Dietary Induction Models of Nutritional Disorders in Rodents

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Bases for Translational Studies

Edited by

Jailane de Souza Aquino,
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CHAPTER 1 - PRESENTATION

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Nutritional disorders are at the heart of the development of a wide range of medical conditions including malnutrition, obesity, diabetes, dyslipidaemia, and hypertension. These diseases together represent the main cause of death in the world, regardless of the development level of a country. The high social and economic cost generated by metabolic diseases has led the scientific community to redouble efforts to try to stop the incessant deleterious effects that they cause. Such efforts have focused on understanding the pathophysiological mechanisms involved in the development of these diseases. To do so, the use of rodent-based animal models has represented and continues to represent an invaluable approach. The use of mice and rats have made it possible to gain a large body of knowledge that has helped to better understand the negative consequences of nutritional disorders.

Despite the extensive use of animal models in research on nutritional disorders, there is still debate about them in relation to their correct applicability and translational relevance. Different physiological and behavioural characteristics existing between humans and rodents are often topics of discussion and disagreement among researchers. In the interest of providing a clearer picture, this book discusses the biological plausibility of rodent models as tools for understanding metabolic disorders and eventual translation in humans.

The second chapter discusses the suitability of rodents as models of health and disease. It addresses the advantages and disadvantages of using these animals as models, particularity rodents. The third chapter presents nutritional recommendations for rodents based on recommendations for calories, carbohydrates, lipids, proteins, minerals, vitamins and fibres. This chapter also presents the types of diets and some examples of dietary modifications performed in studies with rodents. Next, chapters 4, 5, 6, 7, and 8 deal with the induction models of malnutrition, obesity, dyslipidaemia, diabetes and hypertension, respectively, validated for rodents at different stages of life, as well as their physiometabolic repercussions and the diagnosis of these diseases, mainly focusing on dietary models that induce these metabolic disorders.

Chapter 9 focuses on the link between studies with animal models and clinical studies, addresses the importance of translational research, the conversion of doses of substances administered in rodents and humans, as well as the equivalence of age and life cycles between rodents and humans, aiming to direct study designs and models that can facilitate advances in this field of scientific research. Finally, chapter 10 discusses the applicability of studies with animal models in the prevention and treatment of metabolic disorders.

In this context, this book is aimed at professionals, undergraduate and graduate students in the health area and all those who may be interested in the importance of non-clinical studies and how they may be permeating our lives.

CHAPTER 2 - RODENTS AS MODELS OF HEALTH AND DISEASE

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The use of animals in biomedical research is important to knowledge in health care, the firsts registry of animal experimentation were observed before Christ in Greece and Egypt, since that time animals experimentation were used to get knowledge in anatomy and physiology (1–3). Some physician-scientists such as Aristotle (384 - 322 b.c), the first to conduct dissection in animals and demonstrated the difference among animals and Erasistratus (304 - 250 b.c), used living animals, he studied the functions of airways and heart in pigs (1,4).

Later, Galen (a.b 130 - 200) performed anatomical dissection in monkeys, pigs and other animals; Galen was considered the originator of research in physiology and anatomy. His dissection of animals and work on monkeys and other animals were models for human anatomy and physiology and the bases for many treatises (3,5).

However, after Galen, dissection of animals and dead people were prohibited by ecclesiastical authorities who wanted to prevent acquisition of knowledge about the natural world that could be considered blasphemous, just in the 1500 century the experimentation began to restart (1).

In the 17th century, the use of animals in research began to increase, based on the ideas of Renee Descartes. In his opinion that animals were like machines, that do not have spiritual elements, which are possessed by humans only (1,3). At 18th, the French Claude Bernard (1813 - 1878) conducted many researches and contributed in the physiology field, besides recognizing the importance of the use of animals for the biomedical research (1,4,5).

On the other hand, Louis Pasteur (1822-1895) used animals' experimentation to study microbiology, contributed to validation of scientific methods. Also, Pasteur study of a scourge of the time in man and animals, anthrax, and later to chicken cholera. It was with chicken cholera that he developed a method of attenuating bacilli and producing a method of control through immunization (1).

The use of animal models in the biomedical sciences is a long-standing practice, and has contributed to the understanding and expansion of our understanding of the variables that influence human health. Presenting an equally relevant effect for animals, because several procedures and treatments in humans can be performed in animals (3,6). A number of scientific advances for human health were only possible due to the use of laboratory animals (Table 2.1).

