Summary

Microbiome perturbations with antibiotics have been reported to affect bone phenotypes (Cho et al., 2012; Cox et al., 2014; Ohlsson & Sjögren, 2015; Sjögren et al., 2012; Yan & Charles, 2017; Yan et al., 2016). Surgical birth, is another stressor of the normal transfer of primordial microbes to the offspring, and it has been connected to accelerated growth in mice (Martinez et al., 2017). However, the effect of surgical birth on bone phenotypical changes is unclear. We hypothesize that surgical birth, interferes with offspring skeletal development, increasing bone mineral content and density, and that the effect is mediated by the microbiome. To test this hypothesis, we determined the effect of surgical birth and restoration with maternal microbes on bone phenotypes. We separated mice into three groups: control vaginal group, surgical birth group, and surgical birth with maternal microbial restoration group. All litters were fostered. We measured body weight weekly. At 6 and 18-weeks and whole-body composition was measured, as well as bone mineral density and bone mineral content (whole body, both sides of femur, both sides of humerus, both sides of tibia, and spine-vertebrae L1 to L6). Surgically born females had increased weight gain during weeks 4-10 (p<0.05) and showed a non-statistically significant trend to higher spine bone mineral density (p=0.064). Restoration after surgical birth, which indicates a microbiome-mediated effect, normalized weight phenotype but
increased whole-body fat percentage (p=0.029) among young females. It also showed a trend to increase whole-body bone mineral content among young males (p=0.054). The non-significant trends in changes in skeletal development should be further examined by to increase the number of animals for greater statistical power. The results supported the hypothesis that microbes interfere with developmental growth.
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As diverse microbiota exists in the human gastrointestinal tract, it maintains the homeostasis which is essential to the regulation of digestive system, immune system, and endocrine system (Cani & Knauf, 2016; Wu & Wu, 2012). The impact of gut microbiota on bone phenotypical changes has been intensively investigated as microbiome related research advanced in the past few years. In recent years, it has been found that compositional disruption on gut microbiome could lead to changes on bone phenotype (Cho et al., 2012; Claes Ohlsson, 2014; Cox et al., 2014; Ibáñez, Rouleau, Wakkach, & Blin-Wakkach, 2019; Ilseung Cho, 2012; Sjögren et al., 2012).

Gut microbiome could be affected by many factors, including gene, nutrients absorption, vitamin synthesis, and gut microbiome composition (Ibáñez et al., 2019). Early life antibiotics exposure and surgical birth are two of the common ways to interfere with subjects’ gut microbiome composition in the experiments. There are studies showing that early life antibiotics exposure could lead to higher bone mineral content and density in mice, and bigger bone area in female mice (Cho et al., 2012; Cox et al., 2014; Nobel et al., 2015). The vaginal-perineal microbiota is acquired by the newborns during vaginal birth (Dominguez-Bello et al., 2010). Surgical birth bypasses this exposure and leads to microbiome changes (Martinez et al., 2017). Studies have found that surgical birth are associated with accelerated weight gain in mice (Martinez et al., 2017). Although there is evidence suggesting an involvement of microbes in developmental growth, studies on how surgical birth would affect bone phenotype are rare. This study is aiming to find the effect of surgical birth on bone phenotype by comparing the offspring born by surgical birth and
vaginal birth. This study would potentially provide data on how different birth mode would affect skeletal developmental growth in mice.
2- Background

The Microbiome

Humans, as plants and animals, have coevolved with microbes (Achtman et al., 1999; Ghoul & Mitri, 2016; Katz & Bhattacharya, 2008). Prokaryotic microorganisms are unique, as they have colonized every kind of environment on Earth. Some microorganisms are even able to live in extreme environments (Pikuta, Hoover, & Tang, 2007; L. J. Rothschild & Mancinelli, 2001). The microbiome also exists in epithelial surfaces of the human body, including the mouth, skin, and gut (Costello et al., 2009; Huttenhower et al., 2012; Methé et al., 2012). Most of the microbiota biomass is in the large intestine, containing 2-4 million genes. This is more than 100 times of a person’s own genes given people have about 20,000 to 25,000 genes, and about 10 times the total number of human cells (Sender, Fuchs, & Milo, 2016). In mammals, microbes are passed onto the offspring during vaginal birth (Dominguez-Bello et al., 2010), and this first inoculum provides the pioneer bacteria that colonize all body sites of the baby. After birth, infants could acquire microbiome from the environment and other humans (Chen, He, & Huang, 2014). The composition of gut microbiomes varies through life span depending on various factors, including age, host genetics, diet, host immune system, and medication intake etc. (Chaudhari et al., 2020; Ibáñez et al., 2019).

In recent years, more and more studies have focused on effects of gut microbiomes on developmental changes. Gut microbiomes are well known for their digestive abilities, as they can convert fibers and polysaccharides into absorbable nutrients (Ibáñez et al., 2019). Recent studies have shown that the gut microbiome could also affect immune, endocrine, nervous and vascular systems, all of which regulate bone development.
(Gopalakrishnan et al., 2018; Ibáñez et al., 2019; Mayer, Savidge, & Shulman, 2014; Ohlsson & Sjögren, 2015; Tilg & Kaser, 2011). The gut microbiome dynamically interacts with their hosts and these interactions could be affected by dysbiosis (abnormal, unhealthy microbiota structure). Comparing with adults’ gut microbiota, infants have lower alpha diversity in their gut microbiota, and the gut microbiota will have adult level alpha diversity with temporal changes during a maturation period in 3 years (Bokulich et al., 2016; Yatsunenko et al., 2012). An infant’s maturing ecosystem is unstable and can easily being perturbed (Ventura et al., 2009). The microbiota plays key physiological functions throughout life, such as inducing immune tolerance (Nutsch et al., 2016), modulating the development of the immune and metabolic systems (Petersen et al., 2019), and promoting systemic T-cell survival (Soto et al., 2017).

