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A 3-week pre-clinical study of 2′-fucosyllactose in farm piglets

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ABSTRACT

One of the most abundant oligosaccharides found in human milk is 2′-fucosyllactose, a trisaccharide composed of fucose and lactose, and multiple studies have demonstrated a health benefit to this compound. Recent advances have allowed for the large-scale production of oligosaccharides via fermentation, including 2′-fucosyllactose. A neonatal piglet model was used to evaluate the tolerability of 2′-fucosyllactose, produced through this process, in order to demonstrate the suitability of this compound for human infants under 12 weeks of age. Crossbred farm piglets, at lactation day 2, were assigned to one of four treatment groups receiving a liquid diet containing 0, 200, 500 or 2000 mg/L of 2′-fucosyllactose. The calculated consumption of 2′-fucosyllactose corresponded to dose levels of 29.37, 72.22 and 291.74 mg/kg/day, respectively, in males and 29.30, 74.31, and 298.99 mg/kg/day, respectively in females. Piglets were administered diet for 3 weeks; and there were no test article-related effects on growth and development (clinical observations, body weight and food consumption), clinical pathology parameters (hematology, clinical chemistry, coagulation and urinalysis), or any histopathologic changes. Therefore, dietary exposure to 2′-fucosyllactose at concentrations up to 2000 mg/L was well tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth.

1. Introduction

There are over 100 major milk oligosaccharides in human milk (Stepans et al., 2006). Mature human milk contains 12–13 g/L of oligosaccharides, representing the third largest solid component, following lactose and fat, and are present at about a 20-fold higher concentration than that found in bovine milk (Asakuma et al., 2008; Zivkovic et al., 2011). Levels of human milk oligosaccharides (HMOs) vary between individuals and over the course of lactation (Asakuma et al., 2008; Chaturvedi et al., 2001; Thurl et al., 1993, 2010), the most abundant being 2′-fucosyllactose, which ranges from 0.06 to 4.65 g/L (Erney et al., 2000).

The protective properties of human milk have historically been attributed to antibodies; however, recent evidence suggests that HMOs may also play a significant role. HMOs have been shown to act as prebiotics, selectively promoting colonization by *Bifidobacterium bifidum*, a bacterium that is especially prevalent in the intestines of human milk-fed infants (Newburg, 2009). Several studies have shown a positive correlation between total bifidobacteria and fecal IgA titers (Mohan et al., 2008; Mullie et al., 2004). These bacteria produce lactic acid and short-chain fatty acids that may contribute to mucosal integrity and maturation and affect gut-associated immune cells, thus inhibiting infection by pathogenic organisms. Reflux of milk may also coat mucosal surfaces of the infant's upper respiratory tract with oligosaccharides, acting as soluble receptor analogs that inhibit the attachment of pathogenic microorganisms (Asakuma et al., 2008; Espinosa et al., 2007; Stepans et al., 2006).

Neonatal piglets represent an excellent model for human infants, especially when comparing the first three weeks of life for piglets with the first three months of life for human infants (Flamm, 2013; Guilloteau et al., 2010; Herfel et al., 2009; Institute of Medicine (IOM), 2004; Odle et al., 2014). During this developmental period, neonatal piglets have many similarities to human infants including the presence of specific digestive enzymes, nutrient absorption, gut closure, dietary requirements, microbial population and gut transit time. This study sought to evaluate the safety of 2′-fucosyllactose after three weeks of administration to neonatal farm piglets, beginning two days after birth, through the evaluation of growth and development (clinical observations, body weights, food consumption), clinical pathology parameters (hematology, clinical chemistry,

Abbreviations: Alb, albumin; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; Baso, basophils; Bil, bilirubin; Ca, calcium; Chol, cholesterol; Cl, chloride; Cre, creatine; Eosi, eosinophils; Ery, erythrocytes; FDA, Food and Drug Administration; GGT, gamma glutamyltransferase; GLP, Good Laboratory Practice; Gluc, glucose; Hb, hemoglobin; Hema, hematocrit; HMO, Human Milk Oligosaccharide; K, potassium; K_3EDTA, potassium ethylene diamine tetraacetic acid; Leu, leukocytes; Lymp, lymphocytes; Mono, monocytes; Na, sodium; Neut, neutrophils; OC, other cells; OECD, Organisation for Economic Co-operation and Development; P, phosphorus; Reti, reticulocytes; Sorb D, sorbitol dehydrogenase; TG, triglycerides; TP, total protein; UN, urea nitrogen.

