. Currently, the precise causes of over 40 % of cases cannot be identified, leading to the term "Unexplained RSA" (URSA) [3,4]. At present, there are no established methods to predict or prevent the recurrence

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of URSA [5]. Thus, research and exploration of the pathogenesis of RSA, especially URSA, hold considerable clinical and societal significance in improving pregnancy outcomes and enhancing the quality of life for affected individuals.

In recent years, with the advancement of biotechnology, increasing attentions have been paid to the importance of the gut microbiota in health and disease [6]. Gut microbiota dysbiosis can negatively impact pregnancy outcomes by disrupting the maternal immune system [7]. The success of pregnancy and pregnancy outcomes are closely related to the diversity of the gut microbiota [8,9]. Consequently, a healthy gut microbiota is crucial for favorable pregnancy outcomes. Notably, research on the role of the gut microbiota in RSA has begun to emerge globally. Results from 16S rDNA sequencing indicate a significant increase in the relative abundance of Prevotella, Bacteroides, and Prevotella in the gut microbiota of RSA patients [10]. Another study demonstrates that intravenous injection of Streptococcus cricetus in pregnant mice did not result in clinical symptoms within three days, while injection of Actinobacillus sp. Led to miscarriage [11]. Differential gut microbiota-derived metabolites, such as indole propionic acid and 1, 4-dimethylimidazole acetic acid, regulate Th1/Th17 balance in RSA [12]. It can be observed that distinct gut microbiota genera and their derived metabolites may serve as potential therapeutic targets for RSA patients.

Short-chain fatty acids (SCFAs) are a group of saturated volatile fatty acids composed of six or fewer carbon atoms. Unlike long-chain fatty acids, SCFAs are generated through glycolysis by anaerobic bacteria or yeast [13]. SCFAs play a role in maintaining intestinal barrier integrity, preventing intestinal inflammation, and participating in the body's inflammatory and immune responses [13]. Bile acids (BAs) are a major component of bile and play a significant role in fatty acid absorption, transportation, secretion, and regulation of cholesterol metabolism [14]. Primary BAs produced by the liver are transformed into secondary BAs by microbial metabolism after entering the intestines. These secondary BAs include deoxycholic acid, lithocholic acid, glycine-deconjugated deoxycholic acid, taurine-deconjugated deoxycholic acid, glycolithocholic acid, and taurolithocholic acid. The roles of secondary BAs in host health and disease are gradually becoming clearer [15].

However, the roles of gut microbiota and their derived BAs and SCFAs in host immunity remain largely unknown in the context of URSA. This study aims to establish a connection between gut microbiota-derived metabolites (BAs and SCFAs) and circulating immune cell subsets, with the goal of providing new insights into the pathogenesis of URSA. The findings of this study potentially offer strategies for the treatment of URSA, with gut microbiota-derived BAs and SCFAs being potential candidate raw materials for clinical therapies.

2. Results

2.1. Participant characteristics

According to the inclusion and exclusion criteria of this study, appropriate participants were selected. All participants had been treated at the Women's Hospital School of Medicine, Zhejiang University within the past three months and they had signed informed consent forms. This study involved 15 healthy postpartum women (healthy control, HC) and 12 patients with URSA (Fig. 1A). Participants provided approximately 2 mL of peripheral blood and approximately 10 g of fresh feces for this study. None of the participants had used any medication in the past six months. None of them has a history of smoking or alcohol consumption, or any

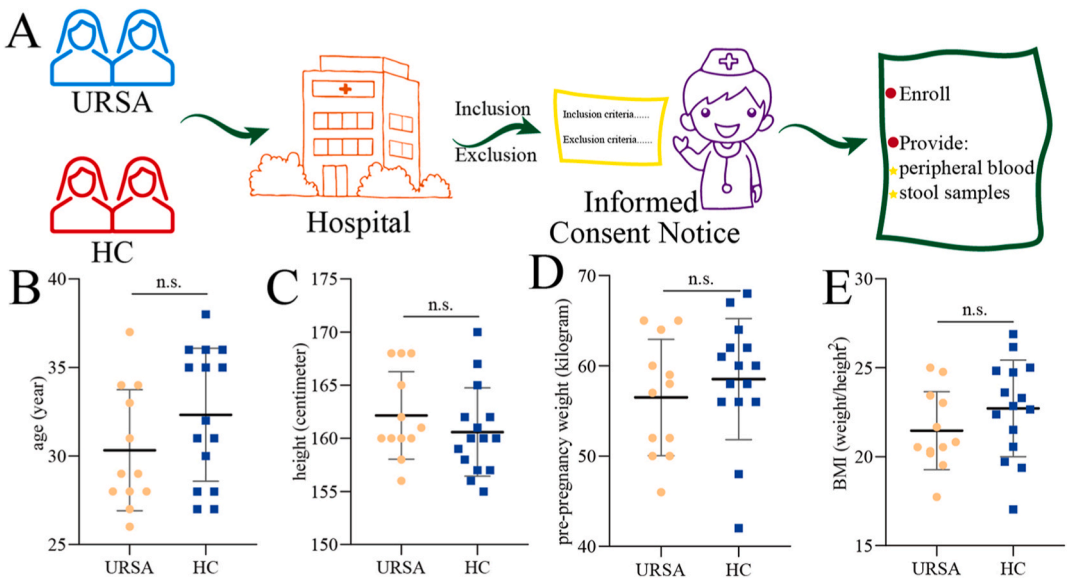

Fig. 1. Participant characteristics. (A) Flow chart for enrolling subjects. (B) Age distribution of subjects. (C) Height distribution of subjects. (D) Body weight distribution of subjects. (E) BMI distribution of subjects. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). Unpaired *t*-test was used for figure B–D, n.s. represents $p > 0.05$ in URSA group compared with HC group.