**Early microbiome disruptions and later risk of disease**

Physiological issues may be caused by impairment in gut microbiome transmission and colonization (Ibáñez et al., 2019; Turnbaugh & Gordon, 2009). For example, disruptions in of gut microbiota associated with cesarean delivery may last throughout childhood (Huh et al., 2012), and it may cause immune-related chronic diseases such as obesity and allergies (Eggesbø, Botten, Stigum, Nafstad, & Magnus, 2003; Huh et al., 2012; Sevelsted, Stokholm, Bønnelykke, & Bisgaard, 2015). These are the same diseases associated with the early use of antibiotics (Lamont, Møller, & Stener Jørgensen, 2020). Certain types of antibiotics can increase the risk of developing different types of chronic diseases (Deng, Li, & Zhao, 2019; Heinsen et al., 2015; Kabbani et al., 2017; Zaura et al., 2015; Zimmermann & Curtis, 2019).
Antibiotics are able to change gut microbiome composition (Zimmermann & Curtis, 2019). Amoxicillin, cephalosporins, macrolides, clindamycin, and quinolones reduce abundance of \textit{E. coli} and increase bacterial abundance of other Enterobacteriaceae (Finegold et al., 1987; Floor et al., 1994; Stark et al., 1996; Zimmermann & Curtis, 2019). These compositional changes could affect host immunity through various ways, such as increasing in antibiotic-resistance genes (Deng et al., 2019) and increasing susceptibility to certain diseases (Looft & Allen, 2012).

**Bone and growth**

Bone is a dynamically active tissue that maintains its mineralization balance and its structural integrity through continuous remodeling (Florencio-Silva, Sasso, Sasso-Cerri, Simões, & Cerri, 2015). Osteoblasts and osteoclasts are active throughout an individual’s life and are essential for bone modeling during growth and remodeling in mature bone. Under normal circumstances, the process of bone formation and resorption are carried out continuously and systematically, and their balance is the key to bone growth and for maintaining normal bone mass and bone density (Feng & Teitelbaum, 2013; Novack & Mbalaviele, 2016).

Normal growth and skeletal development are associated with many factors. Bone growth starts in utero and bone mineral acquisition during intrauterine growth is modulated by factors such as maternal smoking, diet (particularly vitamin D deficiency), and physical activity (Cooper et al., 1995; Mulligan, Felton, Riek, & Bernal-Mizrachi, 2010; Parviainen, Auvinen, Pokka, Serlo, & Sinikumpu, 2017). During childhood and puberty, skeletal development, is also affected by nutrition, mechanical stimulations, but also by other
environment factors (Bilezikian, 2018). Skeletal development during childhood and puberty are both dominated by bone formation with accelerated bone turnover, which results in subtle increases in bone mass (Matkovic, 1996). The increase in height becomes more and more apparent during adolescence because during this period, bones develop much faster at both axial and appendicular sites (Bilezikian, 2018; Grave & Brown, 1976). At the end of puberty, the epiphysis on long bones is sealed and the individual stops growing. Female bones stop developing earlier than those of males, and is one reason the average height of adult women is shorter than that of men (Bilezikian, 2018). Bone mineral content and bone mineral density are two important indexes when measuring the growth of the bones in the experiments. Bone mineral content is measured in grams, and bone mineral density is a volumetric measure (bone mineral content is divided by area). Bone mineral content and bone mineral density are both valid indicators of early skeletal growth status (Kalkwarf, Zemel, Yolton, & Heubi, 2013). Poor childhood growth is directly linked to the later risk of hip fracture and therefore optimizing skeletal growth to achieve peak bone mineral density can be considered a preventive strategy against later life osteoporotic fracture.

**Increased growth in animals with early microbiome disturbances**

Increasing evidence suggests that alteration of gut microbiome composition would affect bone development. The gut microbiome is essential for normal somatic and skeletal growth in individuals with a normal diet (Guss et al., 2017; Poinsot, Schwarzer, Peretti, & Leulier, 2018; Yan & Charles, 2017; Zhang, Lu, Wang, Ren, & Han, 2018). The gut microbiome composition can be affected by antibiotics, which have been used to deplete
the microbiota and facilitate introducing other microorganisms into the gut (Yan & Charles, 2017). In mice, early life antibiotics exposure leads to changes in gut microbiota composition and host metabolism as well as phenotypical changes in skeletal development (Cho et al., 2012; Cox et al., 2014). Cho et al. (2012) and Cox et al. (2014) both studied how low-dose antibiotics affect mice bone phenotype. Cho et al. (2012) gave low dose penicillin, chlortetracycline, and vancomycin to 4-week-old mice for 3 and 7 weeks. They found that increased bone mineral density has increased with antibiotic treatment among which received treatment for 3 weeks but not for 7 weeks. Cox et al. (2014) also gave low-dose penicillin to mice a during their early developmental phase, but for 20 weeks, and found sex differences for the effect of antibiotics on bone mineral content, where male mice had lower bone mineral content, and female mice had higher bone mineral content after antibiotic treatment. Nobel et al. (2015) also confirmed that antibiotic-treated females had higher bone mineral content and bigger bones compared to males, especially the female group treated with amoxicillin. In addition to higher bone mineral content there was also bigger bone area, especially the group treated with amoxicillin.

As for most of the research results that have been reported on aspects of bone mineral content and bone mineral density, bone length is rarely mentioned in bone-antibiotics association experiments. This may suggest that antibiotics associated with phenotypical changes on bone mineral content and bone mineral density are more evident than ones on bone length, and the lengths of the bones do not correlate with strength as much as the data on bone mineral content and bone mineral density. Disturbances in gut microbiome by antibiotics can have an effect based on type of antibiotics, sex, and treatment duration. The mechanisms are unclear, but it is known that inflammation impairs
osteoblast function, which can increase bone mineral density. Tavakoli and Xiao (2019) demonstrated that increased bacterial load caused by antibiotics in mouse model of sickle cell disease can lead to inflammation, which in turn impairs osteoblast function, also increased bone mineral density.