Table 2.1. Some scientific advances for human health that used laboratory animals as models, in chronological order

Reference	Study	Animal model	Year
Crawfor et al. 2013 (7)	Franz Reisinger performed the first experimental corneal transplant	Rabbits Chickens	1818
Nossaman et al. 2010 (8)	Development of the cardiac catheterization technique	Horse	1844
Vecchio et al. 2018 (9)	Insulin used to treat Type I Diabetes was discovered by Frederick Grant Banting and John James Rickard Macleod	Dogs	1921
Pratt et al. 1936 (10)	Sodium thiopental intravenous anesthetic	Rats Rabbits Dogs	1936
Gaynes 2017 (11)	Ernst Boris Chain and Sir Howard Walter Florey first tested penicillin	Mice	1939
Francis et al. 1945 (12)	First flu vaccine	Mice Role Fertilized chicken eggs	1942
DeVita and Chu 2008 (13)	Development of chemotherapeutic agents	Mice	1943
Musso and Gubler 2016 (14)	Isolation and serological investigations of the Zika virus	Monkeys Mice	1947
Baicus 2012 (15)	Testing with monkeys and mice led to the introduction of the first polio vaccine	Monkeys Mice	1954
Mercado et al. 2020; Corbett et al. 2020; Zhang et al. 2020; van Doremalen et al. 2020 (15–19)	Currently available vaccines for SARS-CoV-2	Monkeys Mice	2020

Research with animals provides experimental models that could not be performed using human beings, such as toxicological tests, surgical techniques and vaccine development. Furthermore, some animal species have physiological and genetic similarities with humans that make them relevant for the study of certain diseases. The benefits that animal experiments have brought to biomedicine are undeniable, but it is necessary

to ensure the well-being and proper use of animals, and it is imperative to respect the principles of the 3Rs (Replace, Reduce and Refine) that was proposed by researchers William Russell and Rex Burch in 1959 (3,20–22):

- Replace, whenever possible, the use of animals with methods that employ non-sentient material (*in vitro*, *in silico*, invertebrates);
- Reduce as much as possible the number of animals used to achieve the objective of an experiment;
- Refinement involves adopting experimental procedures to improve the animal's well-being and health, minimizing or eliminating pain and suffering.

Strech and Dirnagl (2019) (23), proposed the addition of additional 3Rs to improve the scientific dimension of animal research, forming the 6R principle:

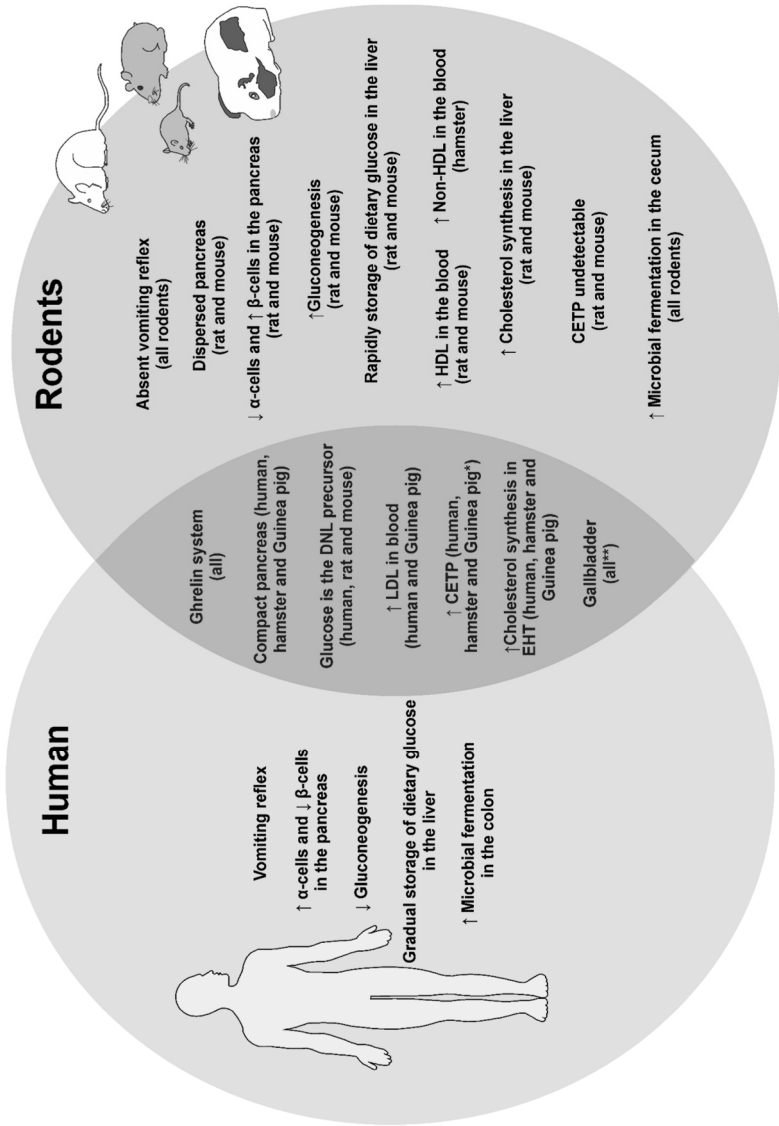
- Robustness - Adequate study design to avoid biases;
- Registration - Accessible reports that include null and negative results;
- Reporting - Related to the registration of study protocols, which allow greater transparency of results.