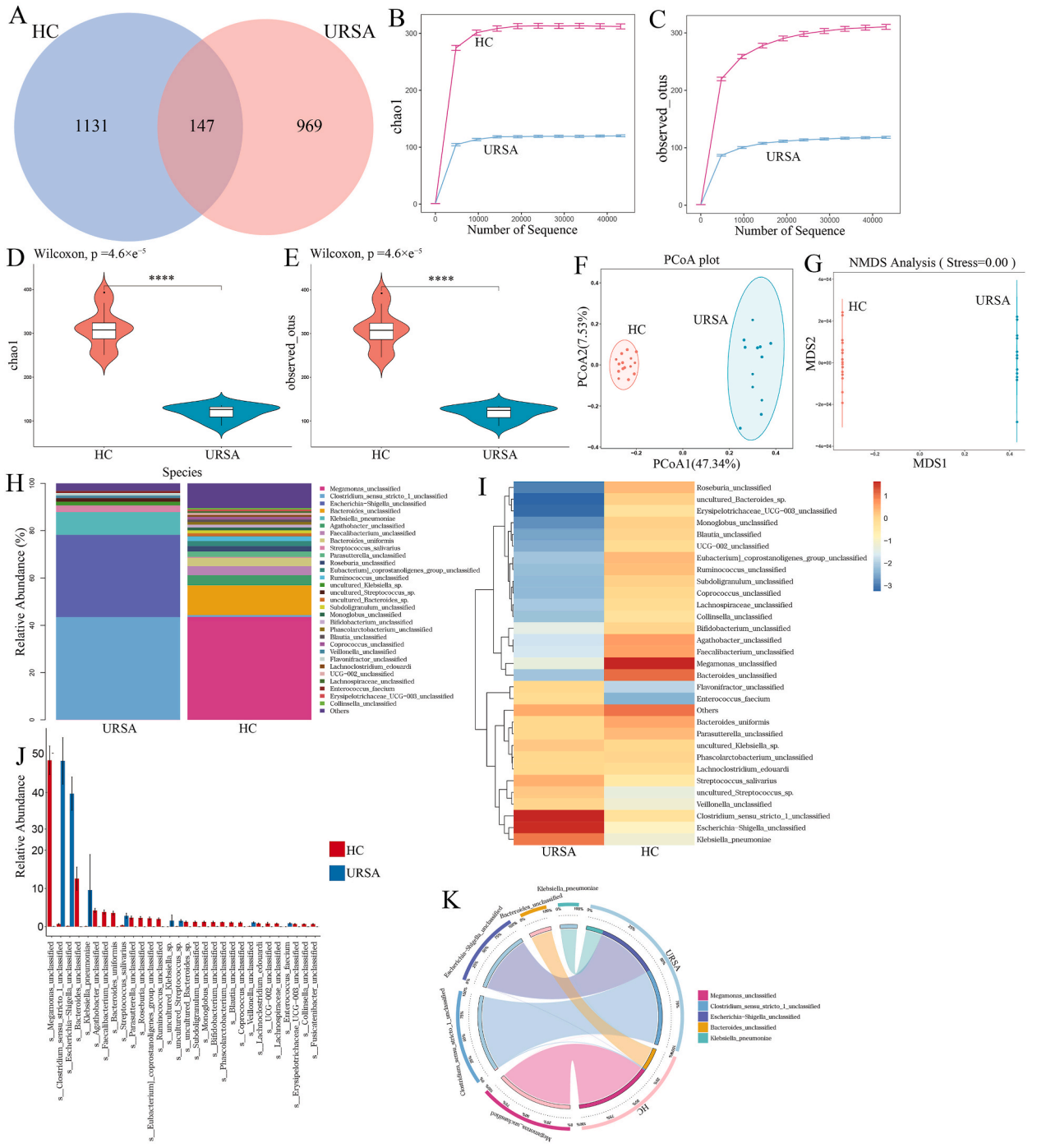

Fig. 2. Gut microbiota diversity decreased and the dominant bacterial species are changed in URSA patients. (A) Gut microbiota species observed in patients with URSA and women with normal pregnancies (HC). (B) The chao1 index of gut microbiota, used to indicate α diversity. (C) The observed_otus index of the gut microbiota, used to indicate α diversity. (D) Violin plot of the chao1 index. (E) Violin plot of the observed_otus index. (F) PCoA analysis of gut microbiota for indication of β diversity. (G) NMDS analysis of gut microbiota for indication of β diversity. (H) Stacked plot of the TOP 30 bacterial species ranked by relative abundance. (I) Heat map of the TOP 30 bacterial species ranked by relative abundance. (J) Histogram of relative abundance ranking for visual display of dominant bacterial species. (K) Circos of the TOP 5 dominant bacterial species ranked by relative abundance, used to display the TOP 5 bacterial species. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). Figure A–K were based on 16S rDNA sequencing data. Unpaired *t*-test was used for figure D and F, *** represents $p < 0.001$ in URSA group compared with HC group.

significant medical history. The average age of the HC group is 32.33 years, and that of the URSA group is 30.33 years, with no statistically significant difference between the two groups (Fig. 1B). The average height of the HC group is 160.8 cm, and that of the URSA group is 162.2 cm, with no statistically significant difference (Fig. 1C). The average pre-pregnancy weight of the HC group is 57.67 kg, and the average pre-pregnancy weight of the URSA group is 56.50 kg, with no statistically significant difference (Fig. 1D). The BMI (kg/m^2) of the URSA group is 21.47 and that of the HC group is 22.71, the difference between the two was not statistically significant ($p = 0.4743$) (Fig. 1E).

2.2. Reduced gut microbiota diversity and altered dominant genera in URSA patients

Fecal samples from 12 patients with URSA (observation group) and 15 healthy postpartum women volunteers (control group, HC) were included in this study based on inclusion and exclusion criteria. The samples were used for 16S rDNA sequencing. 16S rDNA is a high-throughput sequencing technique used to analyze the composition of bacterial communities in specific environments or habitats. It enables the study of microbial community composition, interpretation of microbial diversity, richness, and community structure in

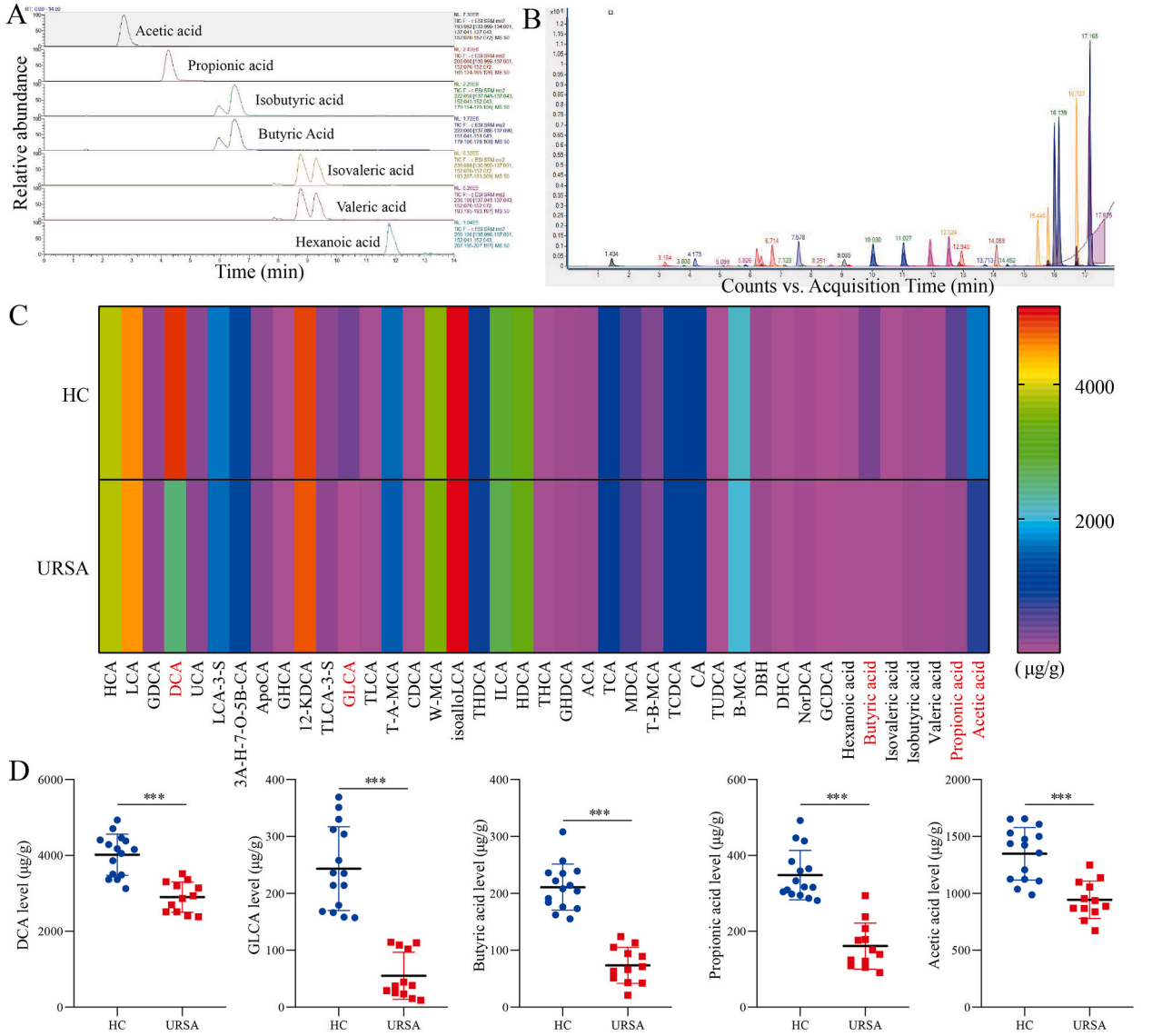

Fig. 3. The levels of gut microbiota-derived BAs and SCFAs were altered in URSA patients. (A) Chromatogram of the standard mixture of seven SCFAs. (B) Chromatogram of the standard mixture of BAs. (C) Heatmap of 34 BAs and 7 SCFAs detected by targeted metabolomics for visualization of level differences. (D) Column diagram with individual values of 5 differential metabolites. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). Unpaired *t*-test was used for figure D, *** represents $p < 0.001$ in URSA group compared with HC group.