**Microbiome immune modulation and bone phenotypes**

The compositional changes of gut microbes could influence bone remodeling activities by affecting immune modulations. In germ-free environments, the immune system is the central control of bone mineral density (Ibáñez et al., 2019). NFκB ligand (also known as RANKL) is the receptor activator that is responsible for activating osteoclasts and is normally produced in bone marrow. However, during inflammation, CD4+ T cells can also be a source of RANKL, which can stimulate osteoclastogenesis (Kong et al., 1999; Nakashima et al., 2011). In addition, Th17 cells also promote osteoclastogenesis in vitro, among CD4+ T cell subsets (Ibáñez et al., 2019). Recently, a study showed that segmented filamentous bacteria, a gut microbe, could promote Th17 cells production and impact skeletal development (Tyagi et al., 2021). The association between Th17 cells and osteoclasts has also been demonstrated in Crohn’s disease in both mice and humans (Ibáñez et al., 2019).

The microbiome can disrupt osteoclastogenesis pathways, and interfere with bone resorption (McGinty & Mallon, 2018). The gut microbiome has a close relationship with the activation of CD4+ T cells and the control of osteoclastogenic cytokine production. Sjögren et al. (2012) showed that bone density is regulated by the intestinal microbiota; they found that the absence of a gut microbiota is associated with reduced frequency of
CD4+ T cells and osteoclast precursor cells in bone marrow (Sjögren et al., 2012). Their results show that osteoclast function was affected in germ-free mice resulting in reduced bone resorption. The cancellous bone (trabecular or spongy bone) and the cortical bone (compact bone) density of female germ-free mice are higher than those of mice fed a normal diet (Sjögren et al., 2012). In addition, there is evidence showing that animals in germ-free conditions have lower numbers of Th17 cells in their spleen and spinal cords (Dobber, Hertogh-Huijbregts, Rozing, Bottomly, & Nagelkerken, 1992; Lee, Menezes, Umesaki, & Mazmanian, 2011; Wu et al., 2010). These data demonstrate that gut microbiota is actively regulating the immune system which regulates bone remodeling (Sjögren et al., 2012).

Recolonization of normal gut microbes will normalize the physiological changes on immune system and bone remodeling activities. When germ-free mice were colonized with a normal gut microbiota at three weeks of age, the bone mass, osteoclasts, CD4+ T cells, and osteoclast precursor cells produced by the bone marrow rise to normal levels (Sjögren et al., 2012). Expression of some inflammatory cytokines in the bone marrow of germ-free mice was also significantly reduced. These cytokines are essential at detecting bone damage and initiating inflammatory responses against infections. Therefore, alterations of the intestinal flora affect the formation of osteoclasts by interrupting the normal immune response within the bone microenvironment, which in turn can disrupt the normal regulation of bone metabolism and development (Sjögren et al., 2012).

Microbiome-mediated metabolic changes and bone phenotypes
Gut microbiomes have a close relationship to the host’s metabolic activities. Bäckhed et al. (2004) found that gut microbiome could promote absorption of monosaccharides and increase deposition of triglycerides in adipocytes, which could affect fat storage in the host. In Chevalier et al. (2015), they found that germ-free mice colonized by microbiota adapted to cold weather would increase the host’s insulin sensitivity and the length of intestine, villi, and microvilli, which help the host manage whole-body energy homeostasis in cold environments. It is suggested by Lafage Proust (2017) that gut microbiome could regulate energy metabolism and bone remodeling activities in the host (Lafage Proust, 2017).

Probiotics are able to regulate metabolic activities through the change of composition of gut microbiota (Parvaneh, Jamaluddin, Karimi, & Erfani, 2014). In Ohlsson et al. (2014), they treated mice, who had received an ovariectomy, with either *Lactobacillus* (L) *paracasei*, or the mix of *L. paracasei* and *L. plantarum* through oral administration for 6 weeks. Mice from both treated groups resulted having higher cortical bone mass than mice who received an ovariectomy but did not receive any probiotics. They concluded that probiotic treatments have suppressed the expression of inflammatory cytokines and increased the expression of osteoprotegerin (OPG), which protected mice from post-surgical bone loss (Ohlsson et al., 2014; Parvaneh et al., 2014). The study by Ohlsson et al. (2014) suggests that the gut microbiome could regulate immune responses caused by sex hormone deficiency. Research by Li et al. (2016) found that sex hormone deficiency can lead to higher gut permeability, and cause higher amount of osteoclastogenic cytokines, but this response is absent in germ-free mice (Li et al., 2016). These results all showed that
gut microbiome is critical in response to sex hormone changes and bone remodeling activities.

