Effects of Rodent Diet Choice and Fiber Type on Data Interpretation of Gut Microbiome and Metabolic Disease Research

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Poor diet reporting and improperly controlling laboratory animal diet continues to reduce our ability to interpret data effectively in animal studies. In order to make the best use of our resources and improve research transparency, proper reporting methods that include a diet design are essential to improving our understanding of the links between gut health and metabolic disease onset. This unit will focus on the importance of diet choice in laboratory animal studies, specifically as it relates to gut health, microbiome, and metabolic disease development. The two most commonly used diet types, grain-based (GB) diets, and purified ingredient diets, will each be described, with particular emphasis on their differences in dietary fiber. A further description of how these diet types and fiber can affect gut morphology and microbiota will be provided as well as how purified ingredient diets may be improved upon.

Keywords: diet • fiber • metabolic disease • microbiota • mouse • rat • purified ingredients

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INTRODUCTION

Many different factors must be considered when designing any laboratory experiment that utilizes animal models. Factors such as the mouse model being used, number of mice per cage, life phase, temperature, and humidity are commonly considered. However, other factors that the animal model is exposed to, such as housing materials (i.e., cage, bedding) and sources of nourishment (i.e., water bottles and diet), are often not considered or simply not reported in the methods section (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; Thigpen et al., 2013). While caging and bedding can modify the phenotype, the diet being fed has the potential to at least be as influential as, if not more influential than, any other environmental factor. Yet, it is very common to find in the methods section of publications vague terms such as 'standard chow', 'standard diet', or 'normal diet' to "describe" the diet. Would we use the term 'standard mouse' to describe a mouse model? The term 'standard diet' tells us nothing and suggests their composition is 1) not important and/or that 2) all diets use similar ingredients, neither of which is true.

Anyone using rodent models (or other animal models) should add "nutritional scientist" to their job description (Ricci & Ulman, 2005). Not only should the researcher in any field know what is being fed to their animals, but they should be able to describe the diet in detail. In order to do this, the diet formula should be open to the public so its composition is known–in other words, one should be able to *report* the complete formula details: the ingredients and their concentrations. Given the long-known importance of diet in toxicity

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studies (Wise, 1982), the diet composition should be clear to both those performing any toxicological research and to those reviewing and reading the manuscript.

Certain dietary components that escape normal digestion come in direct contact with the gut microbiome and become metabolized with specificity, which can lead to changes in bacterial type and amount. Thus, being able to describe each dietary component with confidence should be paramount in studies involving the gut microbiome.

Furthermore, the entire diet composition from one manufactured batch to the next should be easy to *repeat* so that the animal's phenotype and the gut bacteria type and levels remain stable when different batches of the same diet are used. The idea of being able to repeat diet composition over time should lead to more consistent data within and between labs using the same diets. And finally, the diet composition should be easy to *revise* (that is, to change) one nutrient at a time, so that functionality of a specific, purposeful modification can be studied.

Within the metabolic disease field, there is a need to understand how factors in our environment may affect or be affected by the microbiome. Recent research has suggested that the gut microbiome is a central component of overall metabolic health in rodent models and that proper control over dietary components is critical to improving our understanding of this link. This unit will provide the reader with an introduction to the basic diet types used in lab animal research, a protocol for how to report the composition in a publication, and evidence as to why it is important to pay attention to the diet being used in any given study. In particular, an overview of the effect of dietary factors on gut health and microbiome will be provided, with particular emphasis on the role of dietary fiber.

WHAT TYPES OF DIETS ARE AVAILABLE TO RESEARCHERS USING ANIMAL MODELS?

In research with animal models, there are 2 major diet types commercially available to researchers: grain-based (GB) diets and purified ingredient diets (sometimes referred to as "semi-purified diets" or simply "purified diets"). These diet types are very dissimilar from each other in many ways due to the different ingredients used in each.

There are many commercially available GB diets. While it's not possible to define them all (as each may have a very different composition), some factors are common to most. In general, they provide more than adequate nutrient levels for reproduction, growth, and maintenance of animals, are relatively inexpensive, and have a long history of use, each of which makes them attractive for animal husbandry and for the facility purchasing them. However, there are several caveats to their composition that make them a less than an ideal choice in research. Firstly, the ingredients used in GB diets are ill-defined and generally contain multiple nutrients and non-nutrients. As their name implies, GB diets contain ground wheat, wheat middlings (a wheat by-product), ground corn, soybean meal, alfalfa meal, and dried beet pulp. In addition, many contain animal by-products such as porcine meat meal, fish meal, and bone meal, each of which provides sources of protein and fat, but also other potential nutrients and non-nutrients that call into question how these ingredients affect the overall diet composition. For example, soybean meal contains protein, fat, carbohydrate, fiber, vitamins, minerals, and phytoestrogens. Secondly, the ingredient concentrations of GB diets are usually "closed" (i.e., proprietary) and unknown to the research community; thus, one can't *report* their composition. One may ask (and rightfully so), what is the point of formulating and manufacturing a diet for research purposes if the researcher is not allowed to know the composition? That being said, given these ingredients are so ill-defined, this arguably does not matter.