environmental samples. This technique is employed to explore the relationships between microorganisms and their environment or hosts. The results indicated a decreased abundance of gut microbiota in postmenopausal osteoporotic patients. In the control group, we observed a total of 1278 unique Amplicon Sequence Variants (ASVs), while the observation group had only 1116 ASVs, with 147 shared ASVs between the two groups (Fig. 2A). Alpha diversity analysis revealed reduced Chao1 and observed_species indices in the observation group (Fig. 2B–E). Similarly, decreased shannon, Simpson, and pielou-e indices were also observed in the observation group (Supplementary Figs. 1A–F). Principal Coordinates Analysis (PcoA) (Fig. 2F) and Nonmetric Multidimensional Scaling (NMDS) (Fig. 2G) both demonstrated significant differences in microbial composition between the observation and control groups. We selected

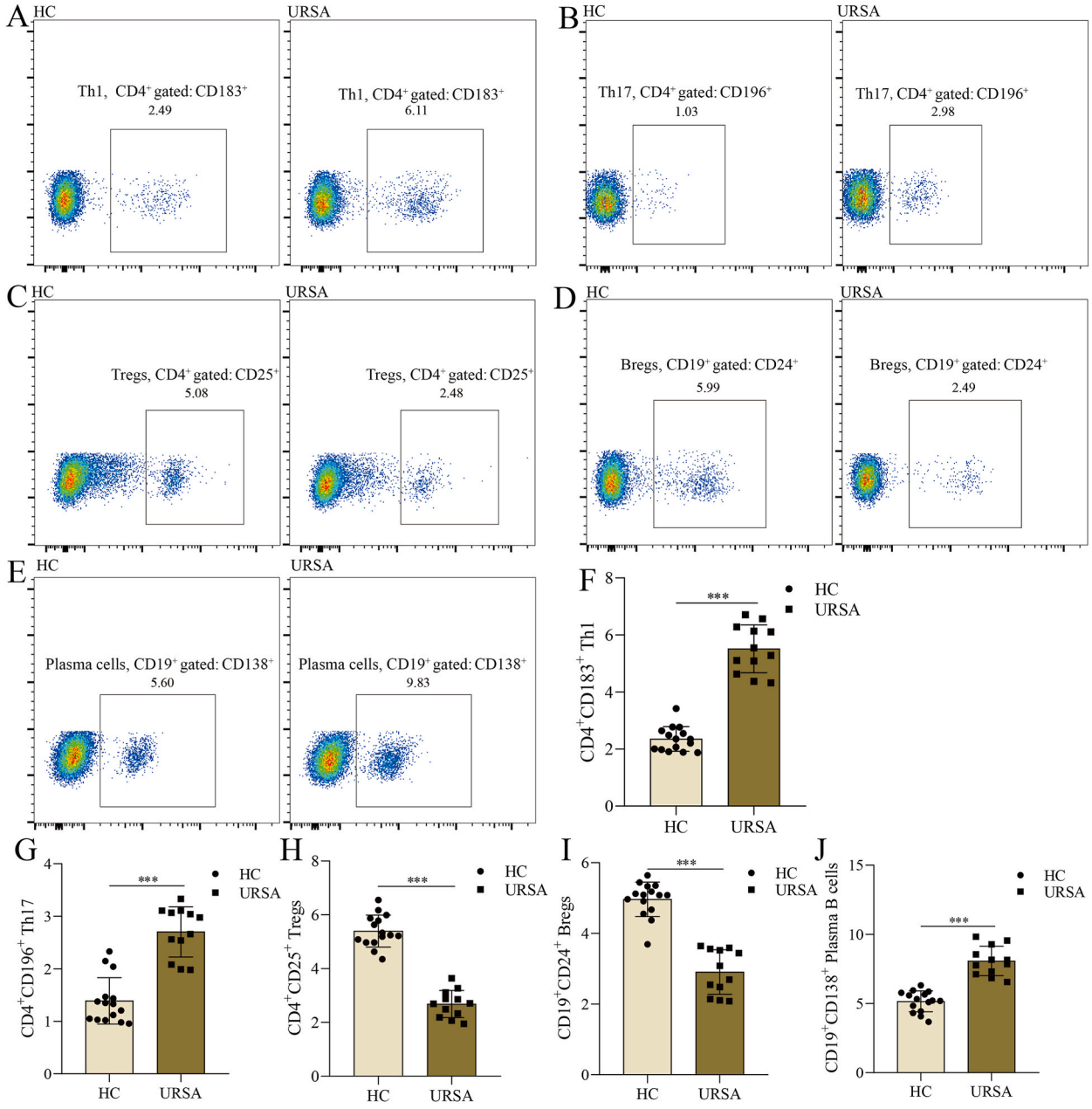

Fig. 4. The frequencies of T cell and B cell subtypes in the peripheral blood of URSA patients were changed. (A) Flow cytometry scatter plot of $CD4^+CD183^+$ Th1 cells. (B) Flow cytometry scatter plot of $CD4^+CD196^+$ Th17 cells. (C) Flow cytometry scatter plot of $CD4^+CD25^+$ Tregs. (D) Flow cytometry scatter plot of $CD19^+CD24^+$ Bregs. (E) Flow cytometry scatter plot of $CD19^+CD138^+$ plasma cells. (F) Histogram of Th1 flow cytometry results. (G) Histogram of Th17 flow cytometry results. (H) Histogram of Tregs flow cytometry results. (I) Histogram of Bregs flow cytometry results. (J) Histogram of plasma cells flow cytometry results. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). Figure A–J were based on flow cytometry results. Unpaired *t*-test was used for figure F–J, *** represents $p < 0.001$ in URSA group compared with HC group.

the top 30 abundant species for classification, revealing significant differences in microbial composition and abundance between the observation and control groups at the phylum (Supplementary Fig. 1G) and species (Fig. 2H) levels. Furthermore, we presented the top 30 abundant species using a heatmap, clearly showing differences at the phylum (Supplementary Fig. 1H) and species (Fig. 2I) levels between the observation and control groups. The top 5 dominant genera in the observation group were *Clostridium_sensu_stricto_1_unclassified*, *Escherichia−Shigella_unclassified*, *Klebsiella_pneumoniae*, *Streptococcus_salivarius*, and *uncultured_Klebsiella_sp*. At the species level. In contrast, the top 5 dominant genera in the control group were *Megamonas_unclassified*, *Bacteroides_unclassified*, *Agathobacter_unclassified*, *Faecalibacterium_unclassified*, and *Bacteroides_uniformis* (Fig. 2J and K).

2.3. Changes in levels of gut microbiota-derived BAs and SCFAs in URSA patients

A portion of the collected fecal samples was used for targeted metabolomic analysis. In brief, using standard references, specific metabolites were selectively and specifically detected and analyzed to validate candidate biomarkers and analyze known target compounds. In this study, we assessed the levels of 45 BAs and 7 SCFAs, detecting a total of 34 BAs and 7 SCFAs. Fig. 3A depicts the chromatogram of the fatty acid standard mixture, with well-separated peaks of the 7 targeted short-chain fatty acid standard metabolites, facilitating accurate quantification of each targeted metabolite. Fig. 3B illustrates the chromatogram of the bile acid standard mixture, demonstrating the effective separation of the various targeted metabolites for accurate quantification. The results revealed significant differences in the levels of acetate, propionate, butyrate, deoxycholic acid (DCA), and glycolithocholic acid (GLCA) in fecal samples from the observation group (Fig. 3C–D).