To improve transparency in animal research reports for reviewers and readers, the ARRIVE guidelines - Research with Animals: Reports of In Vivo Experiments) (24) were created in 2010, being updated in 2020 to ARRIVE 2.0 (25). This guideline consists of a list of items divided into two sets that must be included in manuscripts that report in vivo experiments. The first set describes the basic information that must be included in the manuscript, while the second set contains items that assess the context of the study described.

Implementing the 3Rs or 6Rs in studies requires commitment and encouragement from organizations that support science with laboratory animals, such as the Laboratory Animal Science Council (ICLAS). ICLAS was conceived in 1956 as an international scientific organization that focuses on ethically promoting animal-based science globally and protecting animal welfare (26)

Laboratory rodents, mainly rats, mice, hamsters and Guinea pigs, are widely used in studies to understand human biology and diseases. These rodents have some similarities in anatomy, biochemical and physiology with humans, but each rodent has individual characteristics that limit the extrapolation of different results to humans (Figure 2.1) (27–29).

Figure 2.1. Venn diagram of some anatomical, physiological and metabolic similarities and dissimilarities between humans and rodents



Notes: CETP, cholesterol ester transfer protein; DNL, *de novo* lipogenesis; EHT, extrahepatic tissues. * CETP is detectable in Guinea Pig; ** Except in the rats.

The vomiting reflex is present in humans and rabbits, but not in rodents. This ability may be related to the presence of functional motilin and its receptor in humans and rabbits, but this protein does not exert its function in rodents (30–32). Motilin is a gastrointestinal hormone produced in the small intestine that stimulates motility and gastric emptying to control food intake. The ghrelin is a gastric hormone from the motilin family that is found in both humans and rodents and appears to exert the same functions as motilin in rodents. In addition, ghrelin acts inside and outside the gastrointestinal system, modulating feeding, appetite, endocrine control and energy metabolism (31–33).

The effects of ghrelin on energy metabolism cover the modulation of glucose homeostasis through the stimulation of liver gluconeogenesis, fat mass accumulation, and the improvement of mitochondrial function in the skeletal muscle (34). Gluconeogenesis provides 40–50% and 80% of endogenously glucose, respectively, in humans and rodents with an emphasis on mice (35). The rodents have high energy demand, so they rapidly store dietary glucose as glycogen in hepatocytes (36).

Systemic glucose homeostasis is guaranteed by pancreatic islets through the secretion of insulin and glucagon hormones. Insulin reduces and glucagon increases blood glucose depending on postprandial state and fasting. Insulin and glucagon are secreted by the pancreatic β-cells and α-cells, respectively (37). There are some differences between the pancreas of humans and rodents: a) humans have more α-cells than β-cells in the pancreas, the opposite occurs in rodents; b) glucose transport in β-cells is dependent on glucose transporter protein isoform 2 in rodents, while it depends on glucose transporter protein isoform 1 in humans; c) the pancreas of humans and hamsters are compact and well defined, whereas the pancreas of other rodents is dispersed throughout the intestinal mesentery (38–41).

