Title: Increasing breast milk betaine content modulates offspring *Akkermansia* abundance during early life and improves long-term metabolic health

Authors: Silvia Ribo[1†], David Sanchez-Infantes[1,2,3†], Laura Martinez-Guino[1], Izaskun García-Mantrana[4], Marta Ramon-Krauel[1], Mireia Tondo[5], Erland Arning[5], Miquel Nofrarías[6], Óscar Osorio-Conles[1], Antonio Fernández-Pérez[7†], Pedro González-Torres[8], Judith Cebrià[1], Aleix Gavaldà-Navarro[3,9,10], Empar Chenoll[11], Elvira Isganaitis[12], Francesc Villarroya[3,9,10], Mario Vallejo[7], Joaquim Segalés[13,14], Josep C. Jiménez-Chillarón[1], Teodoro Bottiglieri[5], Ellen W. Demerath[15], David A. Fields[16], María Carmen Collado[4], Carles Lerin[1*]

Affiliations:

[1]Endocrinology Department, Institut de Recerca Sant Joan de Déu, 08950 Barcelona (Spain).

[2]Department of Endocrinology and Nutrition, Institut de Recerca Germans Trias i Pujol, Barcelona (Spain).

[3]CIBER Fisiopatologia de la Obesidad y Nutrición, Madrid (Spain).

[4]Department of Biotechnology, Institute of Agrochemistry and Food Technology-National Research Council (IATA-CSIC), 46980, Valencia, Spain.

[5]Institute of Metabolic Disease, Baylor Scott and White Research Institute, Dallas, TX (USA).

[6]IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra (Spain).

[7]Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas/Universidad Autónoma de Madrid (CSIC/UAM), Madrid (Spain); CIBER Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM).

[8]Bioinformatics and Genomics Program, Centre for Genomic Regulation (CRG), Barcelona (Spain).

[9]Departament de Bioquímica i Biomedicina Molecular, Institut de Biomedicina de la Universitat de Barcelona (IBUB), Universitat de Barcelona, Barcelona (Spain)

[10]Institut de Recerca Sant Joan de Déu, Barcelona (Spain).

[11]Biopolis S.L.-ADM, 46980, Paterna (Spain).

[12]Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, MA (USA).

[13]UAB, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra (Spain).

[14]Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, UAB, 08193 Bellaterra, Barcelona (Spain).

[15]Division of Epidemiology and Community Health, The University of Minnesota School of Public Health, Minneapolis, MN (USA).

[16]Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, OK (USA).

† First co-author

‡ Current address: Centro para el Desarrollo Tecnológico e Industrial (CDTI), Madrid, Spain.

* To whom correspondence should be addressed: Carles Lerin

Endocrinology Department, Institut de Recerca Sant Joan de Déu.

c/Santa Rosa 39, 08950 Barcelona (Spain)

Phone: (+34) 936 009 771 Email: clerin@fsjd.org

One sentence summary: Breast milk betaine content and offspring long-term metabolic health programming

Abstract

Accelerated postnatal growth is a strong and potentially modifiable risk factor for future obesity. To study how specific breast milk components contribute to early growth and obesity risk, we quantified one-carbon metabolism-related metabolites in human milk and found an inverse association between milk betaine content and infant growth. Notably, this association was replicated in an independent and geographically distinct cohort. To determine the potential role of milk betaine in programming offspring obesity risk, we performed maternal betaine supplementation experiments in mice. Higher betaine intake during lactation increased milk betaine content and led to lower offspring adiposity and improved glucose homeostasis throughout adulthood. These effects were accompanied by a transient increase of *Akkermansia* spp*.* abundance during early life and a long-lasting increase in intestinal goblet cell number. *Akkermansia muciniphila* levels in infants were also linked to milk betaine content in the human cohort. Furthermore, administration of *Akkermansia muciniphila* to pups during the lactation period partially replicated maternal betaine effects, including long-term improvements in adiposity and glucose homeostasis. Together, our data demonstrate a link between breast milk betaine content and offspring long-term metabolic health, and indicate that betaine may be a target for early interventions aimed at childhood obesity prevention.

Introduction

Extensive research in humans and animal models indicates that a sensitive period of growth and development both *in utero* and during early infancy strongly influences life-course health outcomes (*1*). A number of perinatal factors, including maternal nutritional status and gestational weight gain, postnatal growth trajectories, feeding patterns, and early-life intestinal microbiota modulate future risk of obesity (*2-5*). Among the risk factors, accelerated growth rate during the first months of life has been consistently associated with higher risk of future obesity and metabolic disease (*5-8*).

Metabolites from the one-carbon metabolism pool have long been recognized as critical nutrients for infant growth and development (*9-11*). Choline is in high demand during early growth, and its levels are especially elevated in milk (*11, 12*). It can be used for phosphatidylcholine biosynthesis or oxidized to betaine. Betaine is a trimethylated derivative of glycine readily available in the diet (*13, 14*). Its circulating levels are inversely associated with BMI and diabetes risk in adult subjects (*15-17*). Betaine acts as a potent osmolyte regulating cellular volume and protecting against external stress in multiple organisms, from bacteria to mammals (*13, 14, 18*). In mammals, betaine is primarily metabolized in the liver, transferring a methyl group to homocysteine to sequentially produce methionine, s-adenosylmethionine (SAM), s-adenosylhomocysteine (SAH), and homocysteine in the methionine cycle (*19*).

Maternal status of one-carbon metabolites during pregnancy has been linked to offspring health outcomes. For instance, lower maternal plasma folate concentration during gestation has been associated with higher offspring obesity risk (*10*), and lower maternal circulating betaine levels during the third trimester of pregnancy have been correlated to higher infant weight and adiposity at birth (*20*). However, despite their importance for infant growth and development, a metabolic profiling of one-carbon related intermediates in breast milk has not been performed, and whether milk levels of these metabolites play a role in modulating infant growth and obesity risk remains unknown. To address these gaps in knowledge, the present study sought to 1) investigate potential associations of

milk one-carbon metabolites with infant early growth; and 2) test whether maternal supplementation with these new potential biomarkers modulates growth, body composition, and glucose homeostasis into adulthood in a murine model.

Results

Breast milk betaine levels are inversely associated with infant growth

To determine whether milk one-carbon metabolites are associated with early growth, we studied a US-based cohort of 34 exclusively breastfeeding mother-infant dyads from the Oklahoma area (*21*) (Cohort I, subject characteristics in Table 1). We quantified choline, betaine, methionine, SAM, SAH, and cystathionine concentrations in human breast milk samples obtained 1 month after birth and examined potential associations of these metabolites with infant growth rate, assessed by changes in weight-for-length z-score (WLZ) from birth to 1 month (Cohort I, Table 2). Statistical models were adjusted for gestational age and WLZ at birth (Model 1), as well as additional maternal variables known to affect infant growth, including pre-pregnancy maternal BMI, gestational weight gain and delivery method (Model 2). We observed a significant inverse association between WLZ change and milk betaine concentration in both models, and only a trend for SAH and cystathionine content (Cohort I, Table 2).

We also studied an independent European-based cohort with 109 exclusively breastfeeding mother-infant dyads from the Spanish-Mediterranean area (*22*), with infant anthropometric data obtained at birth, 1 and 12 months of age (Cohort II, subject characteristics in Table 1). One-carbon metabolites were also quantified in 1-month breast milk samples. Importantly, the association between milk betaine levels and WLZ change at 1 month was replicated in this cohort (Cohort II, Table 2). Furthermore, the longitudinal data from the replication cohort showed that milk betaine content at 1 month was also inversely associated with WLZ change at 12 months of age (Cohort II, Table 2), indicating that the correlation persisted through the first year of life. In this cohort, we also observed associations of infant WLZ change a 1 month with methionine content and, although markedly weaker,

with SAM levels (neither present in Cohort 1), while betaine was the only metabolite showing a significant association with WLZ change at 12 months of age (Table 2). No association was observed for milk betaine content with change in body length or head circumference in either cohort (Table S1).

Dietary betaine transfers into breast milk and decreases adiposity in young mice

To determine whether betaine plays a role in postnatal growth and future obesity, we performed maternal supplementation experiments in mice during lactation. For this purpose, dams were randomly assigned to control (C) or treatment (B, 1% betaine) groups the first day after delivery. Milk samples were collected at day 14 from a subset of dams to determine betaine levels. Supplementation increased milk betaine content by 5-fold (Figure 1A) without altering macronutrient composition (Figure 1B) or energy content (Figure 1C). The increase in milk betaine content translated into elevated plasma betaine levels in suckling 14-day-old offspring (Figure 1D). We next examined the effects of maternal betaine supplementation on offspring growth during the lactation period. Supplementation induced a modest but significant decrease in offspring weight gain during the first week of lactation (Figure 1E). No differences in milk intake were observed between groups (Figure 1F). Notably, body composition analysis at 6 weeks of age revealed decreased fat mass in betaine-exposed mice compared to control mice (Figure 1G). These changes in adiposity were accompanied by decreased mRNA levels of immune markers C-C motif ligand 2 (Ccl2, also known as MCP-1), Toll receptor-like 4 (Tlr4), and Integrin subunit alpha X (Cd11c) in visceral white adipose tissue (vWAT) (Figure 1H); expression of the pro-inflammatory marker Ccl2 was also lower in brown adipose tissue (BAT), liver, and skeletal muscle (Figure S1A). Supplementation decreased circulating levels of interleukin-6 (IL-6), while Ccl2 and plasminogen activator inhibitor-1 (PAI-1) showed a numerical decrease (Figure 1I). Other parameters including glucose tolerance, uncoupling protein 1 (Ucp1) expression in BAT, food intake, oxygen consumption, respiratory exchange ratio, or activity levels did not differ between groups at this age (Figures S1B-S1G). Betaine has been shown to induce hepatic Fgf21 levels in adult mice in a diet-induced obesity context (*16*); however, we observed

no effect of maternal betaine administration on offspring hepatic Fgf21 expression at neither 2 nor 6 weeks of age (Figure S1H).

We next studied the effects of betaine supplementation in a maternal obesity model, which leads to increased offspring early growth and higher adiposity during adulthood in rodents (*23*). For this purpose, diet-induced obese dams were randomly assigned to control (MO-C) or betaine-treatment (MO-B) groups at delivery. Maternal obesity increased weight gain during lactation compared with normal-weight dams (5.5 ± 0.1 g *versus* 4.3 ± 0.1 g during week 1 for MO-C and C groups in Figures 1J and 1E). In this context of accelerated growth, maternal betaine supplementation decreased offspring weight gain throughout the lactation period (Figure 1J) with no differences in milk intake (Figure 1K). At 6 weeks of age, offspring born to betaine-treated dams showed a trend towards lower adiposity compared to control mice (Figure 1L).