2.4. Alterations in frequencies of T cell and B cell subsets in peripheral blood of URSA patients

Flow cytometry was employed to analyze the frequencies of $CD4^+$ T cell and $CD19^+$ B cell subsets in human peripheral blood. The detected $CD4^+$ T cell subsets included: Th1, $CD4^+CD183^+$; Th17, $CD4^+CD196^+$; Tfh, $CD4^+CD185^+$; Tregs, $CD4^+CD25^+Foxp3^+$. The detected $CD19^+$ B cell subsets included: Follicular B cells, $CD19^+CD23^+$; Plasma cells, $CD19^+CD138^+$; Marginal zone B cells, $CD19^+CD21^+$; Bregs, $CD19^+CD21^+CD24^+$. The results indicated an increased frequency of Th1 (Fig. 4A and F), Th17 (Fig. 4B and G), and Plasma cells (Fig. 4E and J), while frequencies of Tregs (Fig. 4C and H) and Bregs (Fig. 4D and I) decreased. Notably, frequencies of Tfh (Supplementary Figs. 2A and D), Marginal zone B cells (Supplementary Figs. 2B and E), and Follicular B cells (Supplementary Figs. 2C and F) remained unchanged.

2.5. Gut microbiota regulates the metabolism of BAs and SCFAs

The gut microbiota regulates the metabolism of bile acids (BAs) in the enterohepatic circulation. Bile acids are synthesized through the oxidation of cholesterol in the liver by cytochrome P450 (CYP), and they undergo conjugation synthesis catalyzed by bile acid-CoA synthetase (BACS) and bile acid-CoA: amino acid N-acyltransferase (BAAT) (Fig. 5) [16]. Bile acids (BAs) secreted by the liver further enter the colon (approximately 10 %–15 %), where these BAs are typically subjected to biotransformation by the gut microbiota, with

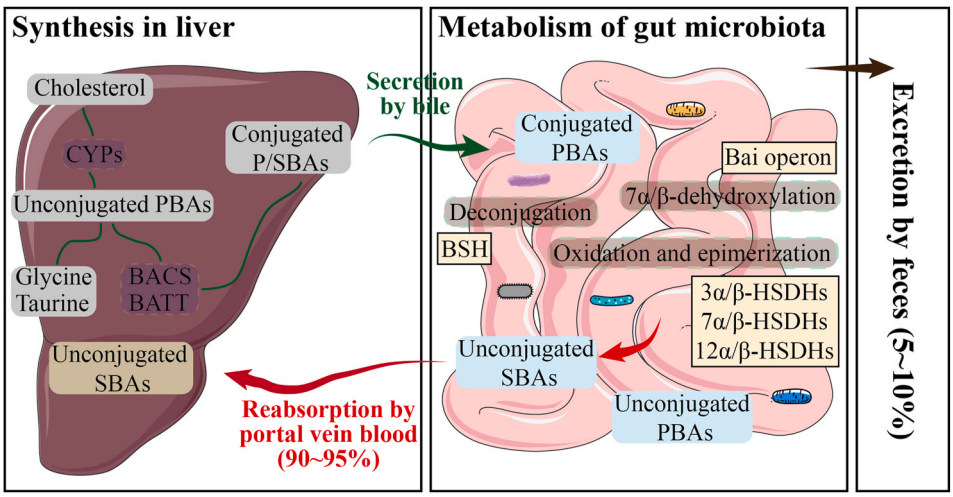

Fig. 5. Gut microbiota regulates BA metabolism in the enterohepatic circulation. BAs are synthesized from cholesterol in the liver via oxidation catalyzed by CYPs and conjugation catalyzed by BACS and BAAT. Subsequently, BAs are secreted into intestine post prandial. Most BAs (90%–95 %) are reabsorbed into liver by portal vein blood, and only a small portion (5%–10 %) are excreted into feces. Gut microbiota inhabiting the intestines carry out biotransformations to convert PBAs into SBAs and convert conjugated BAs into unconjugated BAs. Major microbial biotransformation reactions include deconjugation mediated by BSHs, 7α/β-dehydroxylation mediated by bai gene products, and oxidation and epimerization mediated by hydroxysteroid dehydrogenase.

the transformed ones being excreted in the feces [17]. The gut microbiota metabolizes BAs through processes such as desulfation, 7-dehydroxylation, deconjugation of 3-, 7-, and 12-hydroxyl groups, esterification, oxidation, and epimerization (Table 1) [16,18–20]. Short-chain fatty acids (SCFAs), primarily produced by the gut microbiota through the metabolism of undigested dietary fibers, are closely associated with microbial diversity and dietary fiber intake (Fig. 6) [21,22]. SCFAs play a crucial role in host health and disease, especially acetate, propionate, and butyrate [23]. It is currently believed that the gut microbiota can significantly impact the immunological significance of intestinal immune diseases for distant tissues [24,25]. Acetate primarily originates from acetyl-CoA, a product of acetone conversion [26]. Propionate is generated through the propionic acid ester pathway, using lactic acid as a precursor, or it can be produced via the glycerol pathway using deoxyhexose as a substrate [27]. Butyryl-CoA can be transformed into butyrate through the action of butyryl-CoA transferase; in the reduction of acetoacetyl-CoA to butyryl-CoA, the process involves the conversion of *trans*-butyric acid by *trans*-butyrate synthase and butyrate kinase, leading to the formation of butyrate (Table 2) [28–31]. In this study, we observed that the top 5 dominant genera (Megamonas_unclassified, Bacteroides_unclassified, Agathobacter_unclassified, Faecalibacterium_unclassified, Bacteroides_uniformis) may be associated with differential metabolism of BAs (DCA and GLCA) and SCFAs (acetate, propionate, butyrate).

2.6. Correlation analysis between gut microbiota-derived metabolites (BAs and SCFAs) and peripheral blood immune cell (T cells and B cells) subsets

Gut microbiota-derived BAs and SCFAs have important connections with the body's T/B cells [13,49,50]. Simple linear regression analysis was employed to assess the correlation between gut microbiota-derived BAs/SCFAs and T/B cell subsets [51]. Four conditions were met for the BAs/SCFAs and T/B cell frequencies in this study: (1) both variables were continuous; (2) both variables were paired, originating from the same individual; (3) there were no obvious outliers; and (4) both variables followed a normal distribution or approximate normal distribution. Therefore, simple linear regression was suitable for analyzing the correlation between these two variables in this study. Both gut microbiota-derived BAs and SCFAs exhibited correlations with T/B cell subsets. Interestingly, the levels of gut microbiota-derived DCA were not linearly correlated with the frequencies of Th1, Th17, and Plasma B cells in URSA (Supplementary Figs. 3A–C). The levels of gut microbiota-derived GLCA did not exhibit a linear correlation with Plasma B cell frequency (Supplementary Fig. 3D). Acetate levels did not display linear correlations with the frequencies of Th1, Th17, and Plasma B cells (Supplementary Figs. 3E–G). Propionate levels were not linearly correlated with the frequencies of Th1 and Th17 (Supplementary Figs. 3H–I). In summary (Fig. 7A): DCA positively correlated with Tregs levels (Fig. 7C) and positively correlated with Bregs frequencies (Fig. 7B); GLCA negatively correlated with Th1 frequency (Fig. 7E), negatively correlated with Th17 frequency (Fig. 7F), positively correlated with Tregs frequency (Fig. 7G), and positively correlated with Bregs frequency (Fig. 7D); Acetate positively correlated with Tregs frequency (Fig. 7I) and also positively correlated with Bregs frequency (Fig. 7H); Propionate positively correlated with Tregs frequency (Fig. 8C), positively correlated with Bregs frequency (Fig. 8A), and also negatively correlated with Plasma B cell frequency (Fig. 8B); Butyrate negatively correlated with Th1 frequency (Fig. 8F), negatively correlated with Th17 frequency (Fig. 8G),

Table 1
Summary of BA metabolism by microbial producers (incomplete statistics).