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coagulation, and urinalysis) and macro- and microscopic histologic evaluations. The data presented here demonstrate that 2′-fucosyllactose is well tolerated by neonatal piglets, and therefore would not be expected to have any acute or long-term adverse effects in humans.

2. Materials and methods

2.1. Good laboratory practice and regulatory guidelines

The study was conducted by MPI Research, Mattawan, Michigan in accordance with Good Laboratory Practice (GLP) Regulations following the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58, and the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice, ENV/MC/CHEM(98)17, and any applicable amendments and was based on the following guidelines: Guidance for industry on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (2010), FDA Center for Drug Evaluation and Research Guidance on Nonclinical Safety Evaluation of Pediatric Drug Products (2006), European Medicines Agency Guideline for the Need for Non-Clinical Testing in Juvenile Animals of Pharmaceuticals for Pediatric Indications (2008), and the Consensus Document "The Application of the Organisation and Management of Multi-Site Studies" (2002).

2.2. Animals

A total of 27 male and 21 female Domestic Yorkshire Crossbred Swine (farm pigs) were received two days after birth (designated as Study Day 1) from Bailey Terra Nova Farms, Schoolcraft, Michigan. The piglets were allowed to nurse from the sow at least 48 hours prior to receipt to allow them to consume maternal colostrum for its beneficial effects during the neonatal period (Farmer and Quesnel, 2009; Guilloteau et al., 2010). Prior to receipt at the laboratory, the piglets were given injections of an iron supplement and a broad-spectrum antibiotic (EXCEDE® for Swine), which is standard practice for animals of this age and strain. An additional iron supplement injection was given to all animals approximately one week following the initial injection by the supplier. Additional antibiotics were given via intramuscular injection weekly during the study at a dose of 5 mg/kg. All animals were weighed on the day of receipt and given a detailed physical examination on Study Day 2.

Animals were assigned into treatment groups to make the treatment groups as uniform as possible in terms of age and weight. Due to the imbalance in the number of male and female piglets available at the initiation of the study, animals were assigned to the treatment groups to ensure that the control and highest dose groups had an equal distribution of male and female animals. Thus, 6, 8, 7 and 6 male piglets and 6, 4, 5 and 6 female piglets were assigned to the 0, 200, 500 and 2000 mg/kg treatment groups, respectively. The piglets weighed from 1.8 to 2.8 kg (males) and 1.7 to 3.0 kg (females) upon assignment into study groups. Upon arrival in the testing facility, and throughout the study duration, piglets were housed individually in mobile stainless steel cages with plastic coated flooring and mesh, as needed, in an environmentally controlled room. Supplemental heat was provided by heating pads inside the cage or a heating lamp was used outside the cage, if necessary. Animals from the same group were allowed socialization (at least two per cage) every morning prior to feeding.

2.3. Diets

The diets used in this study included a control diet and three test diets containing 2′-fucosyllactose at 200, 500 or 2000 mg/L. The test vehicle for all four diets was the commercially available Land O'Lakes® ProNurse® Specialty Milk Replacer (Purina Animal Nutrition, Gray Summit, Missouri). The milk replacer was prepared one day prior to use from a powder at a concentration of 119.45 mg/mL in deionized water. The control diet consisted of the control article and test vehicle. Test diets consisted of the test article, 2′-fucosyllactose (Jennewein Biotechonologie, Rheinbreitbach, Germany), and test vehicle. 2′-fucosyllactose was produced through a proprietary fermentation process, with specifications described in Supplementary Table S1. The control diet was offered to Group 1, and the low-, mid- and high-dose diets were offered to Groups 2 through 4, respectively. The concentration of test article in the control, low-, mid-, and high-dose diet was 0, 200, 500, and 2000 mg/L, respectively. To verify formulation concentrations, samples of all diets, including controls, were collected from the first and last dosing formulations, and sent to Abbott Nutrition, Columbus, Ohio, for analysis of 2′-fucosyllactose.