Maternal betaine supplementation improves offspring long-term metabolic health

To determine whether the early-life intervention had long-lasting effects, mice were monitored until adulthood. Offspring from betaine-treated dams under chow diet showed lower body weight during adulthood (Figure 2A). These mice showed decreased visceral and subcutaneous WAT (scWAT) weight with no changes in liver weight at 24 weeks of age compared to control mice (Figure 2B). These effects were accompanied by improved glucose tolerance (Figure 2C) and decreased fasting insulin levels (Figure 2D). Consistent with lower adiposity, adipocyte size (Figure 2E) and pro-inflammatory markers Ccl2 and Cd11c were decreased in vWAT from betaine-exposed mice (Figure 2F). We observed similar long-term effects of betaine supplementation during lactation in the maternal obesity mouse model, with improvements in body weight (Figure 2G), adiposity (Figure 2H), glucose tolerance (Figure 2I), and fasting insulin levels (Figure 2J) in offspring born to betaine-treated dams compared to the control group.

Given that postnatal nutrition is a major determinant of the infant gut microbiota and changes in early-life intestinal bacteria can modulate long-term adiposity in mice (*4, 24-26*), we tested whether gut microbiota contributed to betaine's long-term effects. For this purpose, we co-administered antibiotics to dams during lactation to disrupt the offspring's gut microbiome. Dams were randomly assigned to four groups receiving betaine (1% betaine), antibiotics (1 g/L ampicillin, 0.5 g/L neomycin), antibiotics and betaine, or no supplements; offspring were fed a chow diet after weaning and monitored until adulthood (Figure S2A). Maternal antibiotic administration disrupted the offspring gut microbiota, as shown by the notable decrease in bacterial content in cecal samples from 2-week-old suckling mice (Figure S2B). Remarkably, maternal antibiotic administration during lactation abolished long-term betaine-induced effects on body weight (Figure S2C), adiposity (Figure S2D), glucose tolerance (Figure S2E), and vWAT immune markers Ccl2 and Cd11c (Figure S2F).

Maternal betaine transiently increases offspring Akkermansia spp. abundance during early life

To determine whether milk betaine shapes gut microbiota, we analyzed cecal samples from dams, as well as 2- and 6-week-old offspring. While betaine supplementation did not modify maternal gut microbiota composition (Figures S3A and S3B), principal coordinate analysis (PCoA) of UniFrac distances showed differential microbial community composition in 2-week-old offspring across groups (n=10 mice from 8 different litters per group, Figure 3A – left panel); these changes were transient, as no differences were observed in 6-week-old mice (n=10 from 6 different litters per group, Figure 3A – right panel). Alpha diversity (measured as Chao1 and Shannon indexes) did not differ between groups (Figure S3C). We applied linear discriminant analysis effect size (LEfSe) to identify differentially abundant bacteria at 2 weeks of age and found that *Akkermansia* spp. was the only group significantly altered (Figure S3D and S3E), with higher relative abundance in the betaine-treated compared to the control group (Figure 3B).

We next asked whether breast milk betaine content was linked to *Akkermansia* levels in humans. To answer this question, we determined the absolute concentration of *Akkermansia muciniphila* by

quantitative PCR analysis in a subset of infants from Cohort II from whom fecal samples were available (88 at 1 month and 91 at 12 months of age). As expected from previous studies (*27*), *A. muciniphila* abundance was very low during early life and was only detected in 7 infants at 1 month of age (8% of total samples). However, *A. muciniphila* increased during the first year of life and was detected in 40 infants at 12 months of age (44% of total samples) and its association with milk betaine was examined. For this analysis, infants were categorized into "Low" and "High" betaine exposure groups based on the median value of milk betaine concentration. While we observed no differences in prevalence between groups (it was present in 18 (39%) and 22 (49%) infants from Low- and High-exposure groups, respectively; Chi-square p=0.348), *A. muciniphila* absolute levels were significantly higher in infants that were exposed to higher milk betaine content (Figure 3C). Furthermore, *A. muciniphila* levels showed a significant direct correlation to milk betaine concentration when analyzed as a continuous variable (Spearman's rho=0.35, p=0.026), and a trend towards an inverse correlation to WLZ change from birth to 1 month (Spearman's rho=-0.24, p=0.138) and to 12 months of age (Spearman's rho=-0.25, p=0.112).

Akkermansia spp. abundance has been tightly linked to lower inflammation and ameliorations in metabolic health in mice, partly by increasing intestinal goblet cell number and improving the gut barrier function (*28-31*). Thus, we next performed a histological analysis of the ileum. Ileal villi height and crypt depth did not differ between groups at 2, 6, or 24 weeks of age (Figure S4A). Ileal goblet cell number showed no differences during breastfeeding (2-week-old mice) but was significantly higher at 6 weeks of age in the betaine-exposed group (Figure 3D). Notably, the effect of the early-life intervention on ileal goblet cells was maintained throughout adulthood, as 24-week-old mice born to betaine-treated dams still showed higher goblet cell number than controls (Figure 3D). Intestinal goblet cells are responsible for mucin 2 (Muc2) production to maintain the mucosal barrier. Ileal Muc2 expression paralleled the increase in goblet cells. No major differences were observed in 2-week-old mice between groups (Figure 3E); however, betaine-exposed 6-week-old mice showed significantly higher ileal expression of Muc2 compared to controls (Figure 3F), while other gut barrier markers were

not changed (occludin, Ocln; tight-junction protein 1, Zo1) or showed only a trend towards higher levels (tight-junction protein 2, Zo2, p=0.09, Figure 3F). Expression of these genes was also increased at 24 weeks of age (Figure 3G). Alterations in early-life microbiota can also modulate the development of the immune system by altering T cell differentiation and impacting long-term metabolic health (*26*). Thus, we analyzed whether master transcription factors that drive CD4+ T cell differentiation into the different subset (Th1, Th2, Th17, and Treg cells) were altered by maternal betaine supplementation. We observed no significant changes in ileal gene expression of markers Tbx21, Gata3, Rorc, and Foxp3 at 2, 6, or 24 weeks of age (Figure S4B).

We then tested whether betaine could modulate *Akkermansia* growth by determining the effect of different betaine concentrations on *in vitro Akkermansia muciniphila* growth in anaerobic conditions. Presence of betaine in the media (2.5 and 10 mM) resulted in a modest but significant increase in *Akkermansia muciniphila* growth *in vitro* (Figure 3H). We performed similar experiments with other bacterial species usually present in infant gut microbiota, including *Escherichia coli* (phylum Proteobacteria), *Lactobacillus johnsonni* (phylum Firmicutes), and *Bifidobacterium longum* (phylum Actinobacteria). We observed no significant effects of betaine on growth of these bacteria *in vitro* (Figure S5).

Early-life Akkermansia muciniphila exposure improves long-term metabolic health

To test whether exposure to *Akkermansia* spp. during early life replicated maternal betaine effects, we administered *A. muciniphila* to pups in a mouse model of maternal obesity. For this experiment, we used pasteurized *A. muciniphila* (pAkk) as it has been shown to exert similar effects on metabolic parameters as the live form when administered to humans or mice (*28, 32*). Eleven-day-old offspring from diet-induced obese dams were administered pAkk (MO-Akk group) or PBS (MO-C group) by gavage three times per week until weaning (Figure 4A). pAkk administration induced a 10% decrease in weight-gain over the treatment period compared to controls (Figure 4B). At 6 weeks of age, these mice showed lower scWAT weight and a trend towards decreased vWAT weight (Figure 4C).

Inflammatory markers in vWAT were decreased (Tlr4) or tended towards lower levels in the MO-Akk group (Ccl2 and Cd11c, Figure 4D). Early-life pAkk exposure resulted in higher ileal goblet cell number at 6 weeks of age (Figure 4E), in parallel to increased expression of some gut barrier markers (Muc2, Zo1, Figure 4F). To study the long-term effects of this early-life intervention, we monitored the mice until adulthood. While only a modest numerical decrease in body weight over time was observed in pAkk-treated mice (Figure 4G), 20-week-old mice showed decreased WAT weight (Figure 4H), as well as improved glucose tolerance and fasting insulin levels compared to controls (Figures 4I-4J). Although slightly weaker, the effect of early-life pAkk exposure on the ileum was sustained in time, as ileal goblet cell number (Figure 4K) and expression of some of the gut barrier markers (Zo2, and trends in Muc2 and Ocln) were still increased in adult mice (Figure 4L).

Discussion

Breastfeeding is considered the gold standard of early nutrition and is regarded as a window of opportunity for early-life preventive interventions (*33*). In this study, we quantified one-carbon related metabolites in human milk to shed more light into the potential role of these metabolites in infant growth and obesity risk. Among these metabolites, betaine was associated with changes in WLZ during the first month of life in both the discovery and the replication cohorts. It is worth noting that these cohorts are based in two geographically different regions like Oklahoma (USA) and the Spanish Mediterranean area (Europe), with distinct dietary and lifestyle habits. Furthermore, data from the second cohort showed that the association between milk betaine content and growth was still present in 12-month-old infants. Although studies investigating breast milk composition and infant health are sparse (*34*), a small number of other metabolites and other components have been identified associated with growth. Differences in milk oligosaccharide diversity and hormone levels (insulin and leptin) have been correlated with infant growth, adiposity, and gut microbiota composition (*21, 25, 35*). Furthermore, we recently reported the milk metabolome associated with maternal obesity, with only adenine correlating with both maternal BMI and infant weight status (*36*).

Our data from both cohorts indicate an association between lower milk betaine content and higher infant growth during the first months of life. Interestingly, lower maternal betaine status during pregnancy has been correlated to increased infant birth weight and adiposity (*20*), and maternal betaine supplementation during gestation resulted in lower fetal adiposity in a mouse study (*37*). Together with our results in human milk, these data support a link between lower maternal betaine status during pregnancy and breastfeeding and increased fetal and postnatal growth. Accelerated growth during early infancy (as early as the first weeks of life) has been consistently associated with obesity years later (*5-8, 38, 39*). Therefore, our results suggest a link between lower milk betaine content and higher childhood obesity risk.

Betaine is naturally present in the diet and can also be derived endogenously from choline. A human study showed that providing a choline supplement during lactation increased both plasma and milk betaine concentrations (*12*), suggesting that dietary choline may modulate milk betaine levels. Furthermore, our experiments in mice show that maternal betaine supplementation increased milk levels of this metabolite, indicating that milk betaine content can be readily modulated by dietary intake. Betaine is abundant in whole-grains and some vegetables, and scarce in western dietary patterns (*40*). Further studies are necessary to determine whether consumption of betaine- or choline-rich foods determines milk betaine status.

Despite the fact that early growth trajectories are largely divergent between rodents and humans, our supplementation experiments in mice paralleled the association between milk betaine content and early growth observed in the human studies. Moreover, early-life exposure to betaine had long-lasting metabolic consequences in mice, decreasing adiposity and improving glucose homeostasis during adulthood. A number of animal models clearly support a link between nutritional challenges during early development, growth trajectories, and long-term metabolic risk. For instance, low birth weight or neonatal overfeeding result in accelerated growth, leading to obesity and metabolic dysregulation during adulthood (*41*). Conversely, decreasing early growth by postnatal undernutrition proved

beneficial to prevent future obesity (*42*). Notably, betaine has long been used as a feed additive in weaned livestock animals due to its carcass modifier properties, increasing lean versus fat mass ratio (*43*); in this context, our results indicate that betaine supplementation during the suckling period might suffice for decreasing long-term adiposity.