Third, GB diets have been long known to be neither defined nor free of contaminants (Greenman, Oller, Littlefield, & Nelson, 1980; Wise, 1982). In fact, a number of contaminants have been found in GB diets, including phytoestrogens (from soybean meal and alfalfa meal; Thigpen et al., 1999), heavy metals such as arsenic (Kozul et al., 2008; Mesnage, Defarge, Rocque, De Vendômois, & Séralini, 2015), endotoxins (Hrncir, Stepankova, Kozakova, Hudcovic, & Tlaskalova-Hogenova, 2008), pesticides and pollutants (Mesnage et al., 2015). Often these contaminants are present at biologically relevant levels (Kozul et al., 2008; Mesnage et al., 2015; Thigpen et al., 2013). There is evidence that the levels of contaminants in GB diets vary across batches of the same diet (Greenman et al., 1980; Jensen & Ritskes-Hoitinga, 2007; Thigpen et al., 2007), which could lead to different findings depending on the batch used. Finally, due to complexity of the ingredients, it is impossible to *revise* the composition of GB diets one

Table 1 Typical Sources of Nutrients and Non-Nutrients in Rodent Purified Diets and Grain-Based Diets

Nutrients	Purified ingredient diet-typical sources	Grain based diet-typical sources
Protein	Casein	Soybean meal, ground corn, wheat, and oats whey, alfalfa
Fat	Soybean oil, corn oil	Porcine animal fat, fish meal, meat meal
Carbohydrate	Corn starch, maltodextrin, sucrose	Ground corn, wheat, and oats, wheat middlings
Fiber	Refined cellulose (INSOLUBLE Fiber)	Ground corn or wheat, dried beet pulp, ground oats, alfalfa, wheat middlings (SOLUBLE and INSOLUBLE fibers)
Micronutrients	Mainly vitamin and mineral premixes	Most ingredients, extra micronutrients added
Possible contaminants		
Phytoestrogens	Absent*	Soybean meal (genistein, daidzein), alfalfa meal (coumestrol)
Heavy Metals	Trace/not detectable	Grains and animal byproducts (arsenic, lead, cadmium, cobalt)
Pollutants, pesticides, mycotoxins, mitrosamines, and endotoxins**	Trace/not detectable	Grains (pollutants, mycotoxins) and animal byproducts (pollutants, nitrosamines)

*Unless soy protein isolate is used.
**Endotoxin source unknown, but high in GB diets (Hrncir et al., 2008).

nutrient at a time, and therefore "revisions" to a GB diet are limited to simple additions.

In contrast, purified ingredient diets are made with refined ingredients, each of which supplies one main nutrient (e.g., casein provides protein and corn starch provides carbohydrate), and their ingredient composition is 'open' to the research community. Their refined nature minimizes non-nutrient contaminants, and relative to GB diets, they are a cleaner, more consistent diet from batch-to-batch (Wise, 1982). Finally, the fact that each ingredient provides one main nutrient allows purified ingredient diets to be easily modified nutrient-by-nutrient. Therefore, one can *report* the diet and nutrient composition, *repeat* the nutrient levels from batch-to-batch, minimizing contaminants, and *revise* their composition with confidence. See Table 1 summarizing commonly used ingredients and nutrient and non-nutrient contributions of both purified ingredient diets and GB diets.

The use of purified ingredient diets in research has been extensive and instrumental for the elucidation of nutrient function as well as for determining the estimated nutrient requirements of lab animals. In 1976, what was then the American Institute of Nutrition (AIN) had the goal of developing a purified ingredient diet with an agreed upon formula that would allow for more straightforward comparisons across different labs. The result was the AIN-76A rodent diet (Bieri et al., 1977), which has been extensively used by toxicologists and others to conduct research with a controlled, repeatable diet with minimal contaminants. In fact, the AIN-76A continues to be used for toxicological studies (Kozul et al., 2008; see Table 2). Later, a second AIN committee decided to revise some of the nutritional shortcomings of the AIN-76A diet. They formed 2 diets: the AIN-93M (M for mature) and AIN-93G (G for growth and reproduction; Reeves, Nielson, & Fahey, 1993). They improved upon the AIN-76A formula by reducing sucrose (from 50% to 10%) and replacing it with corn starch, and replacing corn oil with soybean oil, a better omega-3 fatty acid source. In addition, phosphorus was lowered and calcium was raised to increase the calcium to

Table 2 AIN-76A Diet Formula

	g%	kcal%
Protein	20.3	20
Carbohydrate	66.0	68
Fat	5.0	12
Total		100
kcal/gm	3.92	
Ingredient	g	kcal
Casein	200	800
DL-methionine	3	12
Corn starch	150	600
Sucrose	500	2000
Cellulose	50	0
Corn oil	50	450
Mineral mix	35	0
Vitamin mix	10	40
Choline bitartrate	2	0
Total	1000	3902

