

## HUMAN NUTRITION

**Title:** Protein consumption from meat vs. dairy as complementary foods on infant growth, body composition and gut microbiome: a controlled feeding study - **NPB#15-108**

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### Industry Summary.

a. Background

The 1st year of life is very important for later-in-life obesity and heart disease development. Studies have shown that protein intake from dairy is associated with rapid weight gain, which may increase later-in-life obesity risk. However, no studies looked at if this relation between protein and weight gain is true for all protein sources. For example, whether meat protein would also increase weight gain as dairy protein does in infants, is unknown. Moreover, bacteria found in the gut, are recently discovered to play an important role in weight regulation. Limited evidence suggest that meat vs. dairy has different impact on gut bacteria composition. Thus, we would like to address the problem of weight gain during infancy and its association between protein source and gut bacteria.

b. Objective(s)

1. Would different protein sources from meat and dairy result in different weight/fat gain in formula fed infants?
2. Would different protein sources from meat and dairy result in different bacteria profiles in the gut?

c. Methods

Five-month old, health, formula-fed infants were recruited from the metro Denver area. These infants were divided into two groups. One group consumed a meat-based diet and the other group consumed a dairy based diet, from 6 to 12 months of age. During this 7 months of consuming the study diet, blood, urine, and stool samples were collected at multiple time points for analyses of growth biomarkers, glucose metabolism, inflammation, body composition and the gut microbiota. Infant growth (e.g. length, weight, circumferences, skinfolds) were measured every month from 6 to 12 months of age. The same cow milk based infant formula was provided to all participants.

d. Important Findings

- 1) Infant weight and weight-for-age-Z scores increased over time. Both meat and dairy groups have the same amount of increase.

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- 2) Infant length increased. However, length-for-age Z score, a parameter of linear growth status, increased in the meat group and decreased in the dairy group.  
Weight-for-length Z score, a parameter of overweight risk, increased in the dairy group.
- 3) Meat consumption appears to be associated with a gut microbiome profile favoring beneficial strains.

e. Implications/Industry Impact

Currently, there is no emphasis on introducing meat as an important source of protein and micronutrients for older infants. WHO is very explicit for complementary feeding, noting: “Meat, poultry, fish or eggs should be eaten daily, or as often as possible” for older infants. However, survey data on infant feeding patterns in the U.S. confirm the pattern of late introduction of protein foods in general, and meats in particular. The data indicate that < 10% of 7-8 mo olds eat baby food or non-baby food meats. Current baby food market in the U.S. is \$6 billion. Animal flesh protein such as pork, which is an excellent source of high quality protein and micronutrients, has not been promoted, whereas parents are more likely to offer yogurt and cheese as protein sources. Addition of more cow milk protein as complementary foods to a cow milk protein based formula diet may have adverse metabolic effects. The proposed research provides a novel approach via controlled feeding that directly compare meat vs. dairy on growth, body composition, blood biomarkers and gut microbiota changes in formula fed infants starting complementary feeding. Preliminary findings from this study suggest the meat and dairy protein have different effects on growth in formula fed infants, with meat having a potentially stronger linear growth promoting effect and a healthy gut microbiome. These data extend findings from studies in breastfed infants that meat is well tolerated as a complementary food, and may have metabolic and anthropometric advantages.

**Keywords:** protein source, infant growth, rapid weight gain

**Abstract:**

**Background:** High protein intake from infant formula has been associated with rapid weight gain and increased adiposity, but the effect of type of protein from complementary foods has not been prospectively evaluated. Previous research suggest that protein source might play an important role in weight and adiposity regulation.

**Objective:** To test the hypothesis that a meat-based complementary diet does not induce rapid weight gain as a dairy-based complementary diet does.

**Design:** Healthy, term formula-fed (breastfed  $\leq$ 1 month) infants were recruited from metro Denver area and randomized to a meat-based complementary diet group (MEAT), or a dairy-based complementary diet group (DAIRY) from 6 to 12 months of age. Total protein intake (formula + meat or formula + dairy) during the intervention is  $\sim$ 3 g/kg/d, with protein intake from meat or dairy being adjusted monthly based on body weight and formula consumption. Intakes of formula, infant cereal, fruits and vegetables were ad libitum. Three-day diet records were completed at 5, 10, and 12 months of age. Anthropometric measurements were conducted on monthly basis. Blood samples were collected at baseline and end of intervention.