Glucose is the principal supplier of carbon units for hepatic *de novo* lipogenesis (DNL) or de novo fatty acid in humans and rodents, mainly rats and mice (42). *De novo* lipogenesis contributes to the progression of nonalcoholic fatty liver disease through increased secretion and storage of lipids in the liver (43). The lipid metabolism of humans and rodents has differences in some aspects: a) higher plasma concentrations of LDL in human and Guinea pig, HDL in rats and mice, and a non-HDL fraction (mainly VLDL and chylomicron remnants) in hamsters; b) elevated cholesterol synthesis in rat and mouse liver, and in extrahepatic tissues (i.e. gastrointestinal tract) of human, hamster and Guinea pig; c) higher cholesterol ester transfer protein (CETP) activity for hepatic HDL uptake

and reverse cholesterol transport in humans, hamsters and Guinea pigs; d) rats and mice are CETP-deficient species, thus HDL is directly transported to the liver via scavenger receptor B1 (44).

It is worth noting that microbial fermentation occurs predominantly in the cecum of rodents, but in humans, it occurs in the colon (27,45,46). The products of microbial fermentation, mainly short-chain fatty acids, regulate energy metabolism and contribute to the maintenance of intestinal and systemic health in humans and rodents (47,48).

In research with human beings, it is necessary to carry out an ethical evaluation in order to guarantee three basic principles including the research subject, the researcher and society: beneficence, respect for the person and justice. The ethical evaluation must include the qualification of the researchers, the methodological adequacy of the project, the evaluation of the risk-benefit ratio and the informed consent, which is previously evaluated by the ethics committees. This conduct aims to avoid risks to the health of the participants, in addition to reducing costs without their results being really used, due to deficiencies in the method (49).

Before starting research with humans, other possibilities of obtaining data through simulations, use of animal models, cell cultures or others must be exhausted. Once human research is started, privacy and confidentiality of the participants' data are necessary, as quoted in the Belmont Report (50).

Since medical experimentation during the Second World War, there has been a code of conduct in order to avoid disorders in human research, namely the Nuremberg Code (51), this code was the starting point in the fight for the prohibition of abusive experiences with human beings, showing that the human being cannot be considered a simple object for science. Subsequently, in 1964, the Declaration of the World Medical Association of Helsinki brought 12 basic principles for the conduct of human biomedical research (52). Between the main principles, we can observe themes regarding the: basic principles for all medical research; risks, burdens and benefits; vulnerable groups and individuals; scientific requirements and research protocols; research ethics committees; privacy and confidentiality; informed consent; use of placebo; post-trial provisions; research registration and publication and dissemination of results; unproven Interventions in clinical Practice (25). Following all the principles, it is possible to guarantee ethical research with humans.

All protocols and processes for working with humans, from the time available, knowledge of techniques, participants able to research, to financial resources that make the entire project viable, limit the use of humans in research, thus, bringing as an alternative to use of animals.

Due to the similarity with humans in systemic physiology, genetics, organs and pathogenesis, nutritional disorders become possible to be evaluated in rodents, allowing the study of associated diseases, pathophysiological mechanisms, as well as ways of prevention or treatment, in a short space of time (53,54), given that rodents have a shorter life cycle than humans (55). There are several models of induction of nutritional disorders in rodents, such as the use of genetic models (56–58), surgical techniques (59–61) , and induction by chemical substances (62–64). However, the model that most resembles the human reality is the induction of nutritional disorders through dietary induction (65–68).

In this sense, the following chapters of this book will focus on dietary models of induction in rodents of nutritional disorders with the higher incidence and prevalence in the human population, and on the importance of carrying out translational research.

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CHAPTER 3 - NUTRITIONAL RECOMMENDATIONS AND DIETS FOR RODENTS

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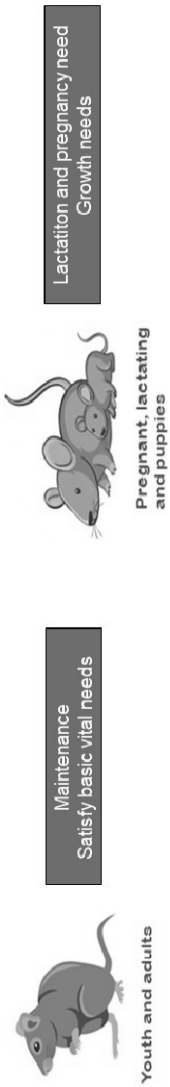
Animal nutrition is one of the essential elements in the production of laboratory animals, influencing their ability to develop, grow, reproduce, fight disease, and respond to experimental manipulation. In addition to ensuring animal welfare, a nutritionally balanced and planned diet affects the quality and reproducibility of experimental results (1). First, scientists neglected the role of diet as a relevant variable for experimental results. As scientists began to realize that the reproducibility of their data was influenced by dietary factors, they began to design their research paying attention to the diet provided to the animals in order to avoid errors and facilitate interpretation of results (2-3).