Gut microbiome is also crucial for the level of insulin-like growth factor (IGF). IGF-1 could promote the osteoblast cell differentiation and have anabolic effect on bone remodeling activities (Giustina, Mazziotti, & Canalis, 2008). Çeliker and Arslan (2000) found that the IGF-1 level in postmenopausal osteoporotic women is significantly lower than that of postmenopausal non-osteoporotic women. Another study shows germ-free mice administrated with one particular strain of lactobacillus could produce higher amount of IGF-1, which promote tissue growth (Pennisi, 2016). These studies suggest that gut microbes could regulate IGF levels, which could affect growth and development. Yan et al. (2016) found that short-term colonization of normal gut microbes in germ-free mice leads to lower bone mass, but long-term colonization of normal gut microbes in germ-free mice would lead to increasing bone mass. Further studies showed that normal mice respond to antibiotic treatment by decreasing IGF-1 levels, which also suppressed bone formation. However, after giving treated mice short-chain fatty acids, IGF-1 levels and bone mass of treated mice was restored to normal (Yan et al., 2016). These results suggest that short-chain fatty acids may be the reason of elevated level of IGF-1, and IGF-1 is crucial for gut microbiome to promote bone formation activities. Although there is no report on the "gut microbes-IGF-1-bone metabolism" regulation axis, in view of the important role of IGF-1 in bone metabolism, it is reasonable to infer that gut microbes may regulate IGF-1 level, which is closely related to bone metabolism, to regulate the status of bone homeostasis in the body.
Most studies have used antibiotics to disrupt the early microbiome (Cho et al., 2012; Cox et al., 2014; Ibáñez et al., 2019; Nobel et al., 2015; Ohlsson & Sjögren, 2015; Yan et al., 2016), and antibiotic-disturbed gut microbiota leads to increased bone mineral density (Cho et al., 2012; Cox et al., 2014) and content (Cox et al., 2014; Nobel et al., 2015). But studies disrupting the early microbiome using surgical birth are scarce (Martinez et al., 2017), with no results on bone phenotypes. Martinez et al. (2017) found that mice born surgically grew bigger, with no difference in fat mass. There are significant differences in the type and quantity of the intestinal flora between infants delivered by caesarean section and vaginal birth. The amniotic membrane is not ruptured at the time when caesarean section is performed, so it prevents mother’s vaginal flora from entering the infant’s body. Therefore, the gut flora of C-section newborns is mainly composed of skin microorganisms such as Staphylococcus, Corynebacterium, and Propionibacterium, while the gut flora of vaginal birth newborns is mainly composed of Lactobacillus, Prevotella, or Sneathia spp. (Dominguez-Bello et al., 2010). In addition, the colonization and development of the C-section infant’s gut flora is also affected. There is evidence showing that the colonization and development of Bifidobacterium is delayed in C-section born infants (Biasucci, Benenati, Morelli, Bessi, & Boehm, 2008; Kabeerdoss et al., 2013; Tsuji et al., 2012). Oliveira et al.(2017) found that Bifidobacterium provides protective functions for periodontitis in rats. One study has shown that rats with periodontitis have increased level of IL-10 and reduced level of RANKL and RANKL/OPG ratio (Oliveira et al., 2017). Therefore, Bifidobacterium could regulate immune response to provide a protective function for bones. Although the specific mechanism of the link between bone phenotype
and gut microbiome is unclear, C-section provides a valid physiological model for studying the effects of early-life change in gut microbiome composition.

Studies of the effect of the early microbiome on body composition and bone phenotypes are important because perturbations to the microbiota are common. C-section, for example, accounts for 33% of the births in the US, with a higher proportion in other countries (Boerma et al., 2018; OECD, 2019). This research will contribute to understanding whether C-section affects skeletal development, which could further provide foundation on C-section related gut-bone axis interactions.
3- Hypotheses and Objectives

Early antibiotics leads to increased growth and fat mass (Cox et al., 2014; Keith A. Martinez II, 2017) and to higher bone mineral density and content (Cho et al., 2012; Cox et al., 2014; Nobel et al., 2015; Yan et al., 2016). Since C-section or surgical birth impairs microbial transmission and also leads to increase developmental weight (Martinez et al., 2017), we hypothesize that surgical birth, interferes with offspring skeletal development, increasing bone mineral content and density, and that the effect is mediated by the microbiome, whose restoration will normalize the phenotypes.

To test this hypothesis, we propose the following Objectives:

Objective 1- Determine the effect of surgical birth on bone phenotypes by comparing bone mineral density and content of offspring born by vaginal birth and surgical birth.

Objective 2- Determine the effect of microbiome restoration after surgical birth on bone phenotype by comparing bone mineral density and content of offspring born by surgical birth and surgical birth with maternal microbe restored right after birth.
4- Design and Methods

The experimental design is shown in Fig. 1. Animals belonged to three birth groups, mice born vaginally, surgically, or both surgically and exposed to maternal vaginal fluids. Animals were weighed and their body composition and bone characteristics were determined at week 6 and week 18.

**Experimental Design**

![Experimental Design Diagram]

Figure 1. Experimental design of the study.

**Animals and procedures**

For this study, we examined Swiss Webster dams who were pregnant at the time of study. Dams were in three groups (Fig. 1):

1- Those that were born normally at E (Embryonic day) 20-21: a pair of mothers gave birth vaginally, and their litters were swapped with each other, so that all of the mice have foster mothers for lactation instead of biological mothers,
2- Those that were delivered surgically: a pair of mothers were staggered 1 day in gestational age, and when the first mother gave birth, the litters were extracted surgically from the second mother (the biological mother) and fostered by the first mother (the foster mother), replacing her own pups by the new litter. The first mother’s litters were euthanized.

3- Mice that were surgically delivered and their exposure to maternal microbiota was restored by gavage of maternal vaginal fluids right after birth. A pair of mothers were staggered 1 day in gestational age, and when the first mother gave birth, the litters were extracted surgically from the second mother (the biological mother) and being restored with maternal microbes. The second mother’s litters were fostered by the first mother (the foster mother), replacing her own pups by the new litter.

The first mother’s litters were euthanized.

The three groups, control-vaginal-foster group, surgical birth group, and surgical birth-restored group were formed after randomly splitting the dams at E(Embryonic day)16, and E14. When E16 gave birth naturally, they either belonged to control mice, or the dam was assigned to be a foster mother, in which case the dam that arrived in the lab at E14 underwent surgical delivery and restored with maternal microbes (see below) or not. (Fig. 2). All animals were raised in plastic cages with 12 hours light cycle and controlled temperature. All mice were fed with normal chow and water.
Figure 2. Experimental animal groups and assessed outcomes. Foster mom 1 and 2 gave birth vaginally and raised each other’s litters. Biological mom 3 gave birth to litter 3 surgically, and foster mom raised them. Biological mom 4 gave birth to litter 4 surgically. Litter 4 was microbial restored and raised by foster mom.