Enzymes/genes mediating biotransformative reactions	BA transformations	Microbial producers	Refs
BSH	conjugated BAs→ unconjugated BAs	*Clostridium* *Bacteroides* *Bifidobacterium* *Enterococcus* *Lactobacillaceae (family)*	[32–38]
Bai genes	CDCA→LCA CA→DCA	*Clostridium scindens* *Clostridium hylemonae* *Clostridium hiranonis (reclassified as Peptacetobacter hiranonis)* *Clostridium leptum*	[20,39–41]
3α-HSDH	DCA→3-oxoDCA	*Eggerthella lenta* *Ruminococcus gnavus* *Clostridium perfringens*	[42–44]
3β-HSDH	3-oxoDCA→isoDCA	*Eggerthella lenta* *Ruminococcus gnavus* *Peptostreptococcus productus (reclassified as Blautia producta)*	[42,43,45]
7α-HSDH	CDCA→7-oxoLCA	*Eggerthella lenta* *Clostridium scindens* *Escherichia coli*	[42,43]
7β-HSDH	7-oxoLCA→urso-DCA	*Ruminococcus gnavus*	[43]
12α-HSDH	DCA→12-oxoLCA	*Clostridium scindens* *Clostridium hylemonae* *Clostridium hiranonis (reclassified as Peptacetobacter hiranonis)* *Clostridium leptum* *Eggerthella lenta*	[42,46,47]
12β-HSDH	12-oxoLCA→epiDCA	*Clostridium paraputrificum*	[43]

Abbreviations: BAs: bile acids; BSH, bile salt hydrolase; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HSDH, hydroxysteroid dehydrogenase.

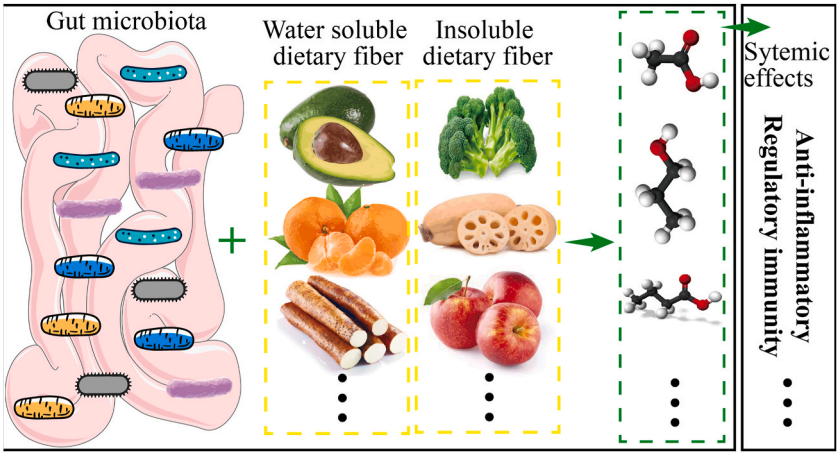

Fig. 6. Major prebiotic sources and production of SCFAs. SCFAs, such as acetate (C2), propionate (C3), and butyrate (C4), are produced from a number of dietary fiber and digestion-resistant starches by the cooperative catabolic activity of commensal bacteria. These bacteria have complex carbohydrate-degrading enzymes and/or the enzymes involved in SCFA-producing pathways, such as the succinate, acrylate, and propanediol pathways. Food rich in dietary fiber enhances the growth of the commensal bacteria that produce SCFAs. Whole grains are a good source of inulin, arabinoxylan, and β-glucan. Fruits are a good source of pectin. Human breast milk is a rich source of oligofructose, which is used to produce SCFAs in infants. Starches engineered to be resistant to digestion also reach the colon for microbial fermentation. Inadequate dietary fiber consumption is common in certain demographic groups in developed countries, leading to SCFA deficiency-related immune insufficiency and dysregulation. Produced SCFAs have strong local effects on the intestine and can exert systemic effects following transport to other organs through the portal vein and blood circulatory system.

Table 2
Biosynthesis of SCFAs in the gut (incomplete statistics).

SCFA	Biosynthesis	Microbial producers	Refs
acetate	from pyruvate in acetyl-CoA pathway	*Akkermansia muciniphila, Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.	[31, 48]
	reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)	*Blautia hydrogenotrophica, Clostridium* spp., *Streptococcus* spp.	
propionate	succinate pathway	*Bacteroidetes* spp, *Roseburia* spp., *Firmicutes, Roseburia inulinivorans, Ruminococus* spp., *Cllostridium* spp., *Clostridiales bactrium, Eubacterium* spp, *Coprococcus* spp., *Dialister succinatiphilus, Phascolarctobaterium succinatutens, Akkermansia muciniphila*	[27, 31]
	acrylate pathway	*Clostridium* sp., *Clostridiales bacterium, Coproccus catus, Clostridium* sp.	
	propanediol pathway	*Roseburia insulinivorans, Ruminococus* spp., *Eubacterium halli, Clostridium* sp.	
butyrate	butyryl-CoA transferase: acetate Co-A pathway	*Roseburia intestinalis, Eubacterium rectale, Roseburia insulinivorans, Clostridiales bacterium, Anaerostripes hadrus, Coprococcus* spp, *Clostridium symbiosum, Faecalibacterium prasnitzii*	[27, 31]
	butyrate kinase pathway	*Bacteroidetes* spp., *Coprococcus* spp.	

positively correlated with Tregs frequency (Fig. 8H), positively correlated with Bregs frequency (Fig. 8D), and negatively correlated with Plasma B cell frequency (Fig. 8E).

3. Materials and methods

3.1. Human sample

Stool samples and peripheral blood from 12 URSA and 15 healthy postpartum women were obtained with ethical approval and informed consent. This study was approved by the Ethics Committee of Women's Hospital School of Medicine, Zhejiang University (approval no. IRB-20230085-R).

3.2. Inclusion and exclusion criteria

URSA inclusion criteria: a. The anatomical structure of the reproductive tract of pregnant women is normal; b. Endocrine

A Simple linear regression analysis of gut microbiota-derived metabolites and circulating immune cell subtypes

Differentiated cell subsets / Differential metabolites	Th1	Th17	Tregs	Bregs	Plasma B cells
DCA	No linear correlation	No linear correlation	Positive linear correlation	Positive linear correlation	No linear correlation
GLCA	Negative linear correlation	Negative linear correlation	Negative linear correlation	Negative linear correlation	No linear correlation
Acetate	No linear correlation	No linear correlation	Positive linear correlation	Positive linear correlation	No linear correlation
Propionate	No linear correlation	No linear correlation	Positive linear correlation	Positive linear correlation	Negative linear correlation
Butyrate	Negative linear correlation	Negative linear correlation	Positive linear correlation	Positive linear correlation	Negative linear correlation

