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Dry Heat Sterilization of a Pelleted, Natural Ingredient Rodent Diet

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Sterilization of rodent feed is recommended to eliminate potential murine pathogens and minimize microbial variability between batches. Most research institutions sterilize feed using steam/pressure (autoclave) or irradiation. Both methods have advantages and disadvantages that contribute to their suitability, including cost, maintenance, availability, and alterations to the exposed product. Dry heat sterilization, which has been in use for over 75 y, uses higher temperatures and longer sterilization times than steam autoclave and is most often used for delicate instruments or products that would be damaged by water such as powders or oil-based liquids. Dry heat sterilization in vivaria has been limited to date but is gaining popularity due to lower initial purchase and ongoing operational costs as compared with steam autoclaves. Little published information exists on the effects of dry heat sterilization on animal feed. We evaluated the sterility and chemical alterations of a natural ingredient, pelleted, rodent diet (NIH-31) after exposure to dry heat. Feed sterility was achieved using a dry heat exposure temperature of 160 °C (320 °F) for 4 h. This exposure resulted in a significant loss of heat-labile vitamins and significantly more acrylamide production as compared with the nonsterile, irradiated, and autoclaved feed.

Abbreviations and Acronyms: BI, biological indicator; BAP, blood agar plate; NIEHS, National Institute of Environmental Health Sciences

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Introduction

Animal feed can be a source of extrinsic variability in research studies, and the quality of feed and the knowledge of potential contaminants should be considered in study design.[20] Microbial contamination of feed is common and unavoidable, especially in natural ingredient diets. Mouse parvovirus a common viral pathogen in research mice, has been reported as likely coming from unsterilized feed.[37] We recently demonstrated the presence of *Clostridium perfringens*, a well-documented pathogen, in nonsterile, natural ingredient laboratory animal feeds.[17] As such, we generally recommend the use of sterilized feed whenever possible, especially for natural ingredient diets in rodent studies, to eliminate pathogenic microorganisms and reduce microbial variability between lots of feed.

The most common methods of feed sterilization used by animal research facilities are γ-irradiation or steam autoclave. Few institutions have the capability to irradiate feed and depend on a third party to perform this service, which can add substantially to the costs. Many institutions autoclave their feed; however, autoclaves are expensive to acquire, operate, and maintain. Sterilization by dry heat has been widely used in the medical and pharmaceutical industries for over 75 y but has not been widely adopted in animal research facilities. Delicate surgical or dental instruments easily damaged by steam and pharmaceutical powders or oil-based compounds that cannot be exposed to water are the most common products sterilized using dry heat.[22,28] Dry heat sterilization generally occurs at temperatures of 160 °C (320 °F) or higher; however, lower temperatures have been validated.[2] The low-moisture content of dry heat is much less efficient for heat transfer compared with steam and thus requires much longer exposure times.[28] Dry heat sterilization has many benefits as compared with steam autoclaves including lower initial procurement cost, smaller facility footprint, and lower maintenance/operational costs. These benefits are appealing to animal research programs that want to save space, lower costs, and reduce energy consumption.

Autoclaving and irradiation are known to affect feed quality.[20] The destruction of heat-labile vitamins, such as thiamine (B_1), vitamin A, and vitamin E, by autoclaving is one of several well-documented effects.[20,23] To compensate for this loss, animal feed manufacturers will increase the vitamin concentrations in their "autoclavable" formulations. Irradiation has been shown to affect vitamin K activity in germ-free mice,[14] vitamin A in feline diets,[5,9] and the concentration of glycosinolates and oxidized lipids in rodent diets, which results in changes in tumor growth.[6,27] Heating feed can also result in the production of acrylamide, a known neurotoxin,[24] genotoxin,[3,7,8,12] and carcinogen,[15,26,36] and acrylamide has been reported in autoclaved rodent feed.[34] We demonstrated that the use of higher temperatures for autoclave sterilization resulted in concomitant increases in the concentrations of both acrylamide in the feed and mouse livers, as well as DNA adducts in the livers of mice fed autoclaved NIH-31 feed for 28 d.[21]

We could not find any peer-reviewed reports on the chemical alterations in rodent feed that was sterilized by using dry heat. One manufacturer of a dry heat oven performed vitamin analysis of 2 different, natural-ingredient rodent diets after dry-heat exposure and reported no notable differences in thiamine (B_1),

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vitamin A, and vitamin E concentrations as compared with nonsterilized feed.[13] We performed the current study to verify those results and to evaluate acrylamide production.

Materials and Methods

Animal feed. All feed used for this study was the open formula, natural ingredient NIH-31, autoclavable formulation.[19] Trial 1 used one lot from US Manufacturer 1. Due to a contractual change in our feed supplier, trials 2 to 14 used one lot (lot 2A) from US Manufacturer 2. Trials 15 and 16, the final trials in which chemical testing was performed, used one lot (2B) from Manufacturer 2 for all 4 feed sterilization methods (nonsterile, irradiated, autoclaved, and dry heat).