phosphorus molar ratio (1.3:1) and minimize the risk of kidney calcinosis, which had been previously observed in weanling female rats fed the AIN-76A, which had an imbalanced ratio (0.75:1; Cockell & Belonje, 2004; Cockell, L'Abbé, & Belonje, 2002). Some of the changes made from the AIN-76A to the AIN-93 series are not necessarily considered 'improvements'. First, while the AIN-93 diets met the recommended level of 3 g of P/kg, they were formulated such that ~half of the phosphorus requirement was met by the casein, with the remainder coming from the mineral mix (in the AIN-76A, the phosphorus requirement was met solely by the mineral mix). Therefore, a dietary modification that reduces casein (e.g., researchers studying a different protein source) could result in a deficient level of P unless the formulator takes care to add back P, leading to the question of what form to add (e.g., sodium phosphate, potassium phosphate). Second, an array of micronutrients were added (e.g., boric acid, ammonium vanadate, lithium chloride, and nickel carbonate), despite no proven requirements for rodents, as acknowledged by the original formulating committee (Reeves et al., 1993). In addition, data suggest that rats fed the AIN-93M diet had a reduced survival rate relative to those fed a GB diet (NIH-31), particularly in calorically restricted rats (Duffy et al., 2002). Both the AIN-76A and AIN-93 diets have been modified with higher fat levels (among other nutrient alterations) for studying obesity, metabolic diseases, and cancer, and during their use in these studies, it was found that even on low fat purified ingredient diets (based on the AIN diets), rodents can still develop mild metabolic disease (increased adiposity and insulin resistance) relative to GB diets in some cases (Benoit et al., 2013; Chassaing et al., 2015). Therefore, while purified ingredient diets do offer advantages over GB diets, data suggest that further improvements are necessary.

FACTORS IN RODENT DIETS THAT AFFECT GUT MICROBIOME RESEARCH

As mentioned above, there are many contaminants in GB diets. Some of these substances (e.g., phytoestrogens, heavy metals) are considered to be endocrine disruptors, which are defined as substances or mixtures that alter function(s) of the endocrine system which may then cause health effects in an organism or their populations. In an effort to control endocrine disruptors, a select few GB diets have been manufactured without soybean meal and alfalfa meal to limit phytoestrogens such as genistein, daidzein and coumestrol. Other GB diets are made with little to no animal by-products, to avoid the presence of nitrosamines. However, the limitation and/or removal of these compounds/ingredients doesn't necessarily eliminate all variables that could affect biological outcomes. By assaying different GB diets across 5 different continents, Mesnage and colleagues (Mesnage et al., 2015) found that GB diets typically contain other potential contaminants including various pesticides, heavy metals, genetically modified grains, polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and dibenzofurans. Their levels in some cases greatly exceeded acceptable daily intakes, according to European standards and those set by the EPA, and were highly variable among these diets. According to these authors, it is conceivable that the presence of these contaminants could be a cause of certain pathological effects observed in laboratory animals that may once have been thought to be 'spontaneous' (Mesnage et al., 2015).

Aside from these possible contaminants, there is another factor that is present in very high levels in GB diets that has until more recently been either ignored or overlooked–dietary fiber. So, what is considered fiber? As

reviewed by Holscher (Holscher, 2017), fiber typically describes most carbohydrate polymers which are neither digested nor absorbed. These polymers enter the lower gastrointestinal tract, some of which can be fermented by trillions of bacteria representing ~1,000 different species. The definitions of fiber vary among different countries but encompass polymers that either include oligosaccharides with 3 to 10 monosaccharide units or only polysaccharides with ≥10 monosaccharide units. These can either be naturally occurring in the food, derived from food by physical, chemical, or enzymatic means, which impart a physiological effect of benefit to health, or are synthesized polymers which have been shown to have a physiological benefit to health as generally accepted scientific evidence (Codex Alimentarius, 2013).

GB diets contain many ingredients which provide dietary fiber. For example, ground corn, whole wheat, ground oats, wheat germ, wheat middlings, alfalfa meal, soybean meal, and dried beet pulp (by-product of sugar beet processing) all provide a significant portion of the total fiber in these diets. To truly define the fiber types and their concentrations in GB diets would require different assay techniques to accurately determine the complete fiber composition present. GB diets have been measured for total, insoluble, and soluble fiber levels with total levels ranging from 15 to 25%, with around 18 to 20% insoluble fiber and around 3 to 5% soluble fiber (Pellizzon and Ricci, unpublished observations). Insoluble and soluble fiber (in large part from plant cell wall material) include a complex array of hemicellulose, cellulose, lignin, and pectin, all of which have been shown to be present–and are variable–in different GB diets (Wise & Gilburt, 1980). The hemicelluloses are defined as a group of polysaccharides that surround cellulose polysaccharides, which include the monosaccharides glucose, arabinose, mannose, xylose, and galacturonic acid, as well as a complex molecular structure with both linear and branched chains. Cellulose itself has a simpler polysaccharide structure in that it consists of linear chains of β-(1,4)-linked glucose units and each chain is bonded together through hydrogen bonds (Ikarashi et al., 2011). Lignin, the second most abundant polymer in nature, is a highly-branched polymer comprised of phenylpropanoid units, which unlike cellulose and hemicellulose, are not polysaccharides, but are covalently bound to fibrous polysaccharides including cellulose and hemicellulose (Kalia et al., 2011). Pectin is also found in plant cell walls and consists of complex polysaccharides comprised of different monosaccharide molecules including galacturonic acid, xylose, rhamnose, and arabinose (Chateigner-Boutin, Bouchet, Alvarado, Bakan, & Guillon, 2014). Typically, GB diets will provide some information regarding the amount of neutral detergent fiber (NDF = combination of hemicellulose, cellulose, and lignin) and acid detergent fiber (ADF = combination of cellulose and lignin), which will allow you to calculate hemicellulose, but not cellulose or lignin separately. Furthermore, while the NDF is a better predictor of total fiber than crude fiber, soluble fibers (i.e., pectin and oligosaccharides) are unknown. Even if this information was available, it has been observed that total, insoluble, and soluble fiber of grains (e.g., ground corn, wheat, and oats) can vary significantly (Stevenson, Phillips, O'sullivan, & Walton, 2012; Vitaglione, Napolitano, & Fogliano, 2008). A review by Vitaglione et al. (Vitaglione et al., 2008) provides ranges of total, insoluble, and soluble fiber in various grains including ground wheat (total: 11.5 to 17%, soluble: 1.4 to 2.3%, insoluble: 10.2 to 14.7%), ground corn (total: 13.1 to 19.6%, soluble: 1.5 to 3.6%, insoluble: 11.6 to 16%), and ground oats (total: 11.5 to 37.7%, soluble: 2.9 to 3.8%, insoluble: 8.6 to 33.9%).