**Results:** 64 infants completed the study (32 MEAT, 32 DAIRY). There was an increase of WAZ over time without difference between groups. However, LAZ increased in MEAT and decreased in DAIRY, lead to a significant group-by-time interaction of WLZ. Circulating IGF-1 concentrations increased in both MEAT and DAIRY.

**Conclusion:** Meat promotes linear growth but does not have the same weight accelerating effect as dairy protein does when included in infants' complementary diet.

## **Introduction:**

Proper guidance during the first 2 years of life, a critical time when the plasticity in rate and quality of growth is high, would yield long-term benefits for optimal growth and obesity prevention. Emerging epidemiologic evidence suggests that rapid weight gain early in life predisposes to later obesity development. Multiple observational studies [1-5] have reported greater weight gain in formula-fed infants compared with breast-fed infants. Because standard cow-milk based formula (~2.2 g protein/100 kcal) has a higher protein content than breast milk (~1.5 g protein/100kcal), the difference in protein intakes has been considered as a key contributor to the greater weight gain in formula-fed infants. Koletzko et al [6] conducted the first large-scale randomized controlled trial (RCT) to examine protein quantity on infant growth, using isocaloric infant formula with high-(2.9 g/100 kcal) or low-protein (1.7 g/100kcal) content from birth to 24 months. Results showed that length-for-age Z score (LAZ) did not differ between groups during the intervention. However, weight-for-length Z score (WLZ), a risk parameter of being overweight, of the high-protein formula group was 0.20 higher than that of the low-protein formula group at 24 months. Potential mechanisms of the relation between dietary protein and WLZ has been suggested to be mediated by regulation of Insulin-like growth factor I (IGF-1). In the study conducted by Koletzko et al [6], values of IGF-I were 40% higher in the high-protein formula group compared with the lower-protein formula group at 6 months, and WLZ at 6, 12 and 24 months was positively associated with IGF-1 at 6 months [7]. These findings have been the basis of current recommendation to limit protein intake to 15% or less of total energy intake for 0-2 years [8], without clear distinction for type of protein. Indeed, Gunther et al [9] followed 203 infants from 6 months to 7 years with diet information at multiple time points, and found that early dairy protein intake, not meat, at 12 months was positively correlated with fatness at 7 years. In addition, In 2.5 year-old boys, habitual milk protein consumption were positively correlated with serum IGF-1 concentration while the same relation was not observed with meat intake [10]. These findings suggest that the effect of protein on weight gain might be source dependent.

Because of different amino acid composition between meat and dairy protein, they may exert distinctive impacts on growth, body composition, blood metabolites and gut microbiome. For example, dairy protein, especially whey, has 26% of BCAA while meat has 18%. Leucine content in whey, dairy and meat are 14, 10 and

7%, respectively [11]. BCAA and leucine, as discussed above, are potential mediators of fat oxidation and insulin resistance. Meat also has a higher content of arginine than dairy. Arginine, in some studies is shown to be significantly associated with IGF-1 and linear growth [12], independent of other amino acids. However, the relation between IGF-1 and different amino acids is still inconclusive [13, 14]. In addition, the gut microbiome might play an important role in the relation between dietary protein and weight regulation. One observational study [15] examined the correlation between long-term dietary factors and microbiome composition in adults, and reported that animal protein, but not dairy protein, was highly associated with the abundance of *Bacteroides*, which is reduced in abundance in obesity. In contrast, another study [16] reported that consumption of a high-protein, meat-based complementary diet from 5 to 9 months of age, resulted in a potentially obesogenic microbiome, with increased abundance of *Clostridium Group XIVa* (an SCFA producer). However, WLZ did not differ between the high- and low-protein groups [17]. More research is needed to investigate the relation between protein source and gut microbiome on weight gain and adiposity in infants and toddlers. Thus, the objective of this study was to compare growth Z scores, blood biomarkers and the gut microbiome between infants consuming a meat- or dairy-based complementary diet from 6 to 12 months of age.

**Objectives:** *From your research proposal.*

The main purpose of this proposal is to request additional funding through NPB for an on-going NPB supported study (13-197) to increase the sample size based on power calculation using preliminary data, and the reviewers' comments to the original proposal which suggested sample size was small. In addition, we are requesting additional time to complete the study because of the increased sample size.