Figure 3.1. Rodent feeding and its relationship with the life cycle



The diet formulations are based on several aspects such as the nutritional needs of the animal, food, diet demonstration format, and expected food intake, in addition to food adequacy according to the life stage and development of the animal (Figure 3.1) (4).

The diets used in scientific research with rodents aim to meet the nutritional requirements of each stage of life and try to place them between the minimum value of maximum recommended value in order to favor criteria of growth, reproduction, longevity, nutrient storage in the body, and all functionality of the animal's biology. Likewise, the types of diets and purposes can also change (1).

The first dietary pattern for rodents was proposed by the American Institute of Nutrition - AIN in 1976, and since then the AIN-76 diet has been used in nutritional and toxicological studies (5). However, this diet presented some problems, including a vitamin K deficiency. In order to resolve the issue, the Committee on Animal Nutrition recommended increasing the amount of vitamin K to 500 mg/kg of diet and using a stabilized form of menadione. In addition, it has been suggested to add an antioxidant to corn oil and to modify the sucrose content if the researchers perceive the need (6). Over the years it was detected that this AIN-76 purified diet had nutritional and technical problems, with emphasis on the tendency to cause renal calcification in female rats, which led to reformulation and new diets being proposed (7).

The changes between the first formulation, AIN-76 and its reformulation (renamed as AIN-93), occurred in several aspects from its composition to the ingredients used. A conference named "FASEB Workshop - Federation of American Societies for Experimental Biology" was held in 1989, organized by Forest Nielsen and Philip Reeves to review the diet formulation and make new recommendations (based on new concepts). The recommendations discussed at this event were then tested until the AIN-93 diet was released to the scientific community in 1993 (8).

Among the changes from the AIN-76 diet to AIN-93, we can highlight:

- Carbohydrate source (sucrose, cornstarch and dextrinized cornstarch);
- Addition of L-Cysteine in the protein source;
- Soybean oil as a source of fat;
- Addition of the tert-butyl hydroquinone (TBHQ) antioxidant;
- Change in chemical form and amount of vitamins and minerals;

- Subdivision of recommendations according to life stage (growth and maintenance)

Diet preparation is also an important point for rodent nutrition. Ingredients must be of good quality, purchased from reliable suppliers and from a single batch, while storage must be in a dry place, away from moisture without contact with the ground, and other fat-source ingredients must be stored in a place with refrigeration.

3.1 Diet types

There is a diversity of diets used for laboratory rodents. Researchers should consider what kind of diet will be fed to the animals before conducting an experiment because dietary factors can influence phenotypes, ranging from health to disease (3-9).

The chosen diet should be based on the experimental objectives to be achieved. In addition, care should be taken to ensure a palatable diet that meets the nutritional needs of the animals (1). Diet formulations are classified according to the degree of their ingredients into chemically defined diets, purified diets and diets with natural ingredients (commercial feed).

3.1.1 Natural ingredient diet

Natural ingredient diets are used in most bioterium and are manufactured and marketed under vendor trade names. They are formulated with agricultural products and industry by-products, such as wheat grains, corn, bran, fish meal, blood meal and others (4-10), which makes them relatively cheaper to manufacture, as well as being more palatable to most rodents (10).

Diets are manufactured using open or closed formulas. However, natural product diets primarily use “closed” formulas, meaning manufacturers provide a list of ingredients that have been used and nutrient concentrations, but ingredient amounts are not publicly available (Table 3.1). Furthermore, variations in the composition of ingredients can occur due to their complex nature, which can influence the concentration of nutrients, and may vary from batch to batch of the diet (Table 3.2) (11). These variations can affect the interpretation of experimental data and their reproducibility.