Surgical birth

Surgical birth was performed on E14 dams between 24-48 hours after E16 foster dam gave birth naturally. Surgical birth dams were euthanized by CO2 asphyxiation and with cervical dislocation. The abdomen was sprayed with antiseptic solution and cut with sterile scissors. Uterine horns were removed, and pups were removed from uterus and placentas, and amniotic fluid was cleaned away from each pup’s face, nostrils, and mouth with a sterile gauze. Pups were massaged until they began gaining pink color and their mouth opened. Born pups were kept on the warm pad until all pups in the litter were delivered. Foster moms’ feces were wetted and picked up to rub foster pups. After rubbing, pups were placed into the foster mother’s nest.
Microbial restoration

Pups from the surgical-restored group were subject to microbial restoration. A sterile cotton swab was inserted into the biological mother’s vagina after euthanasia, removed and placed into 1.5uL centrifuge tube containing 250uL of sterile saline. Centrifuge tube was vortexed for 5 second. Once all pups were delivered, 7uL of the vaginal saline solution was inoculated into each pup’s mouth. Pups were in a separate part of the cage until inoculation was completed.

Outcome Measurements

We measured body weight, body composition, and bone phenotypes (Fig. 2). At week 6 (P42) and week 18 (P126), 1 male and 1 female per family were euthanized, weighted and analyzed by DXA (Dual-energy X-ray absorptiometry). 56 mice were measured at week 6, and 49 mice were measured at week 18 (Table 1).

Table 1. Number of 6 weeks and 18 weeks old animals in the current study.

<table>
<thead>
<tr>
<th>Week</th>
<th>Number of animals analyzed</th>
<th>Number of Families</th>
<th>Total number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 6</td>
<td>Surgical birth</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Control vaginal Birth</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Surgical birth-restored</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>56</td>
<td>28</td>
</tr>
<tr>
<td>Week 18</td>
<td>Surgical birth</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Control vaginal birth</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Surgical birth-restored</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49</td>
<td>25</td>
</tr>
</tbody>
</table>

Body weight

The body weight of each animal was measured weekly using a 4-decimal weighing scale.
**Body morphometry and soft tissue body composition**

Lean, and fat mass were determined by dual energy x-ray absorptiometry (DXA) at week 6 (P42) and week 18 (P126), after mice were euthanized by CO\textsuperscript{2} asphyxiation. Corpses were stored at -80 Celsius degree until dissection. Corpses were defrosted overnight, and before dissection, whole-body composition was examined (Lunar PIXImus, GE Lunar Corp.). Data on lean mass, fat mass and whole-body fat percentage were calculated.

**Bone phenotypes**

Bones dissected in this study include the spine (vertebrae L1 to L6), the left and right femur, the left and right humerus, and the left and right tibia. Each bone was placed on a Delrin plate and then measured by DXA (Lunar PIXImus, GE Lunar Corp.). Bone mineral density and bone mineral content of femur, humerus, tibia, and spine were measured.

**Bone length Measurement**

A ruler with precision of 0.1 centimeter was used for measurements. All bones were measured on the image produced by the DXA measurement. Four spine bones, eight femur bones, eight humerus bones, and eight tibia bones were randomly taken from the collection and measured for the actual length using ruler with precision of 0.1 centimeter. The ratio of actual length to image length was calculated. The actual length of the rest of the bone was calculated using this ratio.

**Statistics**
SPSS v26 (IBM, Inc.) was used for statistical analysis. The data are not normally distributed according to the Shapiro test. Therefore, non-parametric tests were performed on the data set. The existence of outliers was examined before the performance of Kruskal-Wallis test. Kruskal-Wallis tests are performed to test the significance of each variable. For certain variables, sample sizes needed to increase statistical power were calculated using G*Power (Faul, Erdfelder, Lang, & Buchner, 2007). Alpha was set at 0.05. Data are represented as mean ± SD, unless otherwise indicated.
5- Results

Body morphometry and composition.

Surgically born females showed significant increase in body weight gain in comparison with the control, from week 4 to week 10 (Fig. 4; p<0.05). Yet, surgical birth did not lead to significant increase in total body weight compared to controls in both sex and both time point (Fig. 3; Table 2, Kruskal-Wallis Test, p>0.05). Surgical+restored females and males were not significantly different on weekly weight gain compared to the control mice (Fig. 4).
Figure 3. Body weight in males and females. A) week 6; B) week 18. C) Female (Left) and male (Right) body weight timepoint trajectories and D) FDR (False Discovery Rate) adjusted p-value (bottom) from week 3 to week 15. Numbers of sample are shown under each boxplot. Each sex is divided into three groups as vaginal foster group (Blue),
surgical birth group (Red), and surgical and restored group (Green). Total body weight differences between mouse groups were non-significant (Kruskal-Wallis test, p>0.05).