There was no acclimation period in this study; experimental diets were introduced on the day of animals' arrival to the testing facility, which was designated as Study Day 1. The control and experimental diets were offered orally via a feeding bowl filled by hand six times per day (3 hours ± 15 minutes between each dose) at a dose volume of 500 mL/kg/day for at least 20 consecutive days during the study. The dose volume was based on published literature (Dilger and Johnson, 2010) and recommendations from the product manufacturer (Nutrition, 2014). The length of the study was based on the standard lactation period for swine at the supplier (Bailey Terra Nova Farms), and also corresponds to the point at which the peak of lactation is reached in the mother (Guilloteau et al., 2010). The diets were allowed to warm to room temperature for at least 30 minutes prior to being offered to the animals. Individual doses were based on the most recent body weight measurements and food consumption was measured and recorded daily during the study.

2.4. Animal observations

All animals were observed for morbidity, mortality, injury, and availability of food twice daily. A detailed clinical examination of each animals was performed twice weekly during the study. Body weights were measured and recorded daily for the first week (Study Days 1–7) and every other day thereafter. Food consumption was measured and recorded daily, and food efficiency was calculated for the overall study period.

2.5. Clinical pathology

Blood samples (~3.2 mL) were collected from the anterior region of the superior vena cava through the thoracic outlet from all animals prior to dosing on Study Day 7 and on Study Day 21. The animals were not fasted prior to sample collection. The samples were collected into tubes containing potassium ethylene diamine tetraacetic acid (K_3EDTA) for evaluation of hematology parameters, citrate for evaluation of coagulation parameters, and serum separators with no anticoagulant for the clinical chemistry parameters. Urine samples were collected via cystocentesis at terminal necropsy for urinalysis.

2.6. Organ weights and histopathology

Gross necropsies were performed on all animals at terminal sacrifice on Study Day 22. Organ weights were obtained for the brain, heart, kidney, large intestine (cecum, colon, rectum), liver, small intestine (duodenum, jejunum, and ileum) and spleen. The pH of the intestinal contents of the cecum and colon were recorded. Relative organ weights (organ-to-body and organ-to-brain ratios) were calculated based on body weights measured at study termination. Microscopic examination of fixed hematoxylin and eosin-stained paraffin sections was performed on sections from all study animals for the organs listed above, as well as eye, including optic nerve; gall bladder; stomach; gross lesions; lung with bronchi; mesenteric lymph nodes; pancreas; and Peyer's patch.

2.7. Statistical analysis

Levine's test (Milliken and Johnson, 1992) was used to assess the homogeneity of group variances for each of the following endpoints and for all collection intervals: body weights, food consumption, hematology (except leukocyte counts), coagulation, clinical chemistry, and organ weights (absolute weights, relative to body weights, relative to brain weights). When Levine's test was not significant ($p \geq 0.01$), a pooled estimate of the variance (Mean Square Error or MSE) was computed from a one-way analysis of variance (ANOVA) and utilized Dunnett's comparison (Dunnett, 1955) of each treatment group with the control group. When Levine's test was significant ($p < 0.01$), comparisons with the control group were made using Welch's t test (Welch, 1937) with a Bonferroni correction (Snedecor and Cochran, 1982). Historical data indicate that leukocyte counts (total and differential) are not normally distributed; therefore, these data were log-transformed prior to being analyzed as described above. Historical data for urinalysis endpoints indicate that these endpoints have unpredictable distribution characteristics; therefore, a non-parametric test was used. Specifically, the data were rank transformed and Dunnett's test was used on the transformed data to compare each treatment group having a non-zero sample size with the control group. Results of all pair-wise comparisons were reported at the 0.05 and 0.01 significance levels, and all endpoints were analyzed using two-tail tests.

3. Results

3.1. Growth and food consumption

Dietary administration of 2′-fucosyllactose at 200, 500, and 2000 mg/L in ProNurse® Specialty Milk Replacer was well tolerated by the neonatal farm piglets during the 3-week dosing period beginning 2 days after birth. All animals survived to scheduled necropsy and no definitive test article-related changes were noted in the clinical observations during the course of the study. The male and female piglets in the treatment and control groups showed consistent growth over time based on body weight (Fig. 1A and B, respectively), nor was there any difference in food consumption in either males or females (Fig. 2A and B, respectively).