Table 3.1. Ingredients and composition of commercial diet

Ingredients:		
Whole Ground Corn, Soybean Bran, Wheat Bran, Calcium Carbonate, Dicalcium Phosphate, Sodium Chloride, Vegetable Oil, Vitamin A, Vitamin D3, Vitamin E, Vitamin K3, Vitamin B1, Vitamin B2, Vitamin B6, Vitamin B12, niacin, calcium pantothenate, folic acid, biotin, choline chloride, iron sulfate, manganese sulfate, zinc sulfate, copper sulfate, calcium iodate, sodium selenite, cobalt sulfate, lysine, methionine, BHT.		
Moisture (max)	125	g/kg
Protein	220	g/kg
Ethereal extract	50	g/kg
Mineral Mix (max)	90	g/kg
Fibre (max)	70	g/kg
Vitamins: Vitamin A 13.000UI; Vitamin D3 2000mg/kg; Vitamin E 34.00mg/kg; Vitamin K3 3.00mg/kg; Vitamin B1 5.00mg/kg; Vitamin B2 6.00mg/kg; Vitamin B6 7.00mg/kg; Vitamin B12 22.00mg/kg; Niacin 60.00mg/kg; Calcium pantothenate 21.00mg/kg; Acid folic 1.00mg/kg; Biotin 0.05mg/kg; Choline 600.0mg/kg;		
Minerals: Sodium 2.700mg/kg; iron 50.00mg/kg; Zinc 60.00mg/kg; Copper 10.00mg/kg; Iodine 2.00mg/kg; Manganese 50.00mg/kg; Calcium 10.000mg/kg; Phosphor 6.000mg/kg; Selenium 0.05mg/kg; Cobalt 1.50mg/kg		
Amino acids: DL-Methionine 300.00mg/kg; Lysine 100.00mg/kg.		
Additives: Butylated Hydroxytoluene (BHT)	100mg/kg	

Notes: Commercial food for rodents, Brazilian brand. All values are based on 1 kg of diet

Table 3.2. Composition of four commercial feeds for rodents of different brands

Diet	A	B	C	D
*Energy value (kcal/g)	-	-	3	3.1
Protein (%)	22	23	21	18.6
Lipid (%)	5	4	5	6
Fibre (%)	7	5	4	3
Mineral mix (%)	9	9	6.1	5.3

Notes: Brazilian brand feed (A); Brazilian brand feed (B); International brand feed (C); International brand feed (D). * The energy value was not reported by these feed brands.

Open-formula diets can be reproduced by any manufacturer, as they provide the concentration of all ingredients used in their production. These diets are standardized for laboratory rodents at the National Institutes of Health (NIH) in order to reduce variability in diet formulations. However, these diets also have the same problems with the batch-to-batch nutrient variation found in closed diets (4-12). It is possible to modify diets in their composition and quantity, with this strategy being feasible to induce nutritional disorders in rodents, for example: induction of obesity with diets mixing a commercial ration with refined sugar and condensed milk, which presented 315.26 kcal, 21% of proteins, 4% of lipids, and 48% of carbohydrates (13), induction of obesity using a hypercaloric diet with foods such as peanuts and biscuits ground and chocolate (14).

Dietary modifications of rodents can also serve as models to elucidate the effects of macronutrients and metabolism and physiological processes. As an example, we have diets with changes in the compounds and fats in order to observe the ingestion of trans fatty acids in the intestinal microbiota and in the intestinal microbiota, which can have distinct effects on metabolism and on the intestinal microbiota rats (14), as well as diets containing soy oil, refined or crude buriti oil and its impact on the lipid and vitamin profile of rats (15).

Finally, all of these modified diets need their nutrient values stated from an analysis of the nutritional composition performed by the researchers during the study, thus helping their reproduction in other laboratories.

3.1.2 Purified diet

Ingredients such as casein (protein), sucrose and starch (carbohydrates), and vegetable oil (lipids) are used in the purified diet. These are diets which provide the quantitative composition of their ingredients, allowing researchers to control this environmental variable, as well as evaluate possible effects of diet formulation on research results (16). These diets show less variation in nutrient composition from batch to batch compared to diets with natural ingredients.

The AIN diet was last reformulated in 1993 and follows the current nutritional recommendations for rodents to date. The AIN-93G diet is recommended for the growth, pregnancy, and lactation phases, while the AIN-93M diet is recommended for the maintenance phase (17). The composition of the AIN-93M and AIN-93G diets are shown in Table 3.3.

Table 3.3. Composition of AIN-93G and AIN-93M diets

Ingredients (g/100g)	**Diets**	
	AIN-93G	**AIN-93M**
Casein (≥85% protein)	20.0	14.0
Maize starch	39.7	46.5
Dextrinized starch (90% to 94% polysaccharides)	13.2	15.5
Sucrose	10.0	10.0
Soybean oil (no additives)	7.0	4.0
Fibre (microfine cellulose)	5.0	5.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-cystine	0.3	0.18
Choline Bitartrate	0.25	0.25
Tert-butyl hydroquinone (TBHQ)	0.14	0.08

Notes: AIN-93G, diet for growth; AIN-93M, diet for maintenance.
Source: Adapted from Reeves et al. (1993) (12)