## **Materials & Methods:**

### **Study Design**

This randomized controlled trial was initiated in 2014 to directly compare a meat- vs. dairy-based complementary diet on infant growth from 6 to 12 months in formula-fed infants. This study utilizes semi-controlled feeding. Infant formula, and meat- or dairy-based complementary foods were provided. Participants visit the Pediatric Clinical & Translational Research Center (CTRRC) at Children's Hospital Colorado (CHCO) at baseline and end of the intervention. Both visits include blood, fecal sample and urine collections, diet record, and anthropometrics measures. Anthropometrics (length, weight, head/abdominal/hip circumferences, skin-fold measurements) and diet records were also obtained at monthly home visits.

### **Subjects**

Sixty-four term, formula-fed infants were recruited from metro Denver area and randomized to a meat- or dairy-protein based complementary diet from 6 to 12 months of age, with stratification of sex and race. Formula-fed infants were chosen 1) to increase internal validity because breast- and formula-fed infants are likely to respond differently to complementary feeding; 2) because formula-fed infants are at higher risks for excessive weight gain, at least partially due to the inherently higher dairy protein consumption from formula; and 3) because the majority of infants in the U.S. are formula-fed, especially after 3 months of age [18]. Exclusion criteria include low birth weight; breastfeeding > 1 month; and significant congenital anomalies or known chronic diseases. Infant demographic data include sex, gestational age, birth weight, and brief medical history. Maternal and family medical history, parental weight status, and mode of delivery are also obtained.

### **Dietary Intervention**

During the 6 to 12 months dietary intervention, total protein intake, including formula is targeted at ~3 g/kg/d. Infant consumes standard, intact milk-protein-based formula *ad lib*. The same formula is provided to standardize this exposure. The meat-based diet consisted of commercially available pureed meats and the dairy-based diet of yogurt, cheese and a whey protein supplement (specially packaged for this study). Fruit and

vegetable intakes are not restricted. Parents are provided tailored guidelines to foster attainment of this intake and encouraged to let the infant's appetite dictate the amount of complementary food intakes, as done with previous complementary feeding intervention by our group [19, 20]. Three-day diet records were collected at baseline (5 months), 10 and 12 months of age and analyzed by the CTRC Nutrition Core (NDSR software).

### **Anthropometric Measurements**

Length, weight, circumferences of head, chest, waist, hip and upper mid arm, skinfolds of tricep, bicep, subscapular, suprailiac and upper mid-thigh were measured at 5 months and at each subsequent monthly visit (6, 7, 8, 9, 10, 11 and 12 months). All measurements were performed in triplicates by trained research personnel. Length was measured in recumbent position using an infant stadiometer accurate to 0.1 cm (Holtain Ltc, Crosswell, Crymych, Pembs, UK). An electronic digital balance (Sartorius Corp, Bohemia, NY) was used to obtain naked infant weight. Skinfolds (tricep, bicep, Z scores were calculated based on WHO/CDC breastfed infant growth standards [21].

### **Sample collection and analyses**

Blood samples were collected at baseline and end of the intervention and were immediately centrifuged and stored at – 80 degree C until analyzed. The following markers were analyzed by the Colorado Clinical and Translational Science Institute's Core Lab: insulin-like growth factor 1 (IGF-1, Chemilluminescence, DiaSorin Liaison); Insulin-like Growth Factor-Binding Protein 3 (IGFBP3, Chemilluminescence, Siemen); total lipids, blood urea nitrogen (BUN).

Stool samples were collected at 6, 10, 12 months. At each visit, 2 samples were collected using disposable diapers fitted with biodegradable liners. Clinical grade gloves and sterile fecal swabs were used to avoid microbial contamination. Samples were transferred back to the laboratory on dry ice and stored at – 80 degree C. 16S rRNA sequencing follows standard procedures [22]. In brief, DNA was extracted from stool (MO BIO PowerFecal kit) and subjected to broad-range PCR of the 16S V3V4 region. 16S amplicons are sequenced on an Illumina MiSeq (2x300 bp V3 kit) and 16S reads are classified using the SILVA/SINA pipeline [23]. The taxonomic composition

of gut microbiota between dietary groups or in association with other infant outcomes, such as growth and serum metabolites are identified using this dataset.