The early-life gut microbiome has recently emerged as an important determinant of long-term disease risk (*26, 33, 44, 45*). Mode of delivery and early nutrition are major determinants in shaping the infant intestinal microbiome. In particular, breast milk stimulates the proliferation of a balanced gut microbiome, the development of a healthy intestine, and maturation of the immune system (*44, 45*). The transition from milk to solid food induces broad changes in nutrient availability and metabolism, as well as in the gut microbiota composition and ileal epithelial structure. Microbial dysbiosis during infancy has been associated with a number of diseases years later, including obesity (*4, 24-26, 44, 46, 47*). For instance, antibiotic use during infancy has been associated with childhood obesity (*4, 47*), and maternal low-dose antibiotic administration during lactation in mice led to offspring overweight (*26*).

Our results suggest that the betaine-induced effects on metabolic health are, at least in part, mediated by the early-life gut microbiota. We showed that maternal antibiotic co-administration blunted betaine-induced improvements in offspring metabolic health; however, we recognize that antibiotics induce broad changes in the gut microbiome and exert a wide range of effects on the host, including alterations in intestinal permeability and adiposity that could be independent of betaine's mechanism of action (*26*). Furthermore, we observed that maternal betaine supplementation transiently increased offspring *Akkermansia* spp. abundance during early life, and that betaine is capable of enhancing *Akkermansia muciniphila* growth *in vitro*. Importantly, data from our human cohort further strengthen the link between milk betaine and this microorganism, as exposure to higher milk betaine content during breastfeeding resulted in higher *A. muciniphila* abundance in 12-month-old infants. Finally, administration of pasteurized *Akkermansia muciniphila* to pups during the lactation period partially

recapitulated maternal betaine effects. All together, these data suggest a role for this microorganism in mediating betaine effects. To note, betaine also acts as a methyl group donor in the methionine cycle, ultimately providing SAM and increasing glutathione availability (*16, 19*). Thus, additional mechanisms related to oxidative stress, epigenetic changes, or phosphatidylcholine biosynthesis may also contribute to betaine-induced improvements in metabolic health.

We have considered different mechanisms by which maternal betaine could modulate offspring intestinal *Akkermansia spp*. abundance. Betaine supplementation did not alter milk macronutrient composition or energy content, although it could modify other components, including hormones or immune system elements, that may affect bacterial communities in the offspring. However, our *in vitro* experiments showing enhanced *Akkermansia muciniphila* growth in the presence of betaine support the hypothesis of a direct effect of betaine on this microorganism. Owing to its dipolar zwitterion structure and osmoprotectant properties, bacteria readily use betaine as protection against external stress (*18*). Thus, betaine could be metabolized or used as an osmolyte by *Akkermansia*. Betaine was not equally effective in modulating growth of other bacteria, suggesting some specificity towards *Akkermansia*. However, growth characteristics of a single bacterium *in vitro* can differ in the presence of other bacteria or in different conditions, where symbiotic or competitive interactions can greatly determine growth dynamics of specific bacteria. In this regard, it is worth noting that betaine supplementation increased *Akkermansia spp*. abundance in pups but not in dams, suggesting a context-dependent effect of betaine. Indeed, the structure and diversity of the microbiota varies greatly between newborn and adults, and the early-life gut microbiota could potentially provide a more favorable environment for *Akkermansia* growth in the presence of betaine. Furthermore, *Akkermansia* might also be a constituent of breast tissue microbiota in humans (*48*) and betaine could increase its abundance in breast tissue or milk, ultimately impacting the offspring gut microbiota. Thus, further experiments would be needed to decipher the exact mechanisms by which betaine increases *Akkermansia muciniphila* growth and whether it can exert this effect in different environments.

Akkermansia has received much attention during the last years, mostly due to reports showing that decreased abundance is associated with obesity and metabolic disorders in both humans and animal models, suggesting a beneficial role for this bacterial species (*28-30, 49*). Furthermore, a recent report linked *Akkermansia muciniphila* to increased lifespan in a mouse model of accelerated aging (*31*). Conversely, other studies have reported increased *Akkermansia* abundance in patients with neurological disorders, including multiple sclerosis, Parkinson's disease, and Alzheimer (*50-53*). These data highlight the notion that interactions between microbes and host are highly dependent on multiple factors (environment, genetic background, type of disease) and the specific effects of *Akkermansia* might also be context dependent (*54, 55*).

A number of studies have shown that daily administration of *Akkermansia muciniphila* to adult mice decreases body weight gain and adiposity and improves glucose homeostasis in both diet-induced obese mice (*28-30*) and lean chow diet-fed mice (*56*). Even more, Depommier et al. recently reported results from a clinical trial in which administration of *Akkermansia muciniphila* improved insulin sensitivity in human subjects with obesity (*32*). Notably, administration of pasteurized *Akkermansia muciniphila* to either mice or humans has shown similar effects on metabolic parameters than the live form (*28, 32*), indicating that these effects are independent of bacterial metabolism. Indeed, administration of an *Akkermansia*-specific extracellular membrane protein (Amuc_1100) to mice recapitulated most of the effects of the live bacteria (*28*). Our results show that administration of pAkk to pups during early life partially recapitulated betaine's effects, including lower early weight gain. In humans, we observed a trend towards higher growth with lower *A. muciniphila* abundance. While the mechanistic link between *Akkermansia* and growth remains unclear, lower *Akkermansia* abundance has been associated with shorter breastfeeding duration and increased weight gain in children (*4*). Moreover, increased *Akkermansia* population has been correlated to slower early weight gain in a mouse model of postnatal undernutrition (*57*). Together with these studies, our data add to the evidence that *Akkermansia* abundance during early life may be linked to postnatal growth.

Several studies indicate that the beneficial effects of *Akkermansia muciniphila* are mediated, at least in part, by improving the intestinal barrier function. Daily administration of *Akkermansia muciniphila* to adult obese mice for 4-5 weeks increases ileal goblet cell number and expression of intestinal barrier markers, leading to decreased systemic inflammation and ameliorated metabolic health (*28-30*). Our data show that, in parallel to transiently increasing *Akkermansia* abundance, maternal betaine supplementation induced a long-lasting increase in offspring ileal goblet cell number and expression of intestinal barrier markers, decreased systemic inflammation and adiposity, and improved glucose homeostasis. Interestingly, the increase in goblet cells was observed at 6 weeks of age but not during lactation. The reason for this could be that the effect on goblet cell number might only be evident after weaning or, alternatively, that two weeks of maternal betaine supplementation are able to increase offspring *Akkermansia* abundance but are not sufficient to induce the response in goblet cells. Furthermore, pAkk administration during lactation led to a lasting increase in intestinal goblet cell number after weaning, in parallel to improvements in adiposity and glucose homeostasis during adulthood.

We observed a strong short-term effect of early-life pAkk administration on goblet cells and intestinal barrier markers; however, its long-term effects were more modest than with betaine supplementation. While these data might suggest the existence of additional mechanisms mediating betaine action, it is worth noting that pAkk administration was started at day 11 after birth because *gavage* was not feasible in smaller pups; these first 10 days of life constitute an important developmental window, and it is plausible that pAkk exposure during this crucial period of time would trigger an even more robust long-term effect. The neonatal period offers a window of opportunity in which important processes including the development and maturation of the gut microbiota and intestinal barrier, imprinting of the immune system, or the growth trajectory may influence health outcomes across the lifespan (*8, 33, 44, 45*). Interestingly, exposure to elevated milk insulin and leptin in infants during the first weeks of life has been also proposed to exert beneficial effects by improving the intestinal barrier function (*25*). It is intriguing to speculate that modulating intestinal development and barrier function during this critical

developmental window by means of betaine or *Akkermansia* may have a role in programming life-course metabolic health.

The limitations of this study include that maternal diet during breastfeeding was not controlled and might affect milk metabolite levels. Furthermore, milk metabolites were determined at a single time-point (1 month after birth), while milk composition is dynamic and may change over time. However, and despite these shortcomings, the association between milk betaine levels and growth was robust and sustained adjustment for confounding variables in two independent and geographically different cohorts. The implications of this study might go beyond breastfeeding, as formula-fed infants are at higher risk of accelerated early growth and childhood obesity and might benefit from revisiting one-carbon metabolite concentration in artificial milk. In summary, this study highlights the breastfeeding period as an important window of opportunity for early interventions, and suggests that modulating betaine intake during lactation may hold promise as a strategy for childhood obesity prevention.

Materials and Methods

Human studies

Cohort I: This human cohort was previously reported (*21*). The study was approved by IRB, and informed consent was obtained (ClinicalTrials.gov NCT02535637). Briefly, exclusively breastfeeding mothers and infants arrived between 8:00–10:00 am on the campus of the University of Oklahoma Health Sciences (Oklahoma City, OK, USA). The mother was encouraged to empty the entire breast using a hospital-grade breast pump.

Cohort II: A prospective mother-infant birth cohort from the Spanish-Mediterranean area was also analyzed (*22*). The study was approved by the Ethics committees (ref. 2018/0024 at Hospital Universitario y Politécnico La Fe) and informed consent was obtained from all participants (ClinicalTrials.gov NCT03552939). Women were enrolled at the end of pregnancy and families were followed-up during the first year of life. Participants with singleton pregnancy and exclusive breastfeeding for at least three months were included in

this study. For the standardized milk collection, breast skin was cleaned with 0.5% chlorhexidine solution and first drops were discarded. Breast milk samples were collected during the morning at 1 month postpartum by use of sterile hospital-grade breast pump. Infant weight and length were measured from birth up to 12 months of age. Infant fecal samples at 1 and 12 months were also collected.

For both cohorts, Infant anthropometric measurements were collected by trained staff using standard methods. Weight-for-length z scores were calculated using the WHO child growth tables, using WHO Anthro software (www.who.int/ childgrowth/software/en/).

Mouse studies

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Barcelona (Spain). Mice were housed on a 12-hour light-dark cycle with free access to food and water. Male and female ICR-CD1 mice (Envigo) were mated to obtain progeny, and male offspring was monitored until adulthood. To minimize growth variability, litters were adjusted to 8 pups at birth removing heaviest and lightest mice. For maternal obesity studies, 6-week-old females were fed a HFD with 45% kcal from fat (D12451, Research Diets) for 8 weeks before mating and the same diet was maintained through pregnancy and lactation. In both models, betaine (1% wt/vol, ref. 61962, Sigma-Aldrich) was administered to dams in water during the lactation period. For the antibiotic co-administration experiment, ampicilin (1 g/L) and neomycin (0.5 g/L) (Sigma-Aldrich) were administered to the dams in water during the lactation period. After weaning, mice were fed 2014 Teklad diet (Envigo). Milk intake was assessed by separating 6-day-old pups from their mothers for 2h and returning them to their cages for a 1h feeding period. Pup body weight was measured before and after the feeding, and the difference in weight indicated the amount of milk intake, expressed as grams per initial body weight per hour. Individual data for pups from a single litter were pooled, and one point per litter was used for the analysis. Milk samples (100-300 µL) were collected manually under anesthesia at day 14 after birth, after separating dams

from pups for 4 hours. Body composition at 6 weeks of age was determined by using a 7.0T Bruker Biospect MRI system (Bruker Medical Gmbh, Germany). Indirect calorimetry was measured in metabolic cages (PhenoMaster/LabMaster, TSE Systems GmbH, Germany); O_2, CO_2, food intake, and locomotor activity were monitored for 48h, and data analyzed using 2-way ANOVA for repeated measures. Intraperitoneal glucose (1.5 g/kg) tolerance test was performed after a 16h fast. Mice were sacrificed between 9-11am.