Figure 4. Female (Left) and male (Right) body weight gain trajectories (top) and FDR adjusted p-value (bottom) from week 4 to week 15. Surgical birth (CS) females (but not males) had higher weight gain from week 4 to week 10 compared to the control group (VF), and restoration after surgical birth normalized the phenotype.
Table 2. Significance of Kruskal-Wallis test of each variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week 6 Females</th>
<th>Week 6 Males</th>
<th>Week 18 Females</th>
<th>Week 18 Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>28</td>
<td>28</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Body Weight</td>
<td>0.809</td>
<td>0.513</td>
<td>0.340</td>
<td>0.984</td>
</tr>
<tr>
<td>Fat mass</td>
<td>0.067</td>
<td>0.307</td>
<td>0.525</td>
<td>0.859</td>
</tr>
<tr>
<td>Whole-body fat percentage</td>
<td><strong>0.029</strong></td>
<td>0.675</td>
<td>0.609</td>
<td>0.300</td>
</tr>
<tr>
<td>Lean mass</td>
<td>0.229</td>
<td>0.392</td>
<td>0.268</td>
<td>0.273</td>
</tr>
<tr>
<td>Bone mineral density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body</td>
<td>0.574</td>
<td>0.995</td>
<td>0.247</td>
<td>0.933</td>
</tr>
<tr>
<td>Femur</td>
<td>0.468</td>
<td>0.722</td>
<td>0.551</td>
<td>0.940</td>
</tr>
<tr>
<td>Tibia</td>
<td>0.876</td>
<td>0.504</td>
<td>0.161</td>
<td>0.596</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.142</td>
<td>0.250</td>
<td>0.357</td>
<td>0.879</td>
</tr>
<tr>
<td>Spine</td>
<td>0.064</td>
<td>0.548</td>
<td>0.303</td>
<td>0.669</td>
</tr>
<tr>
<td>Bone mineral content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole-body</td>
<td>0.758</td>
<td>0.054</td>
<td>0.126</td>
<td>0.647</td>
</tr>
<tr>
<td>Femur</td>
<td>0.523</td>
<td>0.370</td>
<td>0.775</td>
<td>0.883</td>
</tr>
<tr>
<td>Tibia</td>
<td>0.577</td>
<td>0.627</td>
<td>0.495</td>
<td>0.505</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.350</td>
<td>0.391</td>
<td>0.259</td>
<td>0.984</td>
</tr>
<tr>
<td>Spine</td>
<td>0.336</td>
<td>0.292</td>
<td>0.273</td>
<td>0.970</td>
</tr>
<tr>
<td>Bone length</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur</td>
<td>0.221</td>
<td>0.609</td>
<td>0.563</td>
<td>0.159</td>
</tr>
<tr>
<td>Tibia</td>
<td>0.457</td>
<td>0.687</td>
<td>0.606</td>
<td>0.826</td>
</tr>
<tr>
<td>Humerus</td>
<td>0.475</td>
<td>0.414</td>
<td>0.791</td>
<td>0.696</td>
</tr>
<tr>
<td>Spine</td>
<td>0.774</td>
<td>0.125</td>
<td>0.168</td>
<td>0.866</td>
</tr>
</tbody>
</table>
Lean mass was not affected by the treatment in both sex at 6 weeks and 18 weeks of age (Fig. 5; Table 2, Kruskal-Wallis test, p>0.05).

<table>
<thead>
<tr>
<th></th>
<th>Week 6 lean mass</th>
<th>Week 18 lean mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Lean (g)</td>
<td>Lean (g)</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 5. Lean mass in males and females. A) week 6; B) week 18. There is no significance due to the treatment.

Treatment groups have no difference compared with the control group in fat mass (Fig 6; Table 2, Kruskal-Wallis, p> 0.05).
Figure 6. Fat mass in males and females. A) week 6; B) week 18. Females grow more fat mass than males. There is no significant difference due to treatments.

Whole-body fat percentage of 6 weeks old females was significantly affected by treatment (Table 2, Kruskal-Wallis test, p=0.029), with surgical-restored group mice have higher whole-body fat percentage than surgical birth group mice (Fig. 7; Table 3, post-hoc with Bonferroni correction, p=0.023).
Figure 7. Whole-body fat percentage in males and females. A) week 6; B) week 18. At Week 6 females surgically born with maternal microbes restored have higher whole-body fat percentage in relation non restored (Kruskal-Wallis test, p=0.029).

Table 3. Pairwise Comparisons of treatment for whole-body fat percentage.

<table>
<thead>
<tr>
<th>Pairwise Comparisons</th>
<th>Adjusted Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – Surgical birth</td>
<td>0.561</td>
</tr>
<tr>
<td>Surgical birth – Surgical-restored</td>
<td>0.023</td>
</tr>
<tr>
<td>Control – Surgical-restored</td>
<td>0.467</td>
</tr>
</tbody>
</table>

*: Significance values have been adjusted by the Bonferroni correction for multiple tests.

Bone phenotypes

Bone mineral density

6 weeks old females showed that there is a trend of surgical birth to higher spine bone mineral density compared to the control group (Fig. 8I, J; Table 2, Kruskal-Wallis test, p=0.064).

Other individual bones including the humerus, femur, and tibia showed no significant effects after treatments (Fig. 8C, D, Fig. 8E, F, Fig. 8G, H, respectively; Table 2, Kruskal-Wallis tests, p>0.05).
Figure 8. Bone mineral density in male and female mice at week 6 and week 18. Whole-body bone mineral density Week 6 (A) and Week 18 (B); Humerus bone mineral density Week 6 (C) and Week 18 (D); Femur bone mineral density week 6 (E) and week 18 (F); Tibia bone mineral density week 6 (G) and week 18 (H); Spine bone mineral density week 6 (I) and week 18 (J).
Figure 9. Bone mineral content in male and female at week 6 (left) and week 18 (right). Whole-body bone mineral content at Week 6 (A) and Week 18 (B); Humerus bone mineral content Week 6 (C) and Week 18 (D); Femur bone mineral content week 6 (E) and week
Bone mineral content

6 weeks old males showed a trend of surgical birth with maternal microbial restoration leads to higher whole-body bone mineral content compared with surgical birth group mice (Fig. 9 A, B; Table 2, Kruskal-Wallis test, p=0.054).

There were no significant differences caused by treatment between the treatment groups and the control group in femur bone mineral content (Fig. 9 E, F; Table 2, Kruskal-Wallis test, p>0.05), tibia bone mineral content (Fig. 9 G, H; Table 2, Kruskal-Wallis test, p>0.05), humerus bone mineral content (Fig. 9 C, D; Table 2, Kruskal-Wallis test, p>0.05), spine bone mineral content (Fig. 9 I, J; Table 2, Kruskal-Wallis test, p>0.05).