## **Statistical approach**

Statistical analyses were performed using SAS (version 9.3; SAS Institute Inc, Cary, NC). Group data are presented as mean  $\pm$  SD. Baseline parameters were compared using independent Student's t test between MEAT and DAIRY groups and parameters that were different at baseline were included in further analyses. Gender was tested as a categorical variable in the subsequent analysis and results remained unaffected. Repeated measures ANOVA (PROC GLM) were used to evaluate the main effects of time, group, and their interactions on the dependent variables. Independent Student's t test was used to compare values between groups as post hoc analysis. Equal variance was checked using Levene's test. Wilcoxon-Mann-Whitney test was conducted when sample was not normally distributed.  $P < 0.05$  was considered statistically significant.

The gut microbiome data are presented as relative abundance of taxa. Taxa that differ in abundance between treatment groups were identified by non-parametric Wilcoxon rank-sum test. Correlations between microbiome variables and continuous infant outcome variables were assessed by Spearman rank correlation tests. Permutation-based analysis of variance (PERMANOVA) tests using common beta-diversity indices (e.g., Unifrac, Jaccard) assess between-group differences in microbial communities. Machine learning approaches, such as Random Forests, were used to build models of infant growth trajectories, with the goal of identifying particular microbial taxa or gene, or host factors, that best predict growth patterns of these infants.

## Results:

### Subject Characteristics

Sixty-four infants completed the intervention (10/2014-8/2016). Baseline characteristics were summarized in **Table 1**. Because participants were stratified for age, race and sex, there was no difference of these parameters between groups. On average, mothers of the participants were overweight.

### Dietary Intake

At baseline (5 months of age), protein intake did not differ between groups. As designed, protein intake increased to ~3g/kg/d when the participants entered the intervention ( $P < 0.01$  over time; **Figure 1**). Solid food intake also gradually increased over time. For example, protein from formula accounted for over 80% of total protein intake at baseline. At 10 months, protein from meat- or dairy-based solid foods accounted for 70% of total protein intake and formula accounted for ~25%. Total energy intake (kcal/d) increased over time for both groups ( $P < 0.01$ ) but did not differ between groups at any time points. ~15% of total energy was from protein at 10 and 12 months for both groups.

### Growth Z scores

Infant weight and length were obtained at 6, 7, 8, 9, 10, 11 and 12 months and growth Z scores are summarized in **Figure 2**. There was no difference of WAZ, LAZ or WLZ between groups at baseline. Mother's height and BMI were considered covariates. WAZ increased over time without difference between groups at any time point ( $P < 0.01$ ). A significant group-by-time interaction was found for LAZ: LAZ increased ( $P = 0.001$ ) in the MEAT group and decreased ( $P = 0.0002$ ) in the DAIRY group; Post-hoc student's *t* test showed LAZ was significantly higher at 10, 11 and 12 months in the meat-based complementary diet group. The changes of WAZ and LAZ lead to a significant group-by-time interaction for WLZ: WLZ did not change in infants consumed a meat-based complementary diet ( $P = 0.45$ ) but increased in infants consumed a dairy-based complementary diet ( $P = 0.004$ ). Post-hoc student's *t* test showed WLZ was significantly higher at 11 and 12 months in the dairy-based complementary diet group.

### Serum Biomarkers<sup>1</sup>

**Figure 3** shows IGF-1, IGFBP3 and blood urea nitrogen (BUN) concentrations between groups at baseline and 12 months. IGF-1 and IGF-BP3 both increased in both groups over time, without differences between groups. BUN increased over time (P=0.001) without group differences and still within the normal range for this age (7-26 mg/dL). BUN was used to crudely evaluate protein intake over time for compliance. The increase of BUN over time is consistent with the increase of protein intake (Figure 1).

### **Gut Microbiome<sup>2</sup>**

(MEAT n=15, DAIRY n=15) At family level, one of bacteria in the Clostridia class were altered by the diet: *Ruminococcaceae* which is related to short-chain fatty acid (butyrate) production in the gut. %*Ruminococcaceae* increased only in MEAT, not in DAIRY (**Figure 4**).

<sup>1</sup>Analyses of glucose, insulin, leptin, lipid panel and inflammation markers are still pending.