In contrast, purified ingredient diets have typically been formulated to contain only around 5% total fiber in the form of a highly refined (>97%) source of cellulose. Due in part to its insoluble nature, cellulose (unlike most soluble fiber sources) is poorly fermented by most gut bacteria in mice and rats, leading to low levels of the major end products of fermentation, the short chain fatty acids (SCFAs) acetate, butyrate, and propionate. These SCFAs have a variety of potential functions, including providing energy for colonocytes and enterocytes (butyrate), being a source of glucose via gluconeogenesis by intestinal cells or in hepatocytes (propionate) and are potentially capable of entering the circulation and crossing the blood-brain barrier (acetate) to alter appetite (Holscher, 2017). In addition, these metabolites of soluble fiber fermentation may serve to help regulate lipid metabolism and immune function (Holscher, 2017). Knowing this, it is not surprising that cellulose-based purified ingredient diets (such as the above-mentioned AIN based diets) with limited fermentability could lead to adverse health effects both at the

level of the gut and overall. Furthermore, lower fermentation would ultimately lead to less bacterial species diversity, which may have a very potent effect on gut health and metabolic disease development (Chassaing, Vijay-Kumar, & Gewirtz, 2017).

There have been several studies that have compared rodents fed GB diets and purified ingredient diets. Given that a typical GB diet contains very high fiber levels, including fermentable fiber which would lead to SCFA production by gut microbiota, switching animals from a GB to a purified ingredient diet could lead to changes in the overall phenotype, particularly in parameters related to (but not limited to) intestinal health. This next section will discuss how fiber type can alter intestinal health and functionality, and how these changes are linked to changes in microbiota.

EFFECTS OF DIET AND FIBER TYPE ON GUT HEALTH/MICROBIOTA

Rodents fed GB diets and cellulose-based purified ingredient diets have clear differences in gut morphology. Decreases in the size and weight of the cecum and colon in wild type CD-1 mice, Wistar rats, and golden Syrian hamsters were very apparent 28 days after animals were switched from a GB diet to purified ingredient diet (modified AIN-76A diet with wheat starch; Rutten & de Groot, 1992). These authors recognized the differences in fiber contents and types as potential reasons for the very different cecum weights, likely due to increased fermentation of soluble fiber in the GB diet. That soluble fiber in the GB diet is likely key to this trophic effect is supported by data in male C57BL/6N mice fed a GB diet with ~18% total fiber and around 3% soluble fiber (Pellizzon et al., 2015). These mice had dramatically larger ceca and colons after 2 weeks relative to those fed a calorically matched 17% cellulose containing purified ingredient diet (Pellizzon et al., 2015).

Soluble fibers with high degrees of fermentability (i.e., prebiotic fibers) such as inulin can dose-dependently (5, 10, and 20% inulin) increase cecal wall weights and contents in Wistar rats in 3 weeks (Levrat, Rémésy, & Demigné, 1991). In conjunction with inulin-induced increases in cecum weights, blood flow through the cecal vein and concentration of cecal SCFAs also increased. More recently, it was found in rats that the addition of pectin to the AIN-93M diet dose-dependently increased measures (weights and lengths) of gut morphology including the small intestine, cecum, and large intestine weights and lengths (Adam, Williams, Garden, Thomson, & Ross, 2015). Furthermore, measures of gut histology, such as jejunum villus height, crypt depth, and distal ileum crypt depth were improved as early as after 8 days of feeding, demonstrating the rapid morphological and histopathological effects of prebiotic fibers (Adam et al., 2015).