Biochemical analyses

One-carbon metabolites (choline, betaine, methionine, SAM, SAH and cystathionine) in human breast milk samples were determined using stable-isotope dilution liquid chromatography-electrospray ionization (ESI) tandem mass spectrometry (LC-ESI-MS/MS) as previously described (*58*). For mouse milk, relative betaine concentrations were determined by liquid chromatography (Acquity UPLC BEH HILIC column, Waters) coupled to mass spectrometry (QqQ/MS 6490, Agilent). Mouse milk samples were diluted 1:3 before macronutrient analysis by mid-infrared spectroscopy in a Miris Analyzer (Miris AB). Plasma insulin was determined by ELISA (Millipore). Plasma MCP-1 (Ccl2) , IL-6 and PAI-1 were measured using Milliplex Map (EMD Millipore, Merck).

Gene expression and histological analysis

Total tissue RNA was isolated from tissues with TRI Reagent (Sigma-Aldrich) and cDNA obtained with High-capacity cDNA kit (Applied Biosystems). Quantitative PCR (qPCR) was performed with SybrGreen (Takara Bio), using Hprt as housekeeping gene. Primer sequences are provided in Table S2. Tissues were fixed by immersion in formalin, paraffin-embedded, and sections stained with hematoxylin/eosin. For WAT, adipocyte area was measured in 30 random microscopic fields per animal (10-20 cells/field). For ileum, cross-sectional cuts were stained with Periodic acid–Schiff (PAS) method. Ten villi per mouse from 5-6 sections were analyzed. Goblet cell number per villus was determined and expressed as

number of cells per 100 µm. Analyses were performed using ImageJ by a researcher blinded to sample ID.

Gut microbiota analysis

Cecal content from dams, 2- and 6-week-old mice from control and betaine-treated groups was collected and stored at -80ºC until bacterial DNA was isolated using the PowerSoil® DNA Isolation Kit (MOBIO Laboratories). Microbial composition was analyzed using Illumina MiSeq System to sequence the V3-V4 region of the 16S rRNA gene following Illumina recommendations, obtaining an average of 24833 reads per sample. Paired-end reads were processed with QIIME v1.9 performing demultiplexing and quality filtering, OTU picking and taxonomic assignment from phyla to species level. Taxonomic assignment was performed using closed reference OTU picking protocol with Greengenes database (http://greengenes.lbl.gov). Unweighted UniFrac distances were used to perform the principal coordinate analysis (PCoA) and compare community structure. Alpha diversity (Chao1 and Shannon indexes) was also determined. Only taxa with a minimum relative abundance of 0.1% in at least one of the samples were analyzed. Linear discriminant analysis (LDA) effect size (LEfSe) algorithm (*59*) was used to identify differences in relative abundance.

In vitro evaluation of microbial growth

Assays for the evaluation of the influence of betaine in *A. muciniphila* growth were performed with cells grown in BHI:MRS-C (10 mL per replicate) broth at 37°C in anaerobic chamber (Whitley A35 anaerobic workstation). Cells were inoculated in BHI:MRS-C broth supplemented with different betaine concentrations (2.5 mM and 10 mM). Medium without betaine supplementation was included as a control. Cells were incubated anaerobically at 37°C until reaching stationary phase. Total DNA was extracted from cell pellets obtained from 1 ml of culture media using “QIAamp DNA Mini Kit” (Qiagen Inc., Barcelona, Spain) according to the manufacturer’s manual. The total amount of *A. muciniphila* was determined by qPCR using specific primers for the species, as previously described

(*27*), with the aid of SYBR® Green PCR Master Mix (Applied Biosystems, Madrid, Spain). Standard curve was constructed with DNA coming from 10-fold diluted standardized DSM 22959^T fresh cells. *Lactobacillus johnsonii* (BPL130) and *Bifidobacterium longum* (CECT7347) were anaerobically grown in MRS medium with cysteine and *Escherichia coli* (ATCC11303) aerobically in LB medium at 37°C. After 17h, cultures were harvested, washed, standardized with McFarland standard and re-suspended in MRS medium for lactobacilli and bifidobacteria and LB for *E. coli*, containing the different concentrations of betaine. Cell suspensions were incubated at 37°C until reaching stationary phase in a microplate reader (Multiskan Ascent) and number of cells per ml were determined by optical density at stationary phase (600 nm). All assays were performed in triplicate.

Akkermansia muciniphila quantitative PCR analysis in infant fecal samples

Sample collection, storage and DNA extraction in Cohort II were described elsewhere (*22*). Available infant fecal DNA samples were used to quantify the presence and levels of *A. muciniphila.* Specific *A. muciniphila* qPCR was performed in a LightCycler® 480 real-Time PCR System (Roche Technologies, Basel, Switzerland) as previously reported (*27*). A melting curve analysis was made after amplification to distinguish the targeted PCR. Standard curve was created using serial 10-fold dilutions of *A. muciniphila* pure culture DNA corresponding to 10^2 to 10^{10} gene fragment numbers. The bacterial concentration of each sample was calculated by comparing the threshold cycle (Ct) values obtained from the standard curve, and expressed as log number of copies per mg of fecal sample.

Akkermansia muciniphila administration to mice

A. muciniphila strain DSM 22959^T (ATCC BAA-835) was initially grown in a previously described mucin-based medium (*60*). Subsequent cultures and assays were done in BHI:MRS-C medium consisted in BHI (80% vol/vol; Oxoid, Basingstoke, UK), MRS (20% vol/vol; Oxoid): (80%:20% vol/vol; Oxoid) supplemented with 0.10% (w/v) cysteine (Sigma-Aldrich). Cultures were incubated at 37°C in anaerobic chamber (H_2/CO_2/N_2 10%/10%/80%; vol/vol). Counts were obtained on BHI:MRS-C agar (Oxoid) incubated in anaerobic chamber at 37°C for 48h. Pasteurized pellet was prepared following

(*28*) with minor modifications. Briefly, cells grown on BHI:MRS-C broth were washed with PBS and concentrated in anaerobic conditions. Pellets were pasteurized at 70°C during 30 minutes and frozen immediately at -80°C. Cells obtained were quantified by qPCR using specific primers (Table S2). A dose of 10^8 CFU in PBS (or the equivalent volume of PBS for controls) was administered by gavage to suckling pups three times per week starting on day 11 until weaning (day 21).

Statistical methods

Demographic and physiologic characteristics of the human subjects are shown as mean (SD). Normality was tested using the Shapiro-Wilk test and non-normal distributed variables were log-transformed before further analysis. Associations between variables were assessed by standard least squares multivariate linear regression with change in WLZ as dependent variable and log-transformed milk metabolite concentration as independent variable. Model 1 was constructed with gestational age and WLZ at birth as covariates, and Model 2 further adjusting for pre-pregnancy BMI, gestational weight gain, and birth method. For mouse experiments, unless otherwise stated, data are presented as mean (sem). Two-tailed *t*-test, Mann-Whitney U test (for non-normal distribution), or one-way ANOVA (for more than two groups) were applied to analyze differences between groups. A $p<0.05$ was considered statistically significant. Statistical analyses were implemented in JMP® v14 (SAS Institute Inc., Cary, NC).

Supplementary Materials

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Author contribution: SR, DS-I, JCJ, and CL designed the experiments, analyzed data, and wrote the manuscript. DAF, EWD, and EI provided the milk samples and infant data from Cohort I, and IG-M and MCC provided the milk samples and infant data from Cohort II, and assisted in data analysis. Milk samples were analyzed by MT, EA, and TB. LM, MR, OO, and JC assisted with mouse experiments. AF and MV performed and analyzed indirect calorimetry and MRI. PG analyzed 16S sequencing data. MN and JS performed ileal histology studies. AG and FV assisted in the pAkk experiment. EC performed *in vitro* microbial growth experiments. All authors participated in manuscript editing and approved the final version.

Competing interests: MR, JCJ, and CL are inventors in patent application 17382270.1-1466 entitled “Betaine for the prevention of obesity”, filed in the European Patent Office (May 12, 2017). EC is an employee of Biópolis S.L.-ADM (Spain). There are no other competing interests.

Data and materials availability: 16S sequence reads will be available at the European Nucleotide Archive (EMBL-EBI) upon manuscript acceptance.

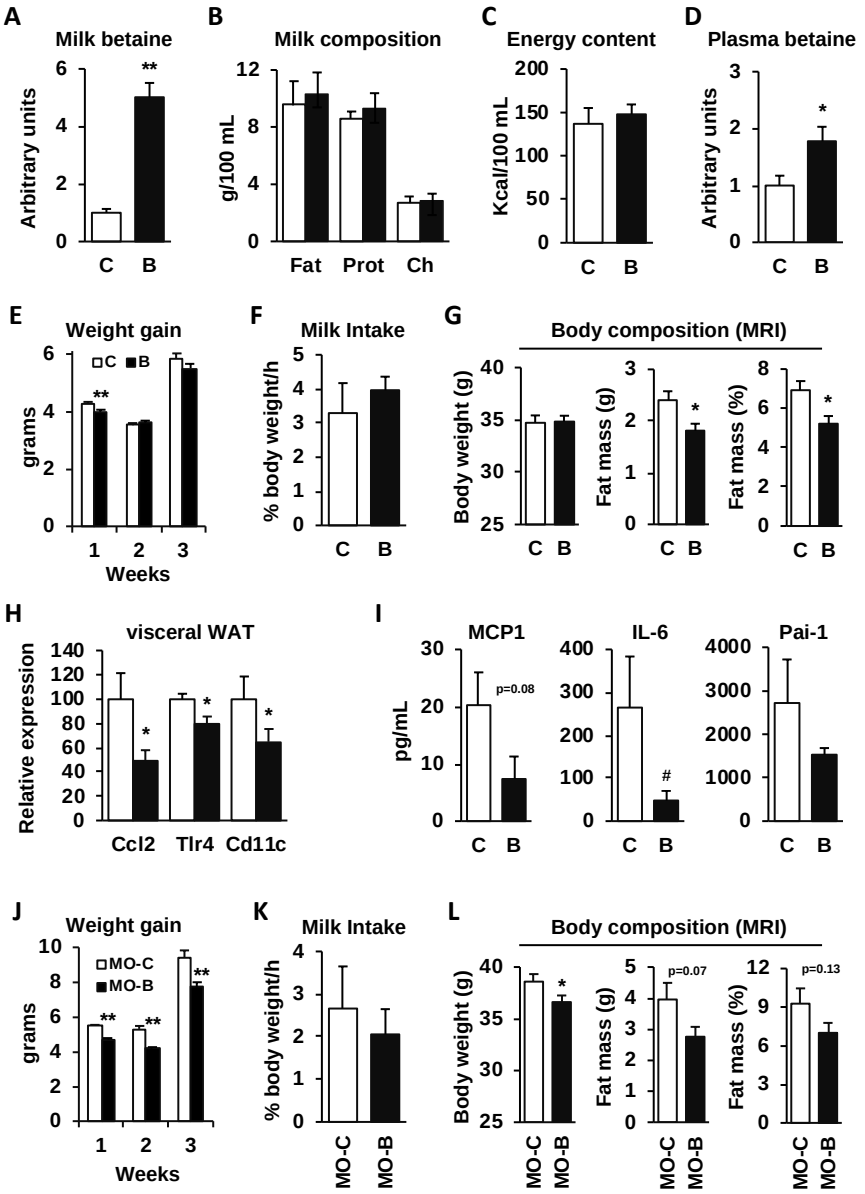

Figure 1. Maternal betaine supplementation decreases adiposity in young offspring.