Sterilization methods. Dry heat sterilization of feed was performed by placing 385 g of nonsterile, NIH-31 feed in the wire hopper of individually ventilated cages (Tecniplast-GM500 Green line, Exton, PA) with bedding (Sani-Chip, PJ Murphy Forestry Products, Ladysmith, WI). Dry heat exposure was performed in a Gruenberg Industrial Oven (model T45H76.88SS, Thermal Product Solutions, New Columbia, PA). The exposure temperatures and times and the number of cages per trial are listed in Table 1. Trial 1 used 2 cages per run and was a pilot trial that used times and temperatures reported to DMK by several US institutions. In trial 2, 30 cages were exposed (5 rows with 6 cages/row). With the assistance of Process Control Solutions, LLC, thermocouples were placed within the feed or bedding of 9 randomly chosen cages dispersed on all rows throughout the 30 total, and feed from all 9 cages was tested for sterility. Trials 3 to 12 were performed using 2 cages per trial with incremental increases in temperature and/or time (Table 1) until feed sterility was achieved. Trials 14 to 16 were performed on 10 cages per trial (5 cages/row), and 4 cages (2/row) located at the center of each row were tested for feed sterility. Biologic indicator (BI) spore strips validated for Dry Heat Sterilization [MesaLabs (Bozeman, MT)–Dual-Species (cat. no. 5-5100T), *Geobacillus stearothermophilus* (10^5 cfu) and *Bacillus atrophaeus* (10^6 cfu), or Crosstex (Rush, NY; cat. No. BG-106), *Bacillus atropheaus* (10^6 cfu)] were buried in the center of the feed in the same feed tested cages, and a chemical indicator strip (Crosstex Dry Heat Indicator Strip, cat. no. DIS-100) was placed on top of the wire bar in the same feed tested cages. For trials 3 to 16, a biologic indicator spore strip and a chemical indicator strip were also placed in a clean, 400-mL glass beaker (no feed, exposure control). After the designated exposure times (Table 1), the cages were allowed to cool for 20 min before removal from the oven.

Gamma-irradiation of feed, used in trials 1, 15, and 16, was performed on 25 lb feed bags by a third-party company. The feed was exposed to a cobalt-60 source with a total exposure of 25 to 50 KiloGray and was then delivered to NIEHS and stored at 12.7 to 15.5 °C (55 to 60 °F) for approximately 1 wk before use.

Autoclaving feed, used in trials 1, 15, and 16 was performed by placing 385 g of nonsterile, NIH-31 feed in the wire hopper of individually ventilated cages (Tecniplast) with bedding (Sani-Chip). The cages were autoclaved using an Amsco Scientific Series, Stage 3 Autoclave (Steris Life Sciences, Mentor, OH). The sterilization temperature was 121.1 °C (250 °F). Sterilization exposure time was 20 min, the drying time was 5 min, and purge time was 1 min with 4 pulses. A VERIFY Dual Species Self Contained Biologic Indicator [cat. no. S3061, *Geobacillus stearothermophilus* (1 to 5×10^5 cfu) and *Bacillus atrophaeus* (10^6 cfu), Steris, Mentor, OH] was buried in the center of feed, and a 3M Comply (SteriGage) Chemical Integrator (3M, St. Paul, MN) was placed on top of the wire bar in select test cages. In the trial 1 pilot study, 2 cages were autoclaved, and feed from both were tested for sterility. Ten cages per trial (5 cages per row) were used for trials 15 and 16, and feed from 4 cages (2/row) from the center of the rows was tested for sterility.

Microbiological testing. Total feed microbial content was measured in 10 gm of nonsterile feed that was aseptically ground using a commercial blender and a sterile, stainless-steel jar. The ground feed was placed into 100 mL of sterile phosphate-buffered water and mixed. Serial dilutions were aliquoted to sterile 100 mm petri dishes (Falcon 351029, Corning Life Sciences, Corning, NY) and overlayed with tryptone glucose extract agar (total plate counts) that had initially been melted at 100 °C then cooled to 55 °C before use. Once samples were mixed and agar solidified, the plates were inverted and incubated at 37 °C. After 48 h, colony counts were performed.

Feed sterility was tested by aseptically placing 25 g of feed into 250 mL of sterile thioglycolate broth and incubating at 37 °C. Both positive and negative control thioglycolate broth cultures were run concurrently with all sterilization trials. These broth cultures were monitored daily for turbidity. Once turbidity was noted, the broth culture was gently swirled, and approximately 25 µl was transferred aseptically to duplicate, 100-mm blood agar plates (BAPs). One BAP was incubated aerobically at 37 °C, and the other BAP was incubated anaerobically at 37 °C in a sealed BD BBL GasPak container system with BD BBL GasPak EZ Anaerobe Sachet with Indicator (Franklin Lakes, NJ). BAPs were examined for bacterial growth at 24 (aerobic) or 48 (anaerobic) hours. If no turbidity was noted in the thioglycolate broth cultures, they were transferred to BAPs as described above after 7 d of thioglycolate inoculation (trials 1 to 14) or days 7 and 14 after thioglycolate inoculation (trials 15 and 16). The diet sample was considered sterile if the 7-d BAPs (trials 1 to 16) and 14-d BAPs (trials 15 and 16) were negative for bacterial growth.

After their exposure to a sterilization method, the biologic indicators were processed as instructed by the manufacturer. The VERIFY biologic indicators were incubated at 55 °