Cecum and colon morphology and weight were maintained at levels found in GB diet-fed C57BL/6 mice when some of the cellulose (10% or 20%) in a purified ingredient diet

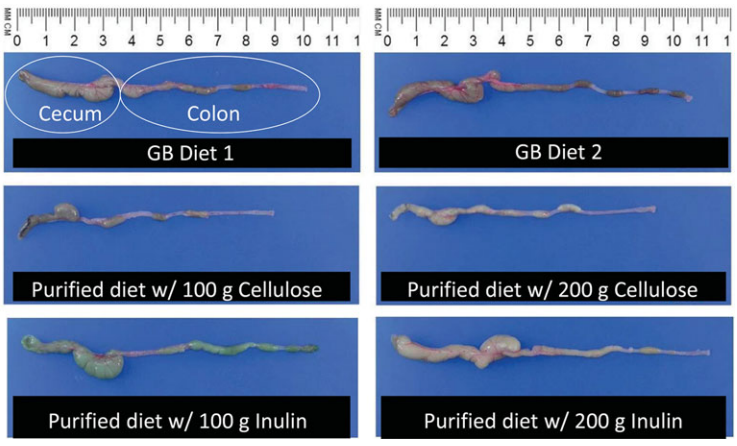

Figure 1 Cecum/colon morphology of C57Bl/6J mice fed 2 GB diets (Diet 1: 18.7% Total, 15.9% Insoluble, 2.8% Soluble Fiber; Diet 2: 18.2% total, 14.9% Insoluble, 3.3% Soluble Fiber) relative to mice fed purified ingredient diets with either cellulose or inulin at 100 or 200 g per 4084 kcals (100 g: 9.3 wt% [cellulose] or 9.6 wt% [inulin]; 200 g: 17 wt% [cellulose] or 18 wt% [inulin]). Groups fed fructooligosaccharides at same concentrations (not shown) had similar morphology to those fed inulin. Data presented at Digestive Disease Week 2015 (Pellizzon et al., 2015).

Table 3 Purified Ingredient Diets with Different Concentrations and Types of Soluble Fiber (i.e., Inulin and FOS) and Insoluble Fiber as Cellulose

	100 g cellulose		100 g soluble fiber		200 g cellulose		200 g soluble fiber	
Fiber contents	g%	kcal%	g%	kcal%	g%	kcal%	g%	kcal%
Protein	18.8	20	19.5	20	17.2	20	18.4	20
Carbohydrate	61.1	65	69.3	65	56.0	65	71.1	65
Fat	6.5	15	6.7	15	5.9	15	6.3	15
Total		100		100		100		100
kcal/gm	3.78		3.92		3.46		3.69	
Ingredient	g	kcal	g	kcal	g	kcal	g	kcal
Casein	200	800	200	800	200	800	200	800
L-cystine	3	12	3	12	3	12	3	12
Corn starch	390.5	1562	353	1412	390.5	1562	315.5	1262
Maltodextrin 10	110	440	110	440	110	440	110	440
Dextrose	150	600	150	600	150	600	150	600
Cellulose	**100**	0	0	0	**200**	0	0	0
Inulin or FOS*	0	0	**100**	150	0	0	**200**	300
Soybean oil	70	630	70	630	70	630	70	630
Mineral mix S10026	10	0	10	0	10	0	10	0
Dicalcium phosphate	13	0	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, 1 H_2O	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0	2	0
Total	**1080.50**	**4084**	**1043.00**	**4084**	**1180.50**	**4084**	**1105.50**	**4084**
Total fiber (%)	9.3		9.6		16.9		18.1	
Total insoluble (%)	9.3		0		16.9		0	
Total soluble (%)	0		9.6		0		18.1	

*Caloric value of inulin and FOS is 1.5 kcal/gm and replaces kcals from corn starch.
Inulin: Orafti HP ~94.5% Insulin (Average DP >=23, DP = 2–60) DP = Degree of Polymerization.
Fructooligosaccharide: NutraFlora P-95 ~90% FOS, 4.75% Sugars (Glucose + Fructose + Sucrose) DP = 3–5.

was replaced with the soluble fibers inulin or fructooligosaccharides (FOS, chains of 3 to 5 fructan units; Chassaing et al., 2015; Pellizzon et al., 2015; see Figure 1 for morphology data and Table 3 for diet formulas). However, gut bacterial phylum changes with inulin or FOS were dramatically different than those fed the GB diets, which included a significantly lower *Firmicutes* and increased *Bacteroidetes*, *Verrucomicrobia*, and *Actinobacteria,* (Fig. 2), the latter which was mainly due to increases in the genus *Bifidobacterium* (Pellizzon et al., 2015). These data suggest that dietary fiber changes that induce similar gut morphology do not necessarily induce a similar gut microbiota profile as compared to GB diets.