Bone length

There were no significant differences caused by treatment between the treatment groups and the control group in humerus length (Fig. 10 A, B; Table 2, Kruskal-Wallis test, p>0.05), spine length (Fig. 10 C, D; Table 2, Kruskal-Wallis test, p>0.05), femur length (Fig. 10 E, F; Table 2, Kruskal-Wallis test, p>0.05), and tibia length (Fig. 10 G, H; Table 2, Kruskal-Wallis test, p>0.05).
Figure 10. Bone length in male and female at week 6 (left) and week 18 (right). Humerus length at Week 6 (A) and Week 18 (B); Spine length Week 6 (C) and Week 18 (D); Femur length week 6 (E) and week 18 (F); Tibia length week 6 (G) and week 18 (H).
Summary of Results

The results of this work are summarized in Table 4. Surgical birth does have effects on phenotypes in a sex-dependent way. Microbial restoration after surgical birth normalizes weight and lean mass phenotypes but leads to increased bone mineral content and density.

Table 4. Summary of results

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Effect of surgical birth</th>
<th>Microbial restoration after surgical birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (Weekly)</td>
<td>Increased in females during week 4-10</td>
<td>Normalized after restoration</td>
</tr>
<tr>
<td>Lean mass</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fat mass</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Whole-body fat percentage</td>
<td>Normal</td>
<td>Increased in week 6 females in relation to non-restored</td>
</tr>
<tr>
<td>Whole body bone mineral content</td>
<td>Normal</td>
<td>Trend to increase in week 6 males</td>
</tr>
<tr>
<td>Spine bone mineral density</td>
<td>Trend to increase in week 6 females</td>
<td>Normal</td>
</tr>
</tbody>
</table>
6- Discussion

**Body weight trajectory**

Our results on body weight do not entirely reproduce previous results at NYU (Martinez et al., 2017), in which C-section-born fostered females and males showed significantly higher weight starting at week 6. In this study, females only grew bigger, but the effect was temporary. Other than genetic factors, gut microbiome composition is considerably affected by the environment, and if the facilities are too clean of environmental bacteria, it affects animal phenotypes (Jin, Touyama, Yamada, Yamazaki, & Benno, 2014; D. Rothschild et al., 2018; Shaw et al., 2017; Zhou et al., 2018).

In humans, a systematic review and meta-analysis on human C-section and obesity (Darmasseelane, Hyde, Santhakumaran, Gale, & Modi, 2014), concluded that age is associated with effect-size, with younger populations being more likely to be affected by C-section birth mode, which leads to higher chance of childhood overweight, and it is consistent with our results on female weight gain (Barros et al., 2012; Darmasseelane et al., 2014; Kuhle, Tong, & Woolcott, 2015; Mueller et al., 2015). During the developmental process, people are exposed to more and more obesity risk factors in addition to c-section-associated gut microbial disruption, which would reduce the ultimate effect of C-section on body weight and whole body fat percentage (Darmasseelane et al., 2014).

**Whole-body fat percentage**

In humans, changing in gut microbiome composition is related to changing in body composition (Le Roy et al., 2019; Remely et al., 2015). Multiple studies have shown the association between early-life exposure of antibiotics and childhood overweight/obesity
Despite the higher body weight in females surgically born, our data shows that females born surgically and restored have higher whole-body fat percentage at six weeks of age, which is unexpected. Other studies have shown increased fat mass in antibiotics (Cho et al., 2012; Cox et al., 2014). Further studies need to better assess body composition during development, in the context of exposure to early stressors.

**Bone Mineral Content**

Our results indicated that whole-body bone mineral content was not affected by C-section procedures but did marginally affected by the restoration process in this experiment. This effect was not found in individual bones (Table 2, p>0.05). The statistical power of this trend is low (0.573), for a power of 0.8 we would need at least 50 total samples, which is 17 samples per group (Figure 11). In future studies, MicroCT scans are needed to segment other bones, such as cranial bones, to determine which bone of surgical birth and restored with maternal microbes group tend to have higher bone mineral content under microbial disruption.
Bone Mineral Density

In our experiment, there is a trend that surgical birth group leads to higher spine bone mineral density compared to the control group among females at week 6. The statistical power of this trend is very low (0.464), and for a power of 0.8 we would need at least 60 samples, or 20 samples per group. Early disruption on gut microbiota have been associated with phenotypical changes in bone mineral density in several studies (Cho et al., 2012; Cox et al., 2014; Nobel et al., 2015). Cox et al. (2014) have found that early-life antibiotics treated female mice have higher bone mineral density, and this change was not observed in males. Cho et al. (2012) have found that antibiotics treated mice had higher bone mineral density at week 3, and this effect was not observed at week 7. All of these results indicated that changing in gut microbiome composition will affect bone mineral density, and it depends on sex, genetics, and many other factors.
Overall limitations and strengths

Our results have several limitations. One is the low statistical power, with the need of almost duplicating the current sample size per group, to obtain reasonable power.

Second, limitations also derive from the fact that our DXA measurements were only performed after animal euthanasia, and therefore we did not measure bone phenotype at the start of the study or multiple time points. However, we did assess bone at an early and later time point after birth (at week 6 and at week 18). Two-time points measurements provided us sufficient data for developmental comparison. This study is part of a larger study, and further research will be conducted about the relationship between change in gut microbes and skeletal development, which will potentially explain more about the bone growth measured in this study.

Translation to humans
Gut microbiome could affect bone health through many ways. The regulation of immune and metabolic response of hosts by gut microbiome is crucial in linking microbiome and bone remodeling activities. For now, most of study about the relationship between gut microbiome and bone phenotype focuses on the anabolic effects of whole-body/bone, and the molecular mechanisms underlying the effects still need further studies.

In addition, C-section has become more and more common in the U.S., it’s essential to learn how it would change gut flora composition, and how the changes in gut flora composition could lead to physiological changes. Gut microbe and bone are important part of human organs, fully revealing the role and function of intestinal microbes on the bones of the whole body is beneficial in in-depth understanding of the interaction between the human digestive system and the skeletal system, and it could also provide new ideas for explaining the relationship between intestinal microbes and bone health; it is helpful to improve the theoretical knowledge of bone metabolism-related diseases, and provide a new way for health care in bone development.