<sup>2</sup>Results of gut microbiome is preliminary, because 50% of the fecal samples are not analyzed yet. Results of the gut microbiome will be included in subsequent publications, not the first one, which focuses on growth.

## Discussion:

This is the first randomized controlled trial that directly compared dietary protein from two common sources: meat and dairy, on infant growth trajectory and the corresponding serum biomarkers. The main finding of this study is that protein source has a significant impact on infant growth. Compared with a dairy-based complementary diet, a meat-based complementary diet promotes linear growth without increasing the risk of being overweight. Protein source doesn't seem to affect intakes of other foods because both the MEAT and DAIRY groups consumed the same amount of calories, protein and fat, only the source of protein was different between groups. This further strengthens the internal validity of our findings.

An increase of WLZ crossing centiles on the growth chart is considered a warning for becoming overweight. A “normal” growth pattern for infants and toddlers would be that WLZ remains relatively stable over time. In the present study, WLZ increase in the dairy-based complementary diet group was  $+0.75 \pm 0.39$  over 7 months, suggesting excessive weight gain relative to length gain, and possible increased risk of becoming overweight. The WLZ difference between groups at 12 months was  $0.30 \pm 0.19$ . Koletzko et al demonstrated in the large infant cohort [6] a WLZ difference of 0.20 between the high- and low-protein formula groups at 24 months. No WLZ score was directly reported in this RCT [6]. Additionally, this study [6] only controlled protein quantity in formula but not complementary foods. In other words, it only examined the liquid part (formula) of the diet. Solid foods gradually replace formula and become an increasingly important part of infants' diet. Findings from the present study on growth Z scores clearly highlight the impact of quality of complementary foods (solid foods) on infant growth.

Although IGF-1 was considered the key mediator in WLZ increase in the RCT by Koletzko et al [6], it is also the most copious growth factor in human bone [24] and greatly influences linear growth (LAZ). Both animal [25] and human studies [26] showed that IGF-1 is associated with bone growth. LAZ increase may lead to WAZ increase at the same time. An increase in both WAZ and LAZ may result in no change of WLZ and additional risk of overweight. Indeed, one study conducted by the current investigators compared a high- vs, low-protein complementary diet on growth from 5 to 9 months in breastfed infants [17]. The high-protein diet (3g/kg/d) was meat based and the low-protein diet (1g/kg/d) was cereal based. Results showed that the high-protein diet

increased both WAZ and LAZ, compared with the low-protein diet, but WLZ was comparable between groups over time [17]. Another longitudinal observational trial found that formula-fed infants had higher IGF-1 concentration than breast-fed infants, which was positively associated with both weight gain and length gain from birth to 3 months of age [27], although Z scores were not reported. In the present study, the increase of IGF-1 is consistent with the increase of LAZ in infants consumed the meat-based complementary diet (Figure 2). The different impact of regulating IGF-1 concentration between meat and dairy proteins may be due to the different amino acid compositions. For example, meat also has a higher content of arginine than dairy. Arginine, in some studies is shown to be significantly associated with IGF-1 and linear growth [12], independent of other amino acids. However, why LAZ decreased (impaired linear growth) in infants who consumed the dairy-based complementary diet, is still unknown.

Complementary feeding, the inclusion of other foods and liquids besides human milk or infant formula, usually starts at 6 months of age and continue to 24 months of age. This 18-month interval accounts for more than 50% of the “1000 days” and represents a critical window for disease prevention and long-term health formation. Despite this, complementary feeding practices in westernized settings have been largely driven by tradition and marketing rather than a sound evidence base. Recently, findings from the RCT conducted by Koletzko et al [2] became the basis of current effort to reduce protein intake (15% or less of total energy) early in life in order to reduce the risk of later-in-life obesity [8], without clear distinction for types of protein. Protein intake from the present study is 3 g/kg/d which is higher than their habitual protein intake but still at 15% of total energy, suggesting the feasibility of consuming such complementary diets. Meat is considered an excellent source of high-quality protein and micronutrients to promote optimal growth and development and does not need to be avoided during complementary feeding. Findings from the present study further demonstrated the benefits of consuming meat as a first food for promoting linear growth.

In conclusion, our findings suggest that compared with a high-protein, dairy-based complementary diet, a high-protein, meat-based complementary diet promotes linear growth without increasing the risk of being overweight in formula-fed infants.