The increase in healthy bacteria and improvement in gut morphology when switching from cellulose to prebiotic fibers such as inulin and FOS also can change the functionality of the gut and lead to important benefits to overall health to the host. In particular, certain diverse and stable microbiota such as *Bifidobacteria* and *Bacteroidetes* appear to prevent the thinning of the mucosal lining and reduce gap junctions between enterocytes, which reduces

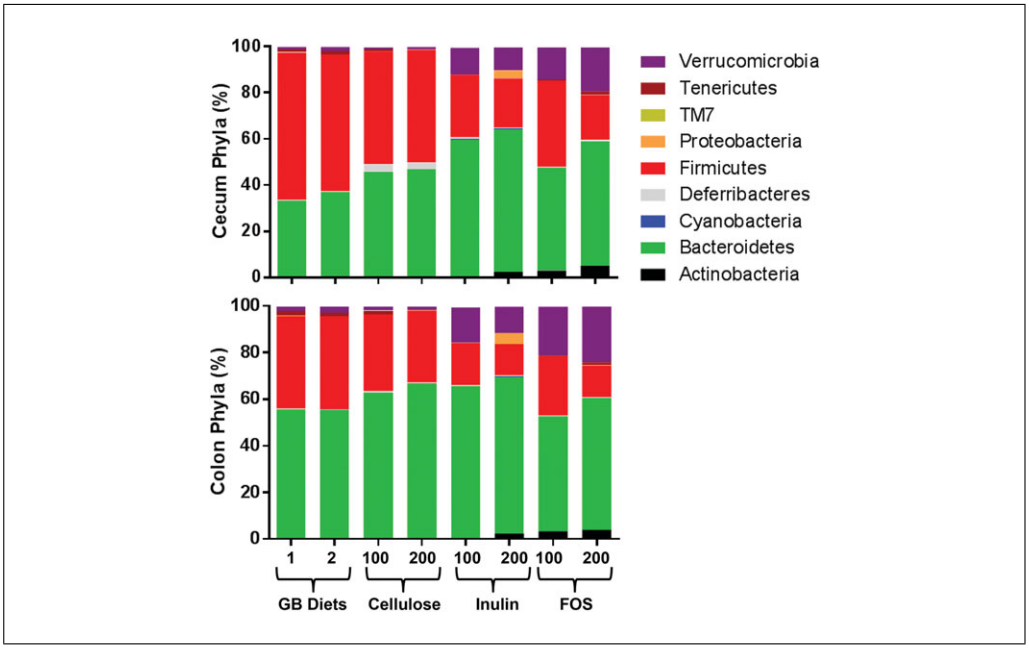

Figure 2 Cecum/colon content Phyla (%) in C57Bl/6J mice fed 2 GB diets (Diet 1: 18.7% Total, 15.9% Insoluble, 2.8% Soluble Fiber; Diet 2: 18.2% total, 14.9% Insoluble, 3.3% Soluble Fiber) relative to purified ingredient diets with 100 g or 200 g fiber per 4084 kcals. Data presented at Digestive Disease Week 2015. Data presented at Digestive Disease Week 2015 (Pellizzon et al., 2015).

permeability (i.e., "leaky gut") and intrusion of bacterial substances such as lipopolysaccharides (LPS) and thereby limits low grade inflammation (Cani et al., 2007; Chassaing et al., 2017). In a study by Desai et al. (Desai et al., 2016), gnotobiotic mice with a synthetic human gut microbiota fed a purified low-fat diet with cellulose had a thinner colonic mucus layer compared to those fed a GB diet. This was driven by increases in mucus-eroding microbiota which feed off the *O*-linked glycans in the mucosal layer, and ultimately disrupted barrier function, leading to low grade inflammation and increased susceptibility to bacterial infection (Desai et al., 2016).

LINK BETWEEN DIET TYPE AND METABOLIC DISEASE DEVELOPMENT LIKELY MEDIATED VIA FIBER TYPE

Over 2,000 years ago, Hippocrates proposed a link between gut health and disease development ("All disease starts in the gut"), and recent studies in rodent models provide some support for this. The type and amount of fiber have been found to be very influential on intestinal health and development of metabolic disease in rodents, and by using purified ingredient diets with higher fat levels and controlled levels of dietary fiber, researchers continue to further our understanding of the relationship between gut and overall health.

As described previously, the beneficial effects of prebiotic fibers on gut health (i.e., improved gut morphology, increased beneficial bacteria and SCFAs, reduced gap junctions) also occur in the context of higher fat diets, such as those with 60 kcal% fat (well above typical low fat diet levels of 10 to 15 kcal% fat). The replacement of cellulose with inulin dramatically increased cecum and colon weights concomitant with reduced adiposity and an increase in fecal SCFAs in both 10 kcal% fat and 60 kcal% fat fed mice (Chassaing et al., 2015; Zou et al., 2017). However, the addition of SCFAs to water in mice fed the high fat purified ingredient diet did not suppress adiposity or improve cecal and colonic weight, (only colon length was increased) suggesting that mechanisms other than SCFAs drove the reduced adiposity by inulin. Indeed, relative to mice fed a 60 kcal% fat diet (mainly lard) with 20% cellulose, Zou et al. found that mice fed 20% inulin had improved insulin sensitivity, glucose tolerance and reduced adiposity with either ablation of the SCFA receptor GPR43 or inhibition of SCFA production (Zou et al., 2017). Instead, they found that the effect of inulin is dependent on a restoration of microbiota and an increase in interleukin (IL)-22, an immune response protein that is

known to be produced by immune cells (NK and T cells) to promote both epithelial cell proliferation and induce antimicrobials, arguing that the addition of soluble fiber impedes normal low grade inflammation (Zou et al., 2017). However, Brooks et al. report contradictory data – that the presence of GPR43 was necessary to reduce adiposity and liver triglyceride levels when animals were fed a lower level of inulin (7.5 g%) in the context of a high fat (21 g% as milk fat) and high sucrose (34 g%) diet (Brooks et al., 2017). Although the reasons for these contradictory data around the importance of the SCFA receptor in mitigating the effects of a high soluble fiber diet are unknown, it's possible that the differences in diet background and fiber level had some influence on the study outcome.