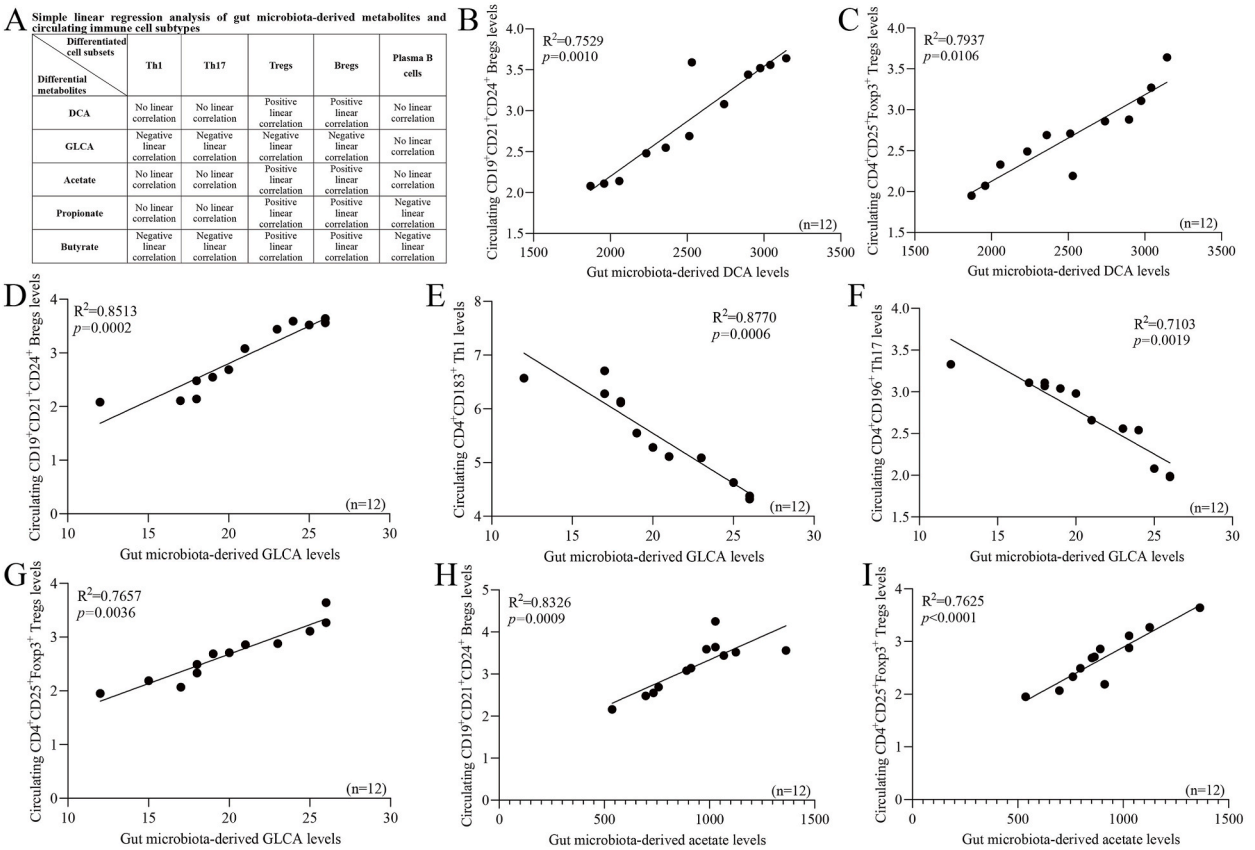

Fig. 7. Simple linear regression analysis of gut microbiota-derived metabolites and circulating immune cell subsets. (A) Summary table of correlations of gut microbiota-derived metabolites with circulating immune cell subsets. (B) Gut microbiota-derived deoxycholic acid (DCA) was positively correlated with the frequency of circulating $CD19^+CD21^+CD21^+$ Bregs. (C) Gut microbiota-derived DCA was positively correlated with the frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs. (D) Gut microbiota-derived glycolithocholic acid (GLCA) was positively correlated with the frequency of circulating $CD19^+CD21^+CD21^+$ Bregs. (E) Gut microbiota-derived GLCA was negatively correlated with the frequency of circulating $CD4^+CD183^+$ Th1. (F) Gut microbiota-derived GLCA was negatively correlated with the frequency of circulating $CD4^+CD196^+$ Th17. (G) Gut microbiota-derived GLCA was positively correlated with the frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs. (H) Gut microbiota-derived acetate was positively correlated with the frequency of circulating $CD19^+CD21^+CD24^+$ Bregs. (G) Gut microbiota-derived acetate was positively correlated with the frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). $p < 0.05$ represents significant statistical significance. R^2 represents the degree of fit, and the closer to 1, the stronger the correlation between the two variables.

examinations such as sex hormones and thyroid function are normal; c. The results of immunology and etiology after pregnancy are normal; d. There is no infection in the whole body and reproductive tract; e. The karyotypes of both husband and wife are normal, and the chromosomes of the embryo are normal; f. is diagnosed as recurrent miscarriage.

Inclusion criteria for the control group: Inclusion criteria: a. Both husband and wife have normal chromosomes; b. No pregnancy complications; c. No history of adverse pregnancy; d. Healthy multiparas.

Exclusion criteria for URSA and the control group: a. age >38 years old or <18 years old; b. smoking, alcoholism, drug abuse, and family history of diseases; c. cardiovascular disease and abnormal liver and kidney function; d. mental illness; e. Taking antibiotics, probiotics, digestive drugs, or hormone drugs within two months.

3.3. 16S rDNA sequencing

16S rDNA sequencing is a common technique used to study the structure of microbial communities. The sequencing process is as follows: (1) DNA Extraction: Total DNA is extracted from fecal samples. (2) PCR Amplification: Selective amplification of the 16S rDNA region is performed using primers designed for highly conserved regions in bacteria and archaea. (3) Gel Electrophoresis: Gel electrophoresis is conducted on PCR products to ensure the successful amplification of the target segment and the absence of contamination. (4) Purification of PCR Products: PCR products are purified to remove primers, residual double-stranded DNA, and other contaminants. (5) Library Construction: The purified PCR products are transformed into a library by linking appropriate DNA sequencing adapters. (6) Sequencing: High-throughput sequencing technologies, such as Illumina, PacBio, or Ion Torrent, are