A) Betaine relative levels in milk from control (white bars) and betaine-treated (black bars) dams (n=8/group). **B**) Milk macronutrient composition and **C**) energy content (n=5 per group); Ch, carbohydrates; Prot, protein. **D**) Plasma betaine levels in 2-week-old mice (n=6 per group). **E**) Offspring weight gain during lactation (n=36 per group). **F**) Milk intake at 1 week of age (pups grouped in litters, with n=4 litters per group). **G**) Body composition in 6-week-old mice from control (n=7) or betaine-treated dams (n=9). **H**) mRNA levels of vWAT pro-inflammatory markers in 6-week-old males (n=8-10 per group). **I**) Plasma levels of pro-inflammatory markers (n=10 per group). **J**) Weight gain during each week of lactation of offspring from obese control (MO-C, n=19) or betaine-treated (MO-B, n=21) dams. **K**) Milk intake at 1 week of age (pups grouped in litters, with n=3 litters per group). **L**) Body composition in 6-week-old mice (n=8 per group). Data are mean ± sem. *, *t*-test p<0.05; **, p<0.0001.

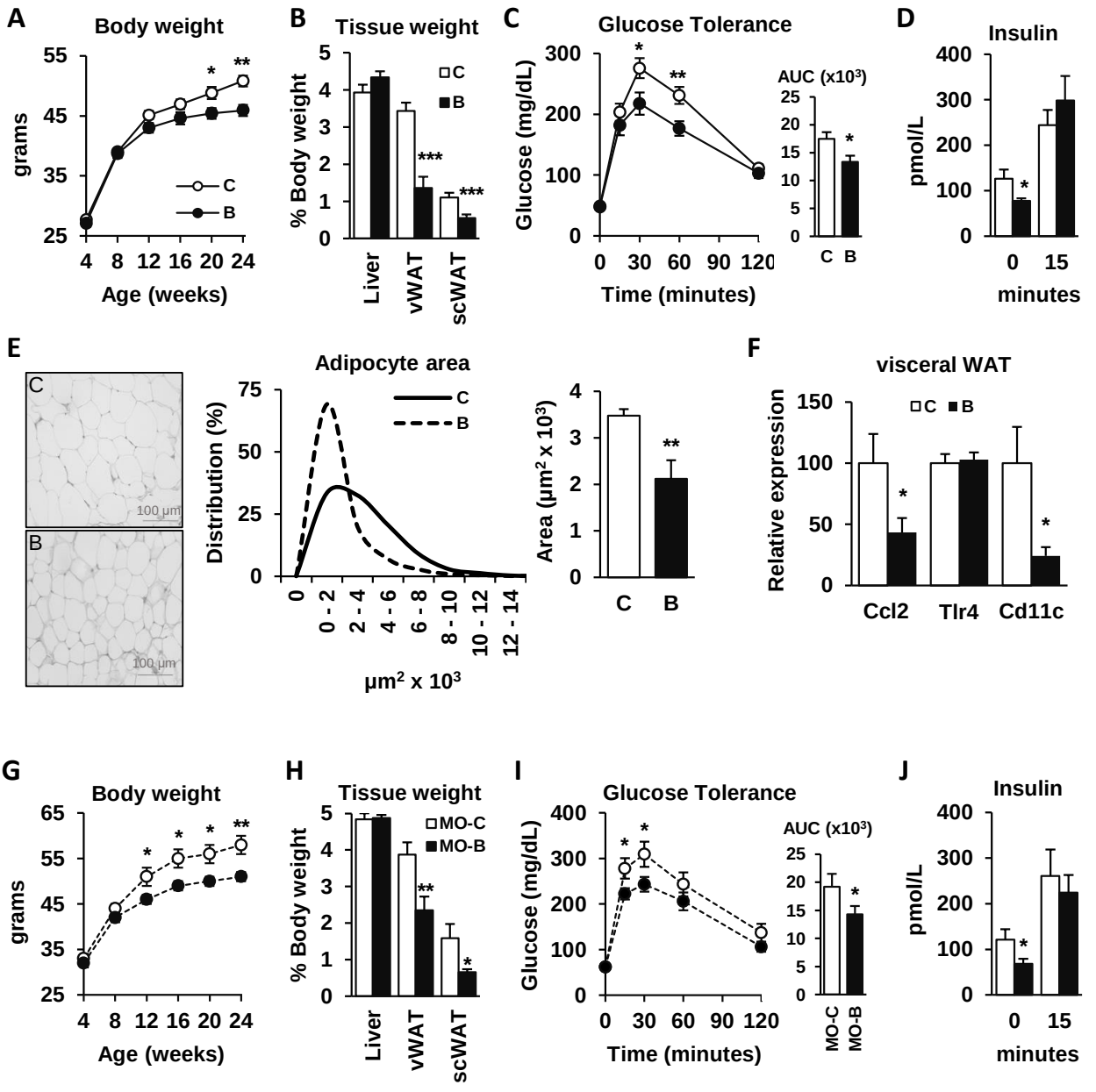

Figure 2. Maternal betaine improves offspring long-term metabolic health.

A-**F**) Offspring from control (white bars/circles) or betaine-treated dams (black bars/circles) were fed a chow diet after weaning (n=16 per group); **A**) Body weight throughout adulthood; **B**) Liver, vWAT and scWAT weight at sacrifice; **C**) Glucose tolerance and **D**) insulin levels at 0' and 15' after glucose load in 20-week-old males; **E**) vWAT adipocyte area and **F**) mRNA levels of pro-inflammatory markers (n=8 per group). **G-J**) Offspring from obese control (MO-C, n=11) and betaine-treated (MO-B, n=13) dams were fed a chow diet after weaning; **G**) body weight was monitored until adulthood; **H**) Liver, vWAT and scWAT weight at sacrifice; **I**) Glucose tolerance and **J**) insulin levels at 0' and 15' minutes after glucose load in 20-week-old offspring. AUC, area under the curve. Data are mean ± sem. *, *t*-test p<0.05; **, p<0.01; ***, p<0.001.

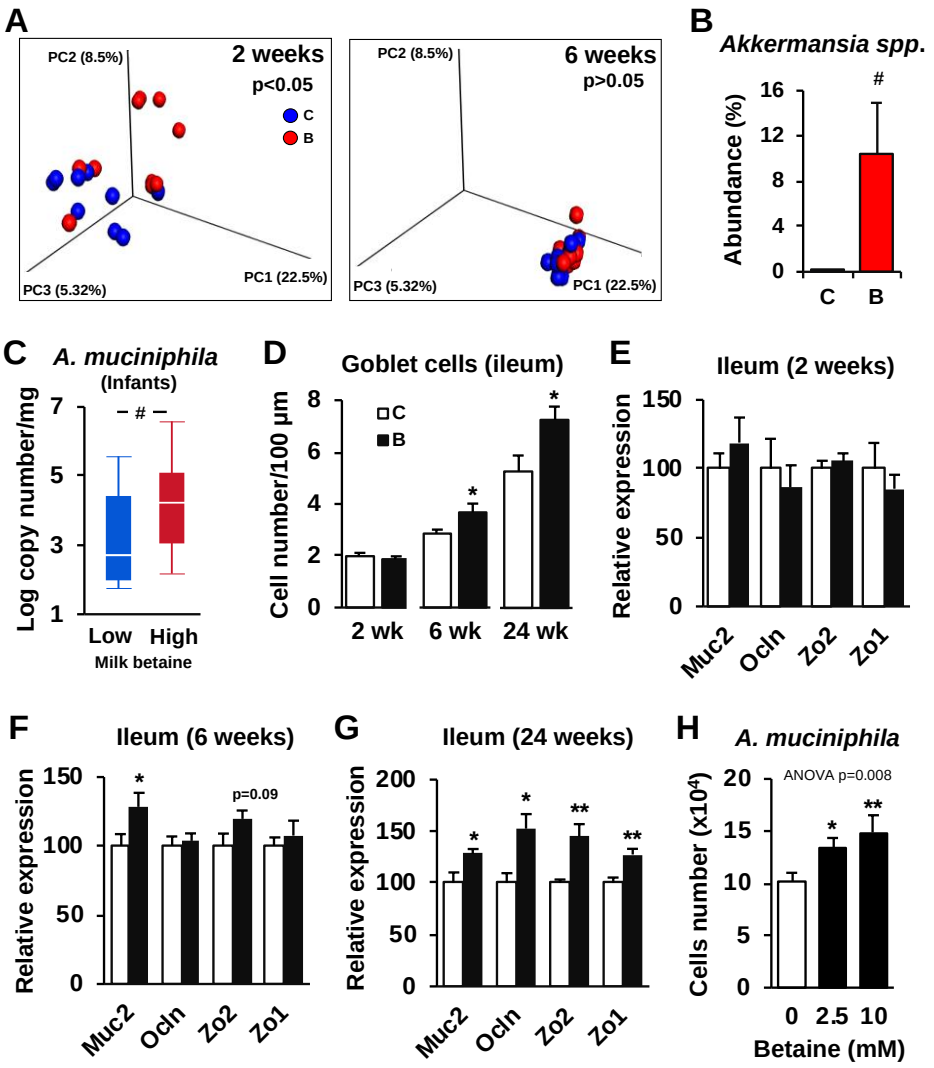

Figure 3. Maternal betaine supplementation increases Akkermansia abundance during early life.