In addition, it's important to consider the type of fructan being used. Liu et al. found that 10% inulin and shorter chain FOS added to a high fat fed (45 kcal% fat) increased cecum crypt depth relative to those fed either 5 or 10% cellulose in the same high fat diet (Liu et al., 2016). In addition, fructan diets also increased *Actinobacteria* and *Verrucomicrobia* (*Akkermansia*) and lowered *Firmicutes* relative to cellulose diets. However, inulin and FOS had differential effects on the integrity of different sections of the cecum and also on colonic barrier function (Liu et al., 2016). Neither fructan reduced body weight despite these changes. Therefore, different fructans that cause similar changes in microbiota may not have a similar influence on overall gut health.

Other fibers such as hemicelluloses derived from wheat and corn, typically found in GB diets, have been found to reduce metabolic disease development in the context of a high fat purified ingredient diet. For example, in a study using a 60 kcal% fat (mainly lard) diet, Neyrinck and colleagues (Neyrinck et al., 2012) found that fermentable fibers such as wheat arabinoxylans added at 7.5% reduced body weight, adiposity, insulin resistance and markers of inflammation in male C57BL/6 mice relative to the same diet with cellulose only. These changes occurred in conjunction with increases in cecal and colonic weights and alterations in microbiota, including increases in *Bifidobacterium* and a reduction in *lactobacillus* as well as an increased mRNA expression of zonula occludens, an important tight junction protein for intestinal barrier integrity. These shifts were inversely associated with plasma inflammatory markers IL-6 and lipopolysaccharides (LPS), which provides further evidence linking soluble fiber-induced changes in microbiota with reduced inflammation. As a side note, a GB diet was used in this experiment, but as alluded to above, this GB diet likely had a significant amount of wheat arabinoxylans (and if not, other plant-based fibers with potential to improve gut health) and should only be viewed as a 'reference diet'.

The corn bran hemicelluloses (oligo- to polysaccharides made up of a complex of heteroxylans, which could be present in GB diets) have been shown *in vitro* to be fermented for longer periods relative to those derived from wheat (Yang, Maldonado-Gómez, Hutkins, & Rose, 2014). The addition of a heteroxylan complex of corn hemicellulose at 5% (in place of cellulose) to a 60 kcal% fat purified diet increased cecal weights, but only in half of the mice studied after 8 weeks (Yang et al., 2016). In the mice that responded to the hemicellulose, SCFA levels were increased in the cecum. This was not driven by a cage effect as responders and non-responders were in the same cages. Like SCFA levels, those responding showed significant shifts in fecal microbiota, including increases in genera *Akkermansia* and *Blautia* as well as lower fasting glucose and insulin levels and improved insulin sensitivity and glucose tolerance relative to cellulose only diets after 7 weeks (Yang et al., 2016). As no metabolic improvements were found in non-responders, this suggested that fermentation of this fiber was responsible for these phenotypical changes and that how each mouse responds may differ for a given corn hemicellulose. It is noteworthy to point out that this group of researchers chose a matched purified ingredient low fat control diet with cellulose, which differed from the high fat diet mainly by an increased level of corn starch calories in place of lard calories (i.e., 10 kcal% fat, 70 kcal% carbohydrate as mainly corn starch). Therefore, we can be certain that the changes between the high fat and low fat group were mainly due to changes in fat and carbohydrate content alone. This is unfortunately not as commonly done and all too often do we find a GB diet used as a 'control' diet for a purified ingredient high fat diet.

CHOOSE THE CONTROL DIET WITH CARE

As described in detail above, purified ingredient diets are very different from GB diets, and as one may expect, these differences

can play a role in data interpretation when both are used in the same study. The problem with using a GB diet as a control diet for a high fat purified ingredient diet has been discussed and recent surveys suggest this problem still can be found in many studies (Fodde, Schmitt, Schewe, & Augenlicht, 2017; Pellizzon & Ricci, 2018; Rendina-Ruedy & Smith, 2016; Warden & Fisler, 2008). In a recent survey of 69 publications including *Cell*, *Nature*, and *Diabetes* using search terms 'mouse high fat', it was found that only 18.8% of studies used a matched purified ingredient low fat control diet for the high fat diet while 40.6% used a GB diet as the low fat 'control' diet, with the remaining 40.6% of papers not providing sufficient data to determine what control diet was used (Pellizzon & Ricci, 2018). A study by Chassaing et al. (Chassaing et al., 2015) brought this issue to light when they used 3 different diets in their study: a GB diet (Purina 5001) and 2 purified ingredient diets, one with 10 kcal% fat and the other with 60 kcal% fat, both with cellulose as the only source of fiber. They observed that C57BL/6 mice fed *either* purified ingredient diet had obvious visual reductions in colon and cecum weights compared to those fed the GB diet. Had they just used the GB diet as the 'low fat control', they could easily have concluded that the reduced cecum and colon morphology was due to the high fat content of the purified ingredient diet. In the same study and another by this same group, these researchers found that the reduced gut morphology and mucosal barrier induced by cellulose based purified ingredient diets was driving obesity and metabolic disorders (Zou et al., 2017). Others have also found that the choice of the low fat comparator diet matters (i.e., GB diet or purified ingredient low fat diet) when interpreting how other metabolic disease parameters such as insulin sensitivity, plasma triglycerides, and certain markers of inflammation are affected by a purified ingredient high fat diet (Benoit et al., 2013).