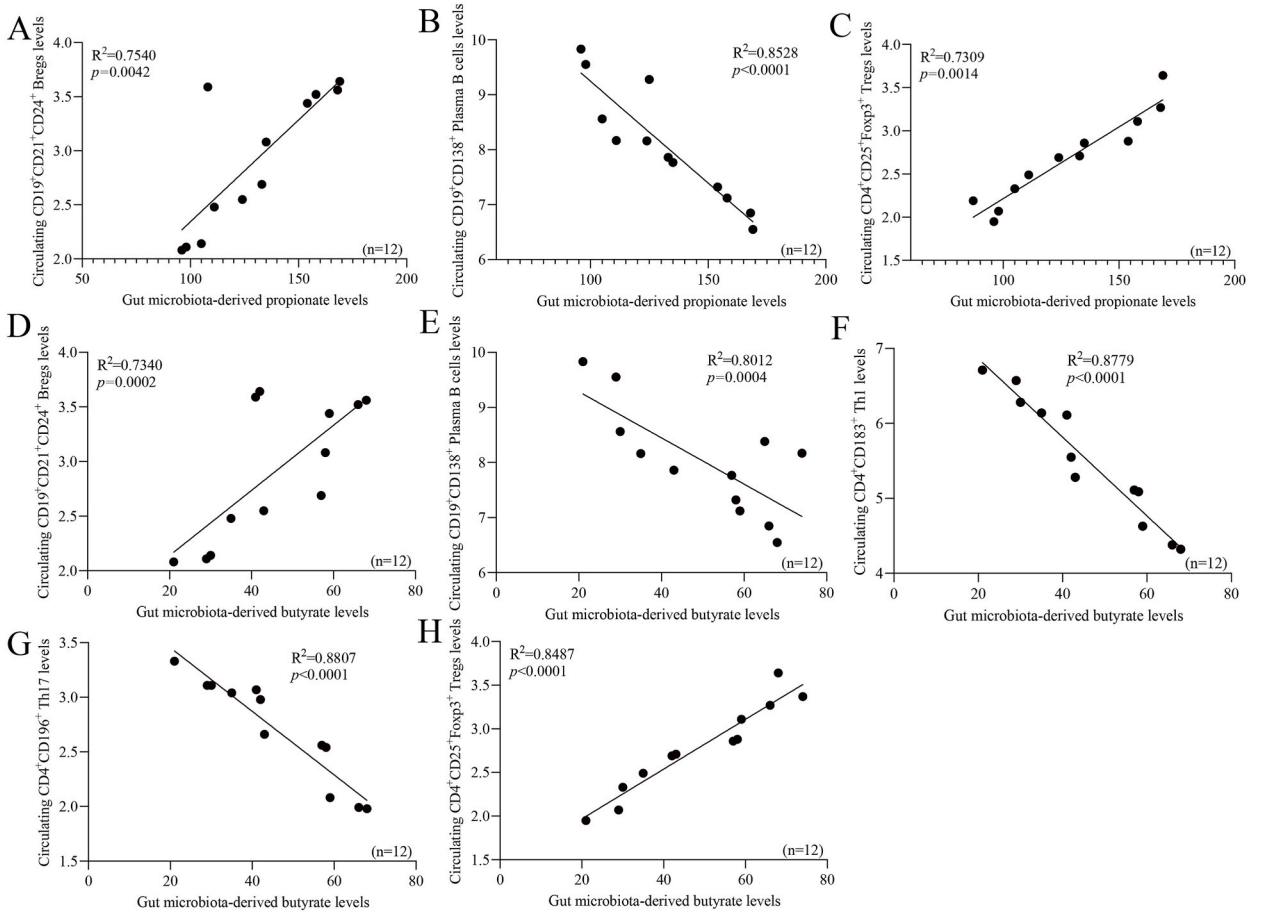

Fig. 8. Simple linear regression analysis of gut microbiota-derived metabolites and circulating immune cell subsets. (A) Gut microbiota-derived propionate was positively correlated with the frequency of circulating $CD19^+CD21^+CD24^+$ Bregs. (B) Gut microbiota-derived propionate was negatively correlated with the frequency of circulating $CD19^+CD138^+$ Plasma B cells. (C) Gut microbiota-derived propionate was positively correlated with the frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs. (D) Gut microbiota-derived butyrate was positively correlated with the frequency of circulating $CD19^+CD21^+CD24^+$ Bregs. (E) Gut microbiota-derived butyrate was negatively correlated with the frequency of circulating $CD19^+CD138^+$ Plasma B cells. (F) Gut microbiota-derived butyrate was negatively correlated with the frequency of circulating $CD4^+CD183^+$ Th1. (G) Gut microbiota-derived butyrate was negatively correlated with the frequency of circulating $CD4^+CD196^+$ Th17. (H) Gut microbiota-derived butyrate was positively correlated with the frequency of circulating $CD4^+CD25^+Foxp3^+$ Tregs. The sample size was 15 for the HC group (n = 15) and 12 for the URSA group (n = 12). $p < 0.05$ represents significant statistical significance. R^2 represents the degree of fit, and the closer to 1, the stronger the correlation between the two variables.

employed to sequence the library. (7) Data Analysis: Bioinformatics analysis of the sequenced data, including the removal of low-quality sequences, elimination of redundant sequences, alignment to the 16S database to identify Operational Taxonomic Units (OTUs). (8) Species Annotation: Potential microbial species identities are assigned to each OTU based on reference databases. (9) Statistical Analysis: Statistical analysis is performed on the differences in microbial communities between samples to reveal variations and similarities among samples.

3.4. Targeted metabolomics analysis

Fecal samples were utilized for targeted metabolomics research to analyze the levels of 7 short-chain fatty acids (SCFAs) and 44 bile acids (BAs) in feces. Targeted metabolomics is a method employed to investigate metabolic processes within an organism, often used to gain deeper insights into disease mechanisms, drug actions, and related aspects. The specific experimental steps are as follows: 100 mg of fecal sample is weighed and added to a grinding tube (previously added with 2 steel beads). Then, 500 mL of 80 % methanol (a mixture of methanol and water for protein precipitation) is added, and the sample is ground for 3 min. After centrifugation at 4 °C and 20000 rcf for 15 min, 100 mL of the supernatant is collected and transferred to an LC injection vial for subsequent UPLC-MS/MS analysis.

3.5. LC-MS/MS analysis

The obtained samples were subjected to LC-MS/MS analysis. In brief, the operational steps are as follows: (1) Liquid Chromatography (LC): The samples pass through a liquid chromatography column where compounds are separated based on their characteristics. This step ensures the separation of impurities before mass spectrometry. (2) Mass Spectrometry (MS): Compounds from the liquid chromatography enter the mass spectrometer. In the mass spectrometer, compounds undergo ionization and fragmentation processes, generating ions used to create a mass spectrum. (3) Tandem Mass Spectrometry (MS/MS): Specific ions are selected for further fragmentation, producing more detailed mass spectral information. This provides more accurate structural information about the compounds. (4) Data Analysis: By comparing experimental data with standard data in databases or using specialized software for data analysis, the compounds present in the sample and their concentrations are determined.

3.6. Flow cytometry

Flow cytometry is used to detect the proportion of immune cell subpopulations in human peripheral blood samples. CD4, CD183, CD196, CD185, CD25, CD19, CD23, CD138, CD21, and CD24 are cell membrane molecules, which were detected by direct staining. First, perforate the cell membrane before staining with antibodies, as Foxp3 is located inside the cells. In short, start by using red blood cell lysis buffer to remove red blood cells, then centrifuge to remove the supernatant (4 °C, 3000 rcf, 5 min). Perforate the cell membrane, incubate with fluorescent antibodies (4 °C, 30 min), wash the cells with PBS, and analyze the cell proportions. Antibodies: BV510 Mouse Anti-Human CD4 (BD Pharmingen; cat. # 742651), PE-Cy7 Mouse Anti-Human CD183 (BD Pharmingen; cat. # 560831), BV421 Mouse Anti-Human CD196 (BD Pharmingen; cat. # 565925), PerCP-Cy5.5 Rat Anti-Human CXCR5 (CD185) (BD Pharmingen; cat. # 562781), APC-R700 Mouse Anti-Human CD25 (BD Pharmingen; cat. # 565107), Alexa Fluor 488 Mouse anti-Human FoxP3 (BD Pharmingen; cat. # 561181). BV421 Mouse Anti-Human CD19 (BD Pharmingen; cat. # 562440), PE-Cy7 Mouse Anti-Human CD21 (BD Pharmingen; cat. # 561374), PE Mouse Anti-Human CD23 (BD Pharmingen; cat. # 555711), FITC Mouse Anti-Human CD24 (BD Pharmingen; cat. # 560992), BV510 Mouse Anti-Human CD138 (BD Pharmingen; cat. # 563091).

3.7. Statistical Analysis

All data are expressed as the mean ± SD. An unpaired *t*-test was used for groups of two. All data met the assumptions of the statistical tests. The number of samples in each group is presented in the figure legends. The results were considered statistically significant at $p < 0.05$. The correlation analysis between the two variables was performed by simple linear regression. Statistical analyses were performed using the GraphPad Prism software (version 8.0.1.244, San Diego, CA, USA) for Windows.

4. Discussion

This study presents a comprehensive investigation into the relationship between gut microbiota-derived metabolites and immune cell subsets in URSA. URSA, characterized by multiple consecutive miscarriages before the 24th week of pregnancy, remains a challenging clinical issue due to its complex etiology and lack of specific clinical manifestations. Addressing this gap, the study sheds light on the potential involvement of gut microbiota-derived metabolites in URSA pathogenesis, providing insights that could potentially lead to new treatment strategies.