A) Principal coordinate analysis of unweighted UniFrac distances of cecal microbiota from 2- and 6-week-old offspring from betaine-treated (B, red circles) or control dams (C, blue circles); p value assessed by adonis test (n=10/group). **B**) Relative abundance of *Akkermansia* spp. in cecal samples from 2-week-old mice. **C**) Box-plot of *A. muciniphila* levels in fecal samples from 12-month-old infants (n=40); infants were categorized into Low- (blue) and High-exposure (red) groups based on milk betaine median values. **D**) Goblet cell number in ileum sections from 2- (n=10/group), 6- (n=16/group), and 24-week-old (n=6-7/group) control (white bars) and betaine-treated mice (black bars). **E**) Ileal mRNA levels of intestinal barrier markers at 2 weeks (n=10/group), **F**) 6 weeks (n=8/group), and **G**) 24 weeks of age (n=8-10/group). **H**) *A. muciniphila* was grown *in vitro* in the absence (white bars) or presence (black bars) of 2.5 or 10 mM betaine until reaching stationary phase (25h of growth); cell number was quantified by qPCR (n=3, mean ± SD; one-way ANOVA with post-hoc Dunnett's test). Data are mean ± sem. *, p<0.05; **, p<0.01 vs. control. #, Mann-Whitney U test p<0.05.

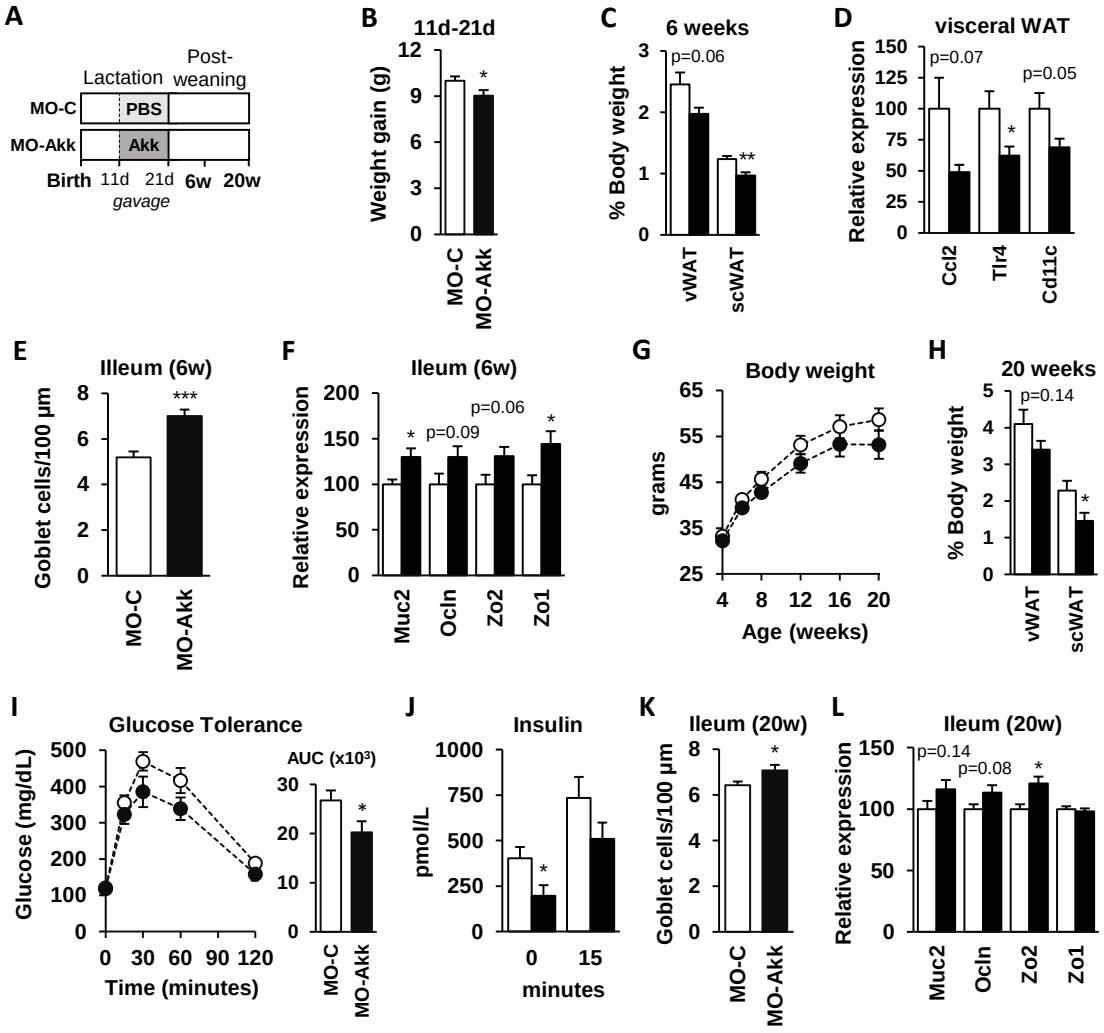

Figure 4. Early-life *Akkermansia muciniphila* exposure improves long-term metabolic health.

A) Eleven-day-old males from obese dams were administered PBS (MO-C, white bars) or pAkk (MO-Akk, black bars) thrice weekly until day 21 (n=16 per group) and sacrificed at 6 and 20 weeks of age (n=8 per group for each time point). **B**) Weight gain through days 11-21 (n=16 per group). **C**) WAT weight and **D**) vWAT mRNA levels at 6 weeks (n=8 per group). **E**) Goblet cell number and **F**) mRNA levels in ileum at 6 weeks (n=8 per group). **G**) Body weight and **H**) WAT weight at 20 weeks (n=8 per group). **I**) Glucose tolerance and **J**) insulin levels at 0' and 15' after glucose load at 18 weeks. **K**) Goblet cell number and **L**) mRNA levels in ileum at 20 weeks. AUC, area under the curve. Data are mean ± sem. *, *t*-test p<0.05; **, p<0.01; ***, p<0.005.

	Cohort I	Cohort II
MOTHER	n=34	n=109
Age (years)	29.1 (5.1)	32.8 (6.2)
Pre-pregnancy BMI (kg/m^2)	27.3 (7.1)	23.5 (3.8)
Gestational weight gain (kg) [a]	12.0 (7.4)	12.1 (4.7)
INFANT		
Birth	n=34	n=109
Gender (female/male)	15 / 19	60 / 49
Gestational age (weeks)	39.6 (1.2)	39.6 (1.1)
Weight (kg)	3.53 (0.48)	3.35 (0.44)
Length (cm)	51.6 (2.3)	50.1 (2.2)
Weight-for-length z score	-0.59 (1.2)	-0.20 (1.15)
1 month	n=34	n=109
Weight (kg)	4.67 (0.71)	4.23 (0.57)
Length (cm)	55.8 (2.1)	54.5 (2.3)
Weight-for-length z score	-0.36 (1.04)	-0.50 (1.17)
12 months		n=107
Weight (kg)	-	9.46 (1.04)
Length (cm)	-	74.8 (2.9)
Weight-for-length z score	-	0.18 (0.87)
MILK METABOLITES	n=34	n=109
S-adenosylmethionine (nmol/L)	1469 (436)	2034 (649)
S-adenosylhomocysteine (nmol/L)	216 (81)	327 (184)
Methionine (µmol/L)	4.39 (2.69)	6.74 (12.36)
Cystathionine (nmol/L)	44.9 (34.3)	129.6 (75.7)
Betaine (µmol/L)	3.12 (2.65)	5.33 (6.13)
Choline (µmol/L)	97 (49)	165 (115)

Table 1. Demographic and metabolic characteristics of mother-infant dyads from Cohorts I and II.

Values represent mean (SD). [a], n=32 for gestational weight gain data in Cohort I.

	COHORT I				COHORT II							
	WLZ 1 month				WLZ 1 month				WLZ 12 months			
	Model 1 n=34		Model 2 n=32		Model 1 n=109		Model 2 n=109		Model 1 n=107		Model 2 n=107	
	B (CI 95%)	p val	B (CI 95%)	p val	B (CI 95%)	p val	B (CI 95%)	p val	B (CI 95%)	p val	B (CI 95%)	p val
SAM	0.09 (-1.08 to 1.25)	0.879	-0.12 (-1.35 to 1.11)	0.841	0.52 (-0.03 to 1.07)	0.066	**0.55 (0.01 to 1.10)**	**0.047**	0.07 (-0.39 to 0.53)	0.765	0.01 (-0.45 to 0.48)	0.964
SAH	-0.95 (-1.91 to 0.01)	0.053	-0.88 (-1.86 to 0.10)	0.077	0.10 (-0.20 to 0.39)	0.525	0.12 (-0.17 to 0.42)	0.407	-0.02 (-0.26 to 0.22)	0.848	-0.06 (-0.31 to 0.18)	0.609
Met	0.10 (-0.59 to 0.79)	0.772	0.39 (-0.36 to 1.14)	0.298	**-0.57 (-0.87 to -0.26)**	**0.001**	**-0.51 (-0.81 to -0.21)**	**0.001**	-0.22 (-0.47 to 0.04)	0.094	-0.18 (-0.44 to 0.08)	0.182
Cysta	-0.33 (-0.68 to 0.02)	0.061	-0.35 (-0.71 to 0.02)	0.061	-0.18 (-0.57 to 0.2)	0.341	-0.22 (-0.59 to 0.16)	0.253	-0.18 (-0.48 to 0.13)	0.258	-0.17 (-0.48 to 0.14)	0.266
Bet	**-0.66 (-1.18 to -0.14)**	**0.015**	**-0.60 (-1.14 to -0.07)**	**0.028**	**-0.56 (-0.91 to -0.21)**	**0.002**	**-0.47 (-0.83 to -0.11)**	**0.010**	**-0.38 (-0.67 to -0.1)**	**0.009**	**-0.34 (-0.64 to -0.04)**	**0.026**
Cho	-0.23 (-0.92 to 0.46)	0.503	-0.26 (-1.03 to 0.51)	0.497	-0.28 (-0.67 to 0.10)	0.148	-0.23 (-0.61 to 0.15)	0.237	-0.21 (-0.53 to 0.11)	0.190	-0.17 (-0.49 to 0.15)	0.287

Table 2. Multivariate regression between milk metabolite concentrations and infant growth.

Least-square regression models were applied to assess the correlation between milk metabolite levels and WLZ change from birth to 1 month or 12 months. B, size effect estimate from the regression model; CI, confidence interval; SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; Met, methionine; Cysta, cystathionine; Bet, betaine; Cho, choline. Model 1, adjusted for gestational age and WLZ at birth; Model 2, model 1 further adjusted by pre-pregnancy BMI, gestational weight gain, and mode of birth. Bold font indicates $p<0.05$.