As long as the control diet is carefully considered, the use of properly matched purified ingredient diets (with low and high fat levels) will continue to provide useful data on how the diet affects the gut microbiome, which in turn influences metabolic health in rodent models. In fact, it will be necessary to continue to use purified ingredient diets in order to control the type of fiber rodents–and ultimately their resident microbiota–are consuming. An example of properly matched low and high fat diets is shown in Table 4.

Given a 'drawback' of many historical purified ingredient diets–that cellulose is typically the only fiber source–researchers (and manufacturers) should consider purified ingredient diets with both insoluble and soluble fiber in an effort to provide a similar 'healthy' gut phenotype induced by GB diets and to minimize the shift in the gut microbiota profile. This approach may also have the benefit of maximizing other phenotypic differences between high and low fat purified ingredient diets (glucose tolerance, hepatic fat levels), something that should encourage the use of defined control diets in place of the variable GB diet 'control'.

HOW TO REPORT A DIET FOR PUBLICATION

It has been recently estimated that around 50% of preclinical research is irreproducible, and this has been attributed to several factors that differ among studies, including biological reagents and reference materials, study design, data analysis and reporting methods, and laboratory protocols (Freedman, Cockburn, & Simcoe, 2015). While there are many environmental factors in lab animal studies that may affect experimental data (and in particular, the gut microbiota profile), diet has been considered one of the most important (Laukens, Brinkman, Raes, De Vos, & Vandenabeele, 2015). Yet, poor diet reporting is still a significant problem in research (Pellizzon & Ricci, 2018). In order to improve our ability to repeat and compare research, it is important to report the details of the diets used in the methods section of any publication. How to report dietary information has been described previously (Institute for Laboratory Animal Research, 2011) and is included (with some mild revision) below.

In addition to the frequency and method of feeding (e.g., ad libitum vs. portioned), effective reports include:

1. Type of diet (i.e., purified ingredient diet, GB diet). Terms such as "Standard Diet", "Standard Chow", "Normal Diet", or "Breeder Chow" are never appropriate;
2. Manufacturer name and location;
3. Complete catalogue or diet number;
4. Complete diet formulation (when 'open' or available, should always be the case for purified ingredient diets but typically not for GB diets);
5. Dietary form (i.e., extruded, pelleted or powder/meal, paste, gel, liquid, precision pellets);
6. Handling and storage methods;

Table 4 Matched Low and High Fat Purified Ingredient Diets

	60 kcal% fat (D12492)		10 kcal% fat matched diet (D12450J)	
	g%	kcal%	g%	kcal%
Protein	26	**20**	19	**20**
Carbohydrate	26	**20**	67	**70**
Fat	35	**60**	4	**10**
Total		100		100
kcal/g	5.2		3.8	
Ingredient	g	kcal	g	kcal
Casein	200	800	200	800
L-cystine	3	12	3	12
Corn starch*	**0**	**0**	**506.2**	**2025**
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275	68.8	275
Cellulose	50	0	50	0
Soybean oil	25	225	25	225
Lard*	**245**	**2205**	**20**	**180**
Mineral mix S10026	10	0	10	0
DiCalcium phosphate	13	0	13	0
Calcium carbonate	5.5	0	5.5	0
Potassium citrate, 1 H_2O	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
FD&C yellow dye #5	0	0	0.04	0
FD&C red dye #40	0	0	0	0
FD&C blue dye #1	0.05	0	0.01	0
Total	**773.85**	**4057**	**1055.05**	**4057**

*Difference only in corn starch and lard kcals where corn starch provides 4 kcal/g and lard provides 9 kcal/g.

7. Any nutrient or non-nutrient analyses performed.

SUMMARY AND CONCLUSIONS

There is a wealth of data that demonstrates the importance of choosing the lab animal diet with care, especially when studying the gut microbiome and its links to various diseases, including (but not limited to) metabolic disease development. The complete ingredient composition should be available in order for researchers to make informed choices and avoid unexpected outcomes. In particular, fiber is a major dietary factor that will impact the gut microbiome, and given the substantial differences in their fiber content, comparisons between GB diets and purified ingredient diets are not appropriate and should be avoided. Rather, GB diets may be considered as reference 'phenotype diets' along-side a purified ingredient low fat control diet. Though the purified ingredient diet typically contains cellulose, it is easy to modify the fiber content in favor of increased gut bacterial fermentation and metabolic health. Further research is still needed to clarify the mechanisms of how fiber improves metabolic health, and such research should be conducted with defined, repeatable diets.

CONFLICTS OF INTEREST

Michael Pellizzon and Matthew Ricci are employees of Research Diets, Inc.

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