The findings of this study demonstrate significant alterations in both gut microbiota composition and the levels of metabolites derived from the gut microbiota in URSA patients. Reduced diversity and distinct bacterial genera were observed in the gut microbiota of URSA patients, suggesting a potential dysbiosis that might contribute to the immune dysregulation associated with URSA. Moreover, targeted metabolomic analysis revealed decreased levels of BAs and SCFAs in URSA patients. These metabolites, which play essential roles in various physiological processes, have been shown to have a significant impact on the immune system.

The study also investigated the peripheral blood Immune cell subsets In URSA patients. The observed increased frequencies of Th1, Th17, and plasma B cells, coupled with decreased frequencies of Tregs and Bregs, reflect an altered immune response in URSA patients. These findings are consistent with the hypothesis that immune dysregulation could contribute to the pathogenesis of URSA. Importantly, through simple linear regression analysis, the study establishes correlations between gut microbiota-derived BAs and SCFAs and the frequencies of various immune cell subsets in circulation. This suggests a potential mechanistic link between gut microbiota-derived metabolites and immune cell dysregulation in URSA.

In this study, we have observed significant alterations in metabolites derived from the gut microbiota, particularly SCFAs and BAs, in patients with URSA. This discovery has prompted an in-depth exploration of the mechanisms by which these metabolites may impact the function of immune cells in peripheral blood. Although our research is still in its preliminary stages, important insights have already emerged regarding the potential mechanisms of interaction between gut microbiota-derived metabolites and immune regulation. Firstly, SCFAs (such as propionic acid, butyric acid, and acetic acid) have been extensively studied because they are significant products of gut microbiota metabolism [52]. These metabolites can influence the immune system through various pathways [53]. One possible mechanism is their modulation of immune cell function through SCFA receptors (FFAR2 and FFAR3) on intestinal epithelial cells [54]. By activating these receptors, SCFAs may promote immune tolerance, reduce inflammatory responses, and affect T cell and B cell differentiation, including the promotion of regulatory T cells (Tregs) development [54,55]. In URSA, we observed a decrease in the frequency of Tregs, which may be associated with reduced SCFAs levels. This finding suggests that the reduction in SCFAs may lead to immune cell dysfunction, subsequently affecting embryo implantation and successful pregnancy. Secondly, BAs are also considered

to play a crucial role in immune regulation [56]. Different types of BAs may impact immune cells by activating specific receptors, such as TGR5, and by influencing the intestinal mucosal barrier [57]. In URSA, we observed a significant decrease in gut microbiota-derived BAs levels, which could be related to alterations in the frequency of immune cell subpopulations. Notably, the negative correlation between BAs and the inflammatory Th1 and Th17 cell subpopulations may indicate the role of BAs in suppressing inflammatory responses. Moreover, the metabolic activity of gut microbiota may affect B cell function, including antibody production. In our study, we observed significant changes in the frequency of B cell subpopulations in URSA patients, which may be linked to metabolites derived from the gut microbiota. This suggests that metabolites, by influencing B cell activity, may impact the immune function of URSA patients. In summary, metabolites derived from the gut microbiota, especially SCFAs and BAs, may impact immune cell function through multiple mechanisms, including the regulation of T cell differentiation, immune tolerance, and the control of inflammatory responses. These findings provide important clues for a deeper understanding of the pathogenesis of URSA and offer new insights for future clinical interventions and treatment strategies. Further research is needed to unveil the details of these mechanisms and identify potential therapeutic targets to improve the pregnancy outcomes of URSA patients.

Interestingly, when exploring the relationship between gut microbiota dysbiosis and immune dysfunction, a classic question often arises: which came first, the chicken or the egg? In other words, does gut microbiota disruption lead to immune dysfunction, or does immune dysfunction trigger gut microbiota dysbiosis? This question becomes particularly important in the context of URSA. One perspective suggests that gut microbiota dysbiosis may act as a trigger for immune dysfunction [58]. The gut is a vital component of the immune system, and gut microbiota is closely intertwined with immune function. Disruptions in the gut microbiota community, such as alterations in microbial composition or reduced microbial abundance, can potentially provoke abnormal immune responses. These aberrant immune responses may result in changes in the frequency of immune cell subpopulations, such as increased Th1 and Th17 cells and decreased Tregs and Bregs cells, all of which have been observed in URSA patients. Thus, gut microbiota dysbiosis may play a pivotal role in initiating or exacerbating the development of URSA. Another viewpoint suggests that immune dysfunction may lead to gut microbiota dysbiosis [59]. Immune system dysregulation can trigger inflammatory responses, impact the integrity of the intestinal mucosa, and alter the microbial environment. In this scenario, immune system abnormalities could be the cause, rather than the result, of changes in the gut microbiota. The observed alterations in T cell and B cell subpopulations in URSA patients may be attributed to immune system abnormalities, subsequently affecting the gut microbiota. In reality, it is highly likely that gut microbiota dysbiosis and immune dysfunction form a mutually reinforcing relationship. Complex interactions exist between gut microbiota and the immune system. Gut microbiota can influence immune system regulation, and the state of the immune system can impact the balance of gut microbiota. This interplay may create a vicious cycle, contributing to the observed anomalies in URSA patients. To gain a deeper understanding of the relationship between gut microbiota dysbiosis and immune dysfunction, further research is necessary. Longitudinal study designs can help determine whether the chicken or the egg came first. Additionally, molecular biology and cell biology research can help elucidate mechanisms, including how gut microbiota regulates immune responses and how the immune system influences gut microbiota. These studies will provide more information for future intervention and treatment strategies to improve the pregnancy outcomes of URSA patients. Whether it's the chicken or the egg, resolving this question will aid in unraveling the intricate mechanisms of URSA.

The study's findings open up several avenues for further research and potential clinical implications. By uncovering the associations between specific gut microbiota genera, their metabolites, and immune cell subsets, the study paves the way for deeper mechanistic investigations. These findings could also potentially be leveraged for therapeutic interventions. The identification of candidate gut microbiota-derived metabolites like DCA, GLCA, acetate, propionate, and butyrate as potential raw materials for clinical URSA therapies offers new strategies for treating this challenging condition.

However, it's important to acknowledge the limitations of the study. The relatively small sample size, cross-sectional design, and lack of mechanistic experiments limit the ability to establish causal relationships. The potential influence of confounding factors, such as diet and lifestyle, should be considered. Furthermore, while correlations have been identified, the functional implications of these associations and their potential for clinical translation need further exploration.

In conclusion, this study provides valuable insights into the intricate connections between gut microbiota-derived metabolites and immune cell subsets in URSA. By establishing correlations between these factors, the study enhances our understanding of the potential mechanisms contributing to URSA pathogenesis. While further research is warranted to validate these findings and establish causality, the study's results hold promise for future therapeutic strategies and emphasize the importance of considering the gut-immune axis in reproductive health.

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Availability of data and materials

The dataset(s) supporting the conclusions of this article are included within the article and its additional files.

Data availability statement

Data will be made available on request.

Ethics statement

This study was approved by the Ethics Committee of Women's Hospital School of Medicine, Zhejiang University (approval no. IRB-20230085-R).

CRediT authorship contribution statement

Zhi Li: Writing – original draft, Methodology, Investigation. **Yongquan Zheng:** Writing – review & editing, Validation, Supervision, Software. **Meng Zhang:** Software, Resources, Data curation. **Kaiqi Wu:** Data curation, Conceptualization. **Long Zhang:** Data curation, Conceptualization. **Yao Yao:** Visualization, Validation, Project administration, Funding acquisition. **Caihong Zheng:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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