Most of the gut disruption experiment were done in mice, but some of the findings could also potentially be applicable to humans. Nutrition and disruption of gut microbiota can cause stunting in children (Dinh et al., 2016). The status of intestinal flora is closely related to the digestion and absorption of food. Vitamin B12 and K are essential for gut microbiome and homeostasis which are key factors to ensure sufficient nutrients are supplied to all parts of the body including the bones (Guss et al., 2019; Lurz et al., 2020). Intestinal microbes help break down complex molecules in food, protect the body from pathogens, and promote the healthy development of the immune system (Kosiewicz, Zirnheld, & Alard, 2011; Tara McGinty, 2018). At present, it is suspected that the intestinal
flora affects the prevalence of stunting to a large extent. Microbes are important factors influencing height (Beard, 2002). Stunted children have enriched inflammogenic gut microbiomes, while normal children have enriched probiotic bacterial species in their gut microbiota (Dinh et al., 2016), although this correlation does not by any mean imply causation. With an altered early gut microbiota, it is plausible that alterations in bone development could be observed with a possible slow development of bones and a consequent stunting (Gehrig et al., 2019). Inadequate skeletal development not only may lead to stunting, but also to greater risk of osteoporosis later in life.
7- Conclusions

The results of this study did not fully support our hypothesis that surgical birth and microbes interfere with offspring skeletal development and partially normalize the altered phenotype observed in the surgical group mice. First, a low statistical power due to small sample size let to non-significant trends in increasing spine bone mineral density in the surgical birth group, and we need to duplicate the current sample size to increase power and see significance. Female mice only -not males- born surgically grew significantly bigger and had marginally higher spine bone mineral density. Restoration after surgical birth show normalized body weights, indicating that microbes modulate phenotypes.

An unexpected finding was the higher whole-body fat percentage among young females in the group that had microbial restoration after surgical birth, in relation to the non-restored, which deserves further study. Finally, that our study results at Rutgers show weaker body weight phenotypes than those observed before in a previous study at NYU (Martinez et al., 2017), suggesting that environmental/diet effects are important for development.
Appendix A. Mean and SD of each variable. Outliers were not detected among the data set.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Female week 6</th>
<th>Male week 6</th>
<th>Female week 18</th>
<th>Male week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>28</td>
<td>28</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.051±1.465</td>
<td>30.777±2.917</td>
<td>45.951±8.250</td>
<td>51.092±5.465</td>
</tr>
<tr>
<td>Whole-body bone mineral density (g/cm²)</td>
<td>0.056±0.004</td>
<td>0.057±0.003</td>
<td>0.072±0.005</td>
<td>0.069±0.006</td>
</tr>
<tr>
<td>Whole-body bone mineral content (g)</td>
<td>0.460±0.076</td>
<td>0.472±0.051</td>
<td>0.602±0.089</td>
<td>0.567±0.053</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>5.904±0.945</td>
<td>7.050±1.585</td>
<td>16.872±5.743</td>
<td>13.492±3.118</td>
</tr>
<tr>
<td>Lean (g)</td>
<td>17.643±1.309</td>
<td>22.679±2.146</td>
<td>25.248±2.779</td>
<td>32.150±3.220</td>
</tr>
<tr>
<td>Femur bone mineral density (g/cm²)</td>
<td>0.062±0.005</td>
<td>0.067±0.007</td>
<td>0.089±0.006</td>
<td>0.087±0.007</td>
</tr>
<tr>
<td>Femur bone mineral content (g)</td>
<td>0.025±0.003</td>
<td>0.029±0.004</td>
<td>0.049±0.005</td>
<td>0.051±0.005</td>
</tr>
<tr>
<td>Femur bone length (cm)</td>
<td>1.196±0.061</td>
<td>1.241±0.085</td>
<td>1.474±0.045</td>
<td>1.459±0.040</td>
</tr>
<tr>
<td>Tibia bone mineral density (g/cm²)</td>
<td>0.052±0.003</td>
<td>0.054±0.008</td>
<td>0.070±0.004</td>
<td>0.073±0.006</td>
</tr>
<tr>
<td>Tibia bone mineral content (g)</td>
<td>0.019±0.002</td>
<td>0.023±0.006</td>
<td>0.031±0.003</td>
<td>0.035±0.004</td>
</tr>
<tr>
<td>Tibia bone length (cm)</td>
<td>1.417±0.078</td>
<td>1.465±0.085</td>
<td>1.578±0.055</td>
<td>1.588±0.455</td>
</tr>
<tr>
<td>Humerus bone mineral density (g/cm²)</td>
<td>0.048±0.004</td>
<td>0.049±0.004</td>
<td>0.063±0.004</td>
<td>0.061±0.003</td>
</tr>
<tr>
<td>Humerus bone mineral content (g)</td>
<td>0.012±0.002</td>
<td>0.014±0.002</td>
<td>0.020±0.002</td>
<td>0.021±0.001</td>
</tr>
<tr>
<td>Humerus bone length (cm)</td>
<td>1.009±0.062</td>
<td>1.054±0.069</td>
<td>1.145±0.030</td>
<td>1.130±0.080</td>
</tr>
<tr>
<td>Spine bone mineral density (g/cm²)</td>
<td>0.061±0.005</td>
<td>0.062±0.006</td>
<td>0.079±0.007</td>
<td>0.07±0.006</td>
</tr>
<tr>
<td>Spine bone mineral content (g)</td>
<td>0.069±0.010</td>
<td>0.077±0.011</td>
<td>0.127±0.022</td>
<td>0.105±0.013</td>
</tr>
<tr>
<td>Spine (cm)</td>
<td>4.725±0.440</td>
<td>5.070±0.374</td>
<td>5.556±0.576</td>
<td>5.340±0.515</td>
</tr>
</tbody>
</table>


Poinsot, P., Schwarzer, M., Peretti, N., & Leulier, F. (2018). The emerging connections between IGF1, the intestinal microbiome, Lactobacillus strains and bone growth. J Mol Endocrinol, 61(1), T103-t113. doi:10.1530/jme-17-0292