Supplemental Figure S1

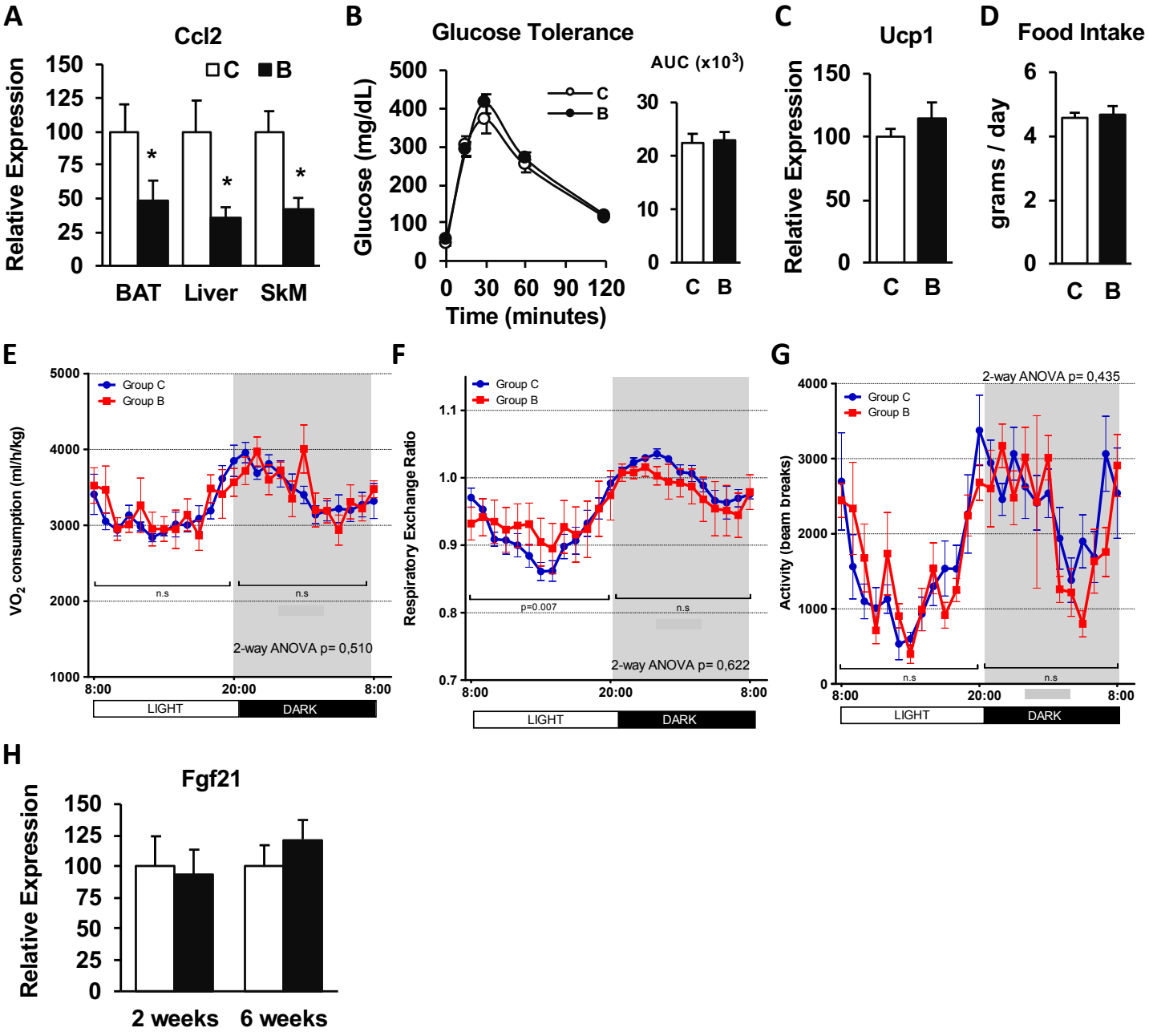

Figure S1. Effect of maternal betaine administration on young offspring.

A) Ccl2 mRNA levels in brown adipose (BAT), liver, and skeletal muscle (SkM) (n=8-10 per group) from control (C, white bars/circles) and betaine-treated (B, black bars/circles) dams. **B**) Glucose tolerance was measured in 8-week-old male offspring (n=12). **C**) Ucp1 mRNA levels in BAT (n=8-10 per group). **D-G**) Food intake, oxygen consumption, respiratory exchange ratio, and activity levels were monitored in metabolic chambers at 6 weeks of age (n=7 and 9 for C and B). **H**) FGF21 mRNA levels in liver from 2- and 6-week-old mice. Data are mean ± sem. *, *t*-test p<0.05

Supplemental Figure S2

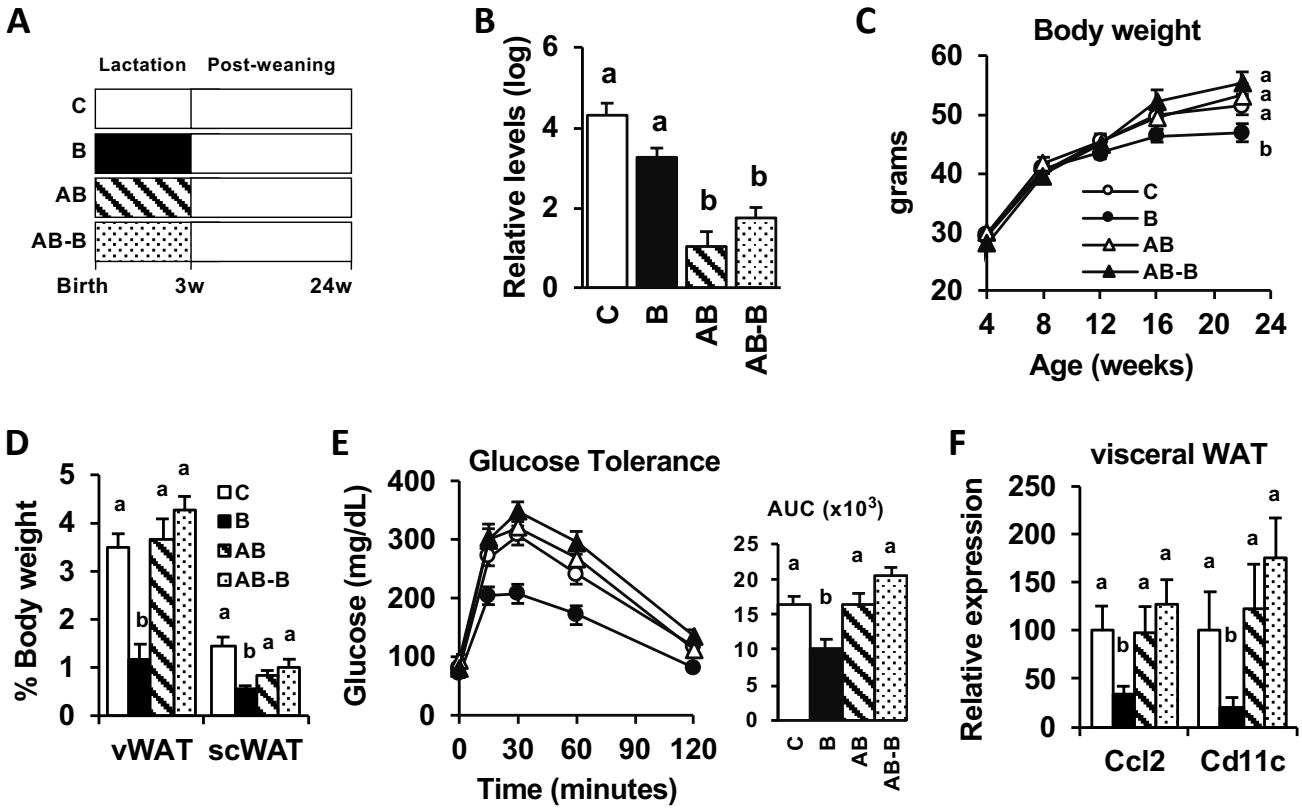

Figure S2. Effects of maternal antibiotic co-administration on offspring long-term metabolic health. Dams were treated with betaine (1%) (B group, black bar), ampicillin (1g/L) and neomycin (0.5 g/L) (AB group, hatched bar), antibiotics and betaine (AB-B group, dotted bar) in the drinking water, or with no supplement (C group, white bar) during lactation. Mice were sacrificed at 2 weeks of age (n=8 from 1 litter per group). Bacterial DNA was extracted from cecal samples and levels of eubacteria 16S gene was determined by qPCR and expressed as log-transformed relative levels per gram of cecal sample. Data are mean ± sem. Different letters indicate significant differences between groups after one-way ANOVA and post-hoc Tukey test ($p<0.05$) .

