Laboratory Animal Diet Considerations for Germ Free Studies

2020 - A commentary on current research considerations

Laura Griffin, Ph.D. Project Manager & Scientist, Research Diets, Inc.
Steven Yeung, M.S Project Manager & Scientist, Research Diets, Inc.

The human gut is host to trillions of microorganisms and is unique to individuals (1). Numerous environmental factors are known to influence the microbiome, including short and long term dietary trends (2). It is now understood that the gut microbiome influences host health and is involved in disease onset (2, 3). As such, the volume of research revolving around the gut-microbiome's involvement in health and disease has escalated in the past two decades (3). Mouse models are the most commonly used mammalian species in mechanistic studies of health and disease. To fully understand the role of the gut microbiome in disease states, it is necessary to perform research studies in a variety of mouse models including germ-free or gnotobiotic colonies (4). Germ free animals are maintained so that their gut microbiomes are uncolonized i.e. they are not hosts to any other living organisms. Similarly, gnotobiotic animals are free of pathogens, but may be host to specific organisms in a controlled environment (5).

With the expansion of germ-free mouse research, there is an increased need for properly controlled, content-stable and modifiable lab animal diets for the study of the gut microbiome. There are two main classes of animal research diets- grain-based 'chow' diets, and purified ingredient diets (6, 7). Grain-based diets are traditionally used as 'maintenance' diets in animal research facilities and are made out of cereal grains and animal by-products such as soybean meal, ground wheat, and fish meal. These ingredients contribute multiple nutrients to the overall diet composition, and often also contain non-nutritive components as well (e.g. phyto-chemicals,arsenic and other heavy metals, endotoxins, mycotoxins, pesticides, pollutants), which are subject to batch-to-batch variability (6). In contrast, purified ingredient diets are composed of highly refined ingredients that each contribute one main nutrient to the overall diet composition and as a result it is straightforward to modify their nutrient content – a crucial consideration when wanting to study the effects of specific dietary components on the gut microbiome. Purified ingredients are subject to little to no variation over time and don't fluctuate in the same way as grain-based diets (8).

Despite the clear differences in composition between these diet types and numerous literature references counseling against comparing one diet type to the other (6, 8–10), the use of grain-based diets as the 'control' diet for a custom formulated purified ingredient diet is unfortunately still quite common (9, 10).

Dalby et al. and Chassaing et al., demonstrated that the gut microbiome is wildly different amongst animals that are fed a grain-based diet compared to either a purified low- or high-fat diet (4), Moreover, the historical lack of soluble fiber in most purified high-fat diets, not just fat level alone, is associated with increased adiposity and gut morphological changes in animal models, and animals on this type of diet should not be compared to those on a fiber-rich, grain-based diet (11). These observations demonstrate the need for more research on how the gut influences metabolic disease, as well as to highlight the necessity to utilize proper control diets for these endeavors (4, 9, 10).

Given that the content of grain-based diets (nutrients, non-nutritive components, fiber) can vary from batch to batch, this could influence phenotype and ultimately lead to misinterpretation of study results. The use of grain-based diets adds an additional problem in germ-free studies. Grain-based chows contain ~100-300 times more lipopolysaccharide (LPS) compared to purified diets and LPS-rich diets can influence development of the immune system in gnotobiotic mice (12–14). In addition, the highly variable levels and types of fiber that can be found in these diets may significantly impact study results, especially if the study design involves comparisons of germ-free mice to conventional or conventionalized mice. As with conventional animal studies, the use of purified ingredient diets in germ-free and gnotobiotic research allows the researcher to have confidence that the diet being fed to the animal is not a source of variation from batch to batch.

Given the need to carefully control diet in germ-free animal studies, it is imperative to use purified ingredient diets for this purpose. In the remainder of this brief review we will summarize some of the considerations that researchers should make especially regarding the use of purified diets used in germ-free studies.