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**Table 1. Subject Baseline Characteristics**

	<b>Meat (n=32)</b>	<b>Dairy (n=32)</b>	<b>P value</b>
Sex	45% male	48% male	
Race	75% white	78% white	
Birth weight (kg)	3.31± 0.37	3.33 ± 0.48	0.85
Birth length (cm)	50 ± 4	48 ± 7	0.37
Gestational age (wk)	39 ± 1	39 ± 1	0.30
Maternal BMI	28 ± 7	27 ± 6	0.45
Maternal height (cm)	167 ± 7	165 ± 8	0.32
Maternal age (kg)	30 ± 6	29 ± 7	0.56

Figure 1.

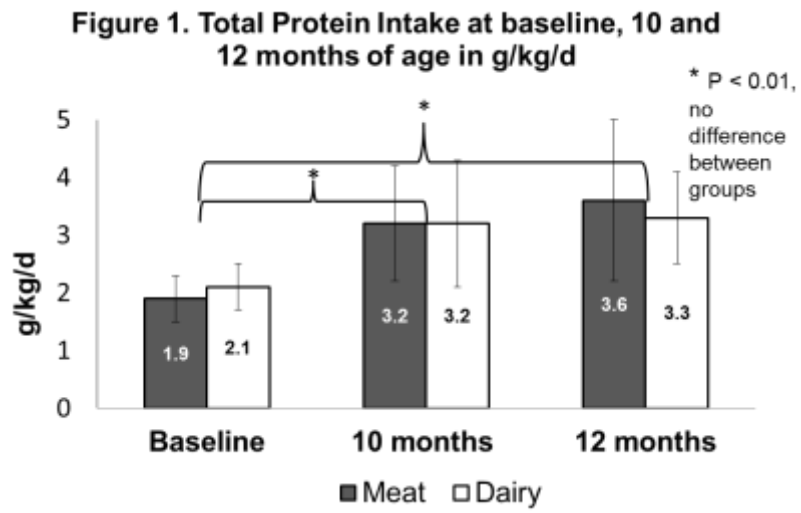
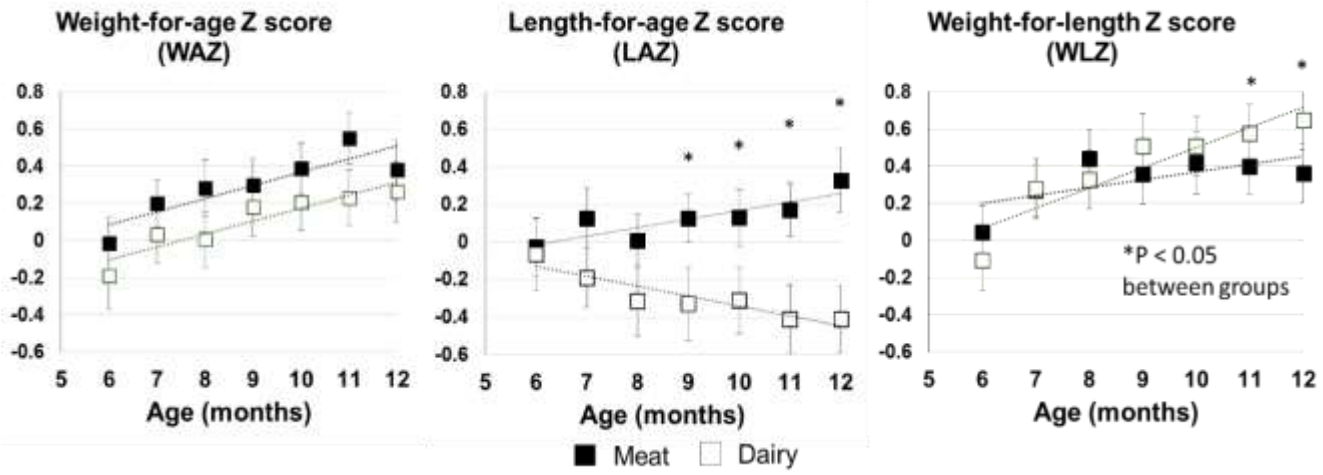


Figure 2.

Figure 2. Growth Z scores during the intervention from 6 to 12 months of age



**Figure 3.**