Supplemental Figure S3

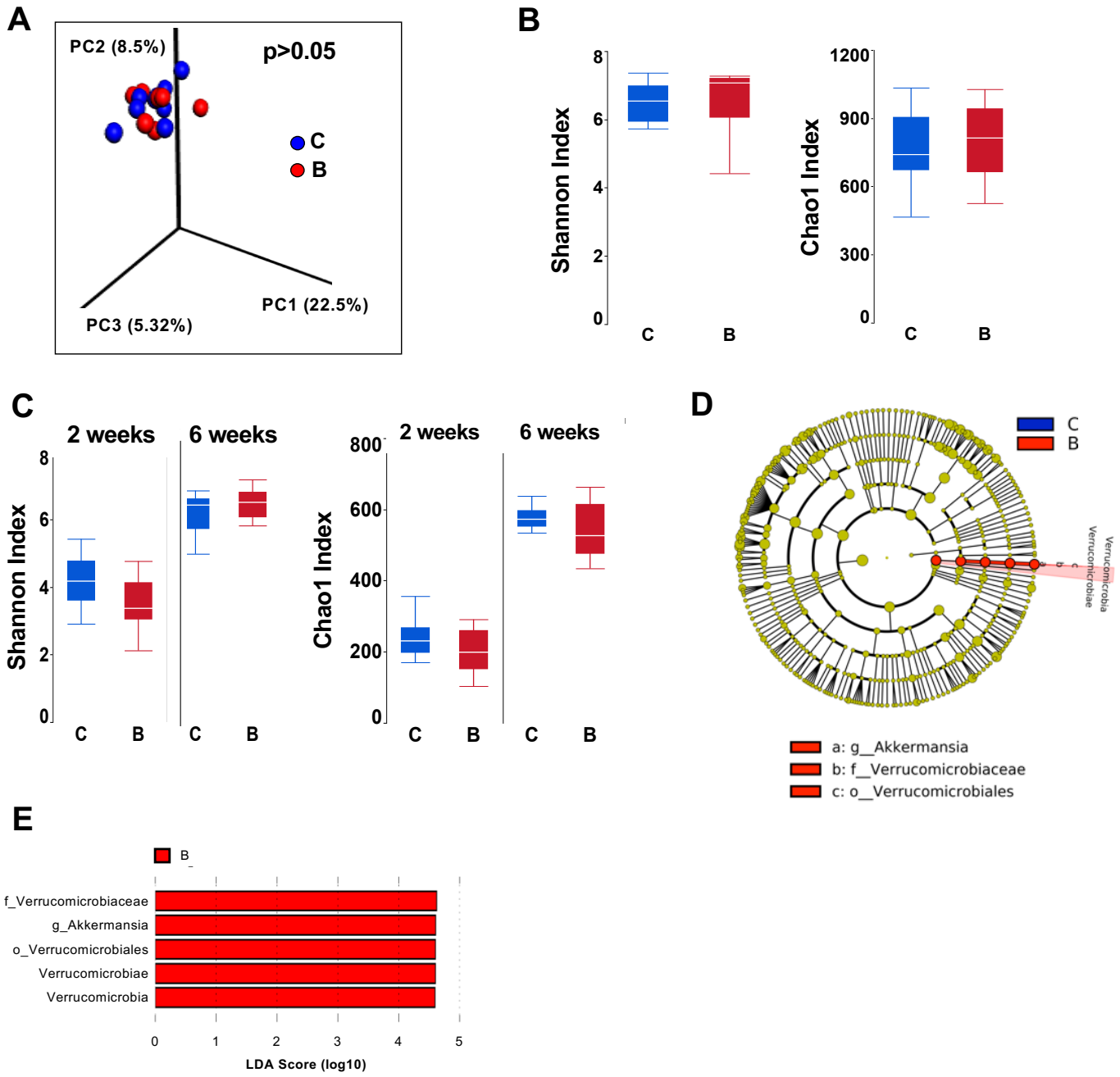

Figure S3. Effect of betaine administration on the maternal and offspring microbiome. Betaine-treated (B group, red circles) or control (C group, blue circles) dams (n=8 per group), and offspring (n=10) were sacrificed at day 14 after delivery, and at 6 weeks of age (n=10). **A)** Principal coordinate analysis of unweighted UniFrac distances of cecal samples; p value assessed by adonis test. **B**) Diversity Shannon and Chao1 indexes from C and B dams. **C)** Diversity Shannon and Chao1 indexes from offspring at 2 and 6 weeks of age. **D**) Cladogram and **E**) LDA scores arising from LEfSe analysis in 2-week-old pups from betaine-treated and control groups.

Supplemental Figure S4

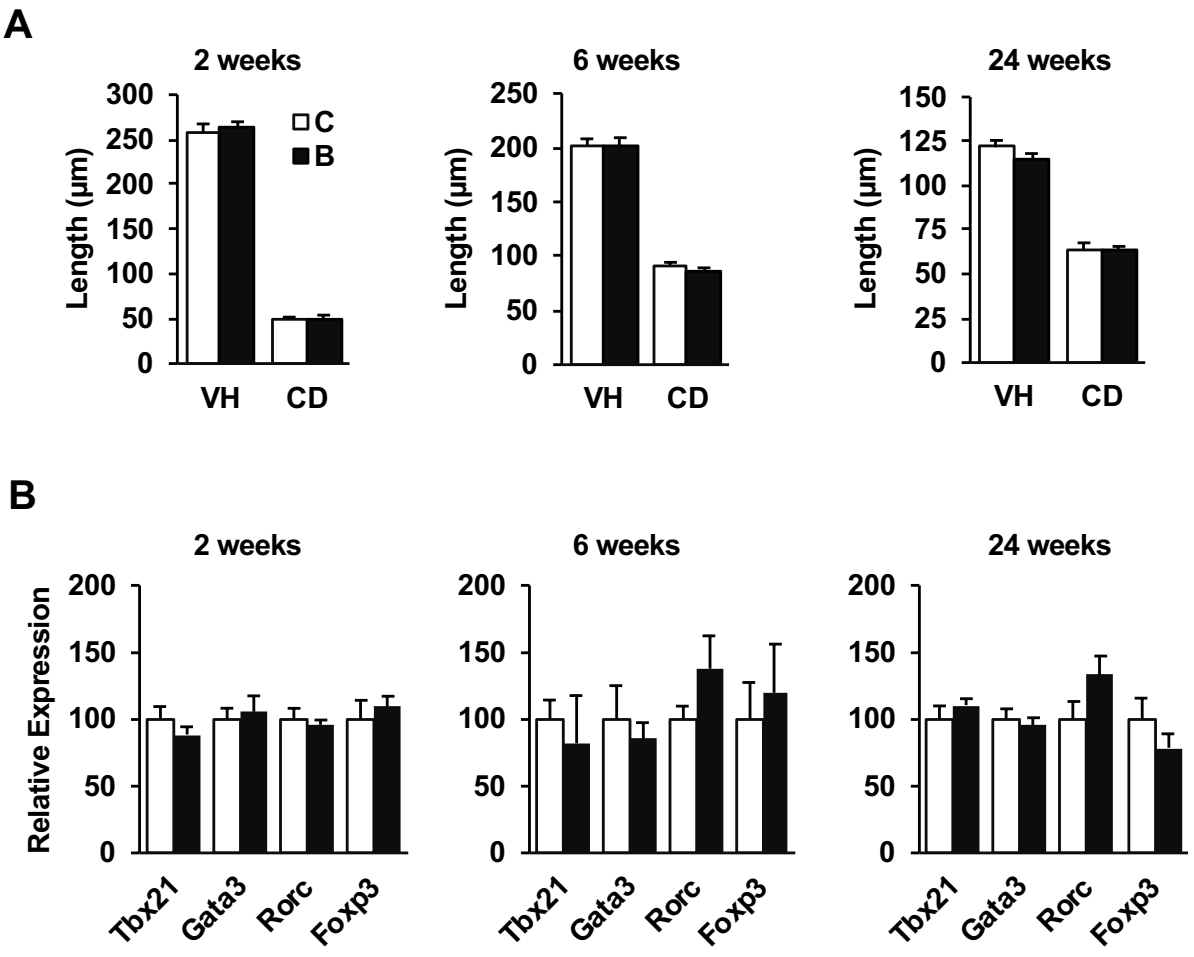

Figure S4. Effect of maternal betaine supplementation on ileum histology and gene expression. **A**) Ileal vili height (VH) and crypt depth (CD) in ileum sections from 2- (n=10 per group), 6- (n=16 per group), and 24-week-old (n=6-7 per group) control (white bars) and betaine-treated mice (black bars). **B**) Ileal mRNA levels of T cell differentiation markers at 2 weeks (n=10/group), 6 weeks (n=8/group), and 24 weeks of age (n=8-10/group). Data are shown as mean ± sem and group differences were assessed by Student's *t*-test.

Supplemental Figure S5

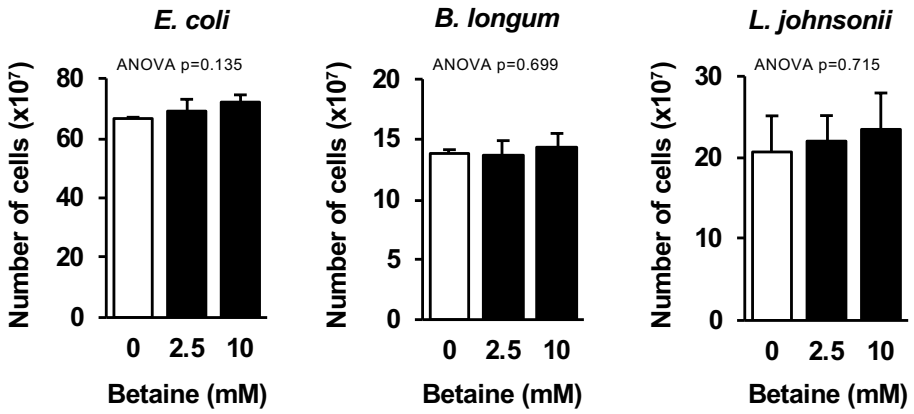

Figure S5. Effect of betaine on bacterial growth *in vitro*. *Escherichia coli*, *Bifidobacterium longum* and *Lactobacillus johnsonii* were grown *in vitro* in the absence (white bars) or presence (black bars) of 2.5 mM or 10 mM betaine in the media until reaching the stationary phase (n=3). Cell number for the indicated bacterial species was determined based on optical density (600 nm) after 8h, 12h, or 15h of growth, respectively. Data are mean ± SD. One-way ANOVA was applied to compare the different groups.

Supplemental Table S1

	Cohort I		Cohort II	
	B (CI 95%)	***P* value**	**B (CI 95%)**	***P* value**
Change in body length z score				
Birth - 1 month	0.01 (-0.57, 0.58)	0.982	0.22 (-0.07, 0.51)	0.138
Birth - 12 months	---		-0.04 (-0.37, 0.30)	0.826
Change in head circumference (cm)				
Birth - 1 month	-0.01 (-0.67, 0.65)	0.973	-0.14 (-0.51, 0.24)	0.478
Birth - 12 months	---		-0.37 (-0.91, 0.15)	0.162

Table S1. No association between milk betaine concentration and change in infant body length z score and head circumference. Least-square regression modelling was applied to assess the correlation between milk betaine level (independent variable) and body length z score change from birth to 1 month or 12 months (dependent variable). Model was adjusted for gestational age, pre-pregnancy BMI, gestational weight gain, birth method, and body length z score at birth (for change in body length) or head circumference at birth (for change in head circumference). B, size effect estimate from the regression model; CI, confidence interval.

Supplemental Table S2

Gene	Forward	Reverse
Hprt	5'-GCCCCAAAATGGTTAAGGTTG-3'	5'-GTCAAGGGCATATCCAACAAC-3'
Muc2	5'-CTGACCAAGAGCGAACACAA-3'	5'-CATGACTGGAAGCAACTGGA-3'
Ocln	5'-ATGTCCGGCCGATGCTCTC-3'	5'-CTTTGGCTGCTCTTGGGTCTGTAT-3'
Zo2	5'-CTAGACCCCCAGAGCCCCAGAAA-3'	5'-TCGCAGGAGTCCACGCATACAAG-3'
Zo1	5'-TTTTGACAGGGGGAGTGG-3'	5'-TGCTGCAGAGCTCAAAGTTCAAG-3'
Ccl2	5'-CAAGATGATCCCAATGAGTAG-3'	5'-TTGGTGACAAAAACTACAGC-3'
Tlr4	5'-GCCTCCCTGGCTCCTGGCTA-3'	5'-CAGGGACTTTGCTGAGTTTCTGATCCA-3'
Cd11c	5'-AGTCTGTTGGGTTCTGTAAG-3'	5'-ACAGTTCTGTTATGACATGC-3'
Fgf21	5'-AGCTCTCTATGGATCGCCTCACTT-3'	5'-ACACATTGTAACCGTCCTCCAGCA-3'
Tbx21	5'-ACGTCTTTACTTTCCAAGAG-3'	5'-GTACATGGACTCAAAGTTCTC-3'
Gata3	5'-TATTAACAGACCCCTGACTATG-3'	5'-CACCTTTTTGCACTTTTTCG-3'
Rorc	5'-CTGTGTTTTTCTGAGGATGAG-3'	5'-GCAGAGATGATGATGGAAAG-3'
Foxp3	5'-AATAGTTCCTTCCCAGAGTTC-3'	5'-GGTAGATTTCATTGAGTGTCC-3'
A. muciniphila	5'-CAGCACGTGAAGGTGGGGAC-3'	5'- CCTTGCGGTTGGCTTCAGAT-3'

Table S2. Primer sequences for qPCR analyses.