Reduction of Bioburden of Diets for Germ-Free Animal Facilities

Since neither the ingredients nor the diet production process are considered sterile, laboratory animal diets can contain variable levels of microbes. Therefore, one of the most important considerations when conducting a study in germ-free mice is the procedure used to reduce the bioburden of the diet. The effects of the procedure on both bioburden and nutritional content of the diet must be considered. Traditional grain-based rodent diets can be autoclaved in small packages prior to placement in germ-free isolators. The autoclaving process adds a great deal of heat and moisture and renders the diet sterile (15). Upon cooling, the diet becomes quite hard and almost impossible to break and distribute amongst animal cages; however, this typically does not alter food intake (16). Moreover, the extreme conditions associated with autoclaving can reduce the nutritional content of the diet and promote lipid oxidation, which limits the severity of the treatment that can be performed (15, 17). Autoclaving purified ingredient diets is not recommended, as this process can cause the pellets to melt and fuse into one large mass, particularly those higher in fat, as well as lead to unfavorable changes to the nutrient contents.

The recommended method of reducing bioburden in purified ingredient diets is gamma irradiation (18). This process works by exposing the diet to a controlled amount of gamma rays from a radioactive source- typically cobalt 60 (17). Exposure of the diet to the radioactive source effectively kills bacteria by destroying bacterial DNA (19). While this procedure does not guarantee complete sterility (as is the case with autoclaving (15)), it is capable of significantly reducing the microbial load in rodent diets. The recommended dose for diets meant for germ-free facilities is approximately 40 – 50 kGy (17).

Our typical dose of irradiation for purified ingredient diets to be used in conventional animal research facilities is 10-20 kGy, which is one irradiation cycle. Given the stricter bioburden criteria for diets to be fed to germ-free animals, and the above-mentioned recommended irradiation dose for germ-free facilities, we examined the effects of multiple cycles of irradiation on the culturable bacteria and the content of certain vitamins in a low-fat and a high-fat purified diet. Three samples from each diet/irradiation treatment were taken and irradiated either once, twice or three times. Culturable bacteria were found in only 1 replicate (5 cfu/gm) from one of the diets that was exposed to only 1 cycle of irradiation (10- 20 kGy). Irradiating the diets either twice (20-40 kGy) or three times (30-60 kGy) resulted in no detectable colony forming units in any replicate of either diet.

Vitamin Supplementation

It is known that gamma-irradiation reduces the vitamin content of laboratory animal diets (17). The extent of reduction may depend on several factors, such as the irradiation dose (19), and not all vitamins may be affected equally. This poses a concern particularly with diets intended for germ-free facilities given the higher doses of irradiation required.

We have conducted several in-house studies on micronutrient losses associated with gamma-irradiation in both low- and high-fat purified diets which were supplemented with 50% more vitamins than the standard concentrations. Standard concentrations added to the diets are slightly higher than what the National Research Council (NRC) recommends for rodents. (For example, the NRC recommended thiamin dose is 5 mg/kg; we ensure nutritional adequacy by supplementing our diets with 6 mg thiamin/kg, or 9 mg thiamin/kg for double-irradiated diets. After three rounds of irradiation (30- 60 kGy combined), even with a fortified vitamin mix, these diets had lower levels of vitamins, including vitamin A, pyridoxine, and thiamine than what the NRC recommends. In each case, the remaining vitamins in the diet did not meet the recommended levels for normal animal growth in both low- and high-fat purified diets. We observed that gamma and delta tocopherol levels were reduced by irradiation, but alpha-tocopherol was not affected. Although this significantly reduced the total vitamin E content in the diets, this may not be terribly impactful on rodent health, as alpha-tocopherol is considered to be the most biologically active form of this vitamin. Another in-house study comparing single irradiation to double irradiation in the same low- and high-fat purified diets (this time without a fortified vitamin mix) indicated that thiamine levels were below the recommended levels after only 2 rounds of irradiation, which further supports the procedure of increasing vitamin concentration if more than 1 dose of irradiation is required.

Since three cycles of irradiation were associated with substantial reduction in vitamin content of the diets and increased peroxide values, our data support that two cycles of irradiation for a total dose of 20-40 kGy sufficiently reduces the microbial load of purified diets, and fortification with 50% extra vitamins sufficiently offsets losses so that basic nutritional requirements are maintained[1]. Of course, other formula changes are possible with purified ingredient diets and further fortifications of certain vitamins may deemed necessary. For example, vitamins such as vitamin K, which are typically synthesized by certain microbial organisms that are absent in germ-free mice, may need to be fortified at a much higher level than what is found in diets for conventional mice (15).

Other Considerations

One of the major advantages of purified ingredient diets is the ability to customize the formula depending on the goals of a research study. Aside from fortifying the vitamin mix as discussed above, here are other topics that a researcher should consider when working with germ-free animals.

[1] We do not routinely assess the microbial bioburden of our diets after irradiation. We suggest that you test the diet regularly as part of your microbial monitoring protocol.

Protein Sources

Traditionally, our purified ingredient diets are made with a casein source that is precipitated by lactic acid bacteria. While it is likely that most of these bacteria are killed during the pasteurization step by the manufacturer, and that any remaining viable bacteria are killed during irradiation of the final diet, it may be possible to detect lactococcus 'parts' by 16S rRNA sequencing in fecal samples of germ-free animals fed diets with lactic casein (20).

This finding may also be observed in conventional animals treated with antibiotics for the purpose of knocking down the gut microbiome. This potential issue can be resolved in two different ways. The less expensive route would be to substitute a mineral acid-extracted casein in place of the lactic acid precipitated version. The alternative option would be to use individual amino acids in place of casein altogether. Overall, it does not appear that gamma-irradiation alters protein digestibility or quality (19, 21).

Packaging

Historically our diets have been packaged in large plastic bags, or double-bagged if irradiated. The diets typically comes in 2.5, 5, or 12.5 kg aliquots depending upon the order size. We do offer a variety of smaller sized bag options, which would allow for smaller amounts of diet to be stored in isolators where space is limited. It also allows for surplus diet to be stored in appropriate conditions (-20C for high-fat diets, for example) and prevents all of the diet from becoming contaminated if a seal is accidentally broken. Heat sealed and vacuum packaging are also available, though we recommend heat sealed bags over vacuum packaging, as the irradiation process can cause the vacuum seal to break. Moreover, the vacuum process can also crush soft pellets (high-fat) and allow low-fat diets with defined edges to pierce the bag during the vacuum process.

Shelf-life

It is known that while irradiation will reduce the microbial load of the diet, thus increasing shelf life in that regard, some detrimental chemical processes that reduce diet quality may be accelerated (19). For example, Maillard browning reactions can be increased in diets that have been exposed to gamma-irradiation. This is more visually noticeable in amino-acid based diets. Additionally, high doses of gamma-irradiation may increase lipid peroxide values, as discussed previously, which may also contribute to a shortened shelf life (19). While proper storage conditions (-20C) for these types of diets can slow some of these shelf-life decreasing effects of gamma irradiation, it is also not ideal to open isolator ports multiple times a week to provide fresh diet. If you are conducting a study that utilizes a diet susceptible to these processes, such as an amino acid diet or a high-fat diet, it is imperative to discuss and plan the frequency of adding new diet to the isolators accordingly. There is no perfect solution at this time, but the risk of opening the isolator port more frequently must be weighed against the possibility of diet degradation at room temperature and the impacts of both on your study outcomes. Regardless, we recommend keeping as much of the diet as possible stored per our recommendation and introducing small amounts into the isolator at a time.

Incorporate Test Compounds

As with any custom diet, it is possible to add test materials to a purified ingredient diet. When it comes to adding compounds to a diet intended for germ-free animals, it is imperative to determine if the compound would be inactivated by the irradiation procedure. It is also worth considering how long the compound might be stable at room temperature (presumably in an isolator) after such a treatment. If you are concerned about your compound withstanding these conditions, one alternative approach that you may wish to consider is introducing the compound into the isolator via sterile filtration at your facility (16). While this approach is more complex than delivery within the animal diet, it may be the best option if you plan to work with a sensitive compound.

The science team at Research Diets Inc. is ready to discuss your specific study details and diet formulations. When you are ready, please contact one of our scientists at info@researchdiets.com to start a conversation about controlling the diet for your germ-free or gnotobiotic study.

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20 Jules Lane | New Brunswick, NJ 08901 USA | Tel: 732.247.2390 Fax: 732.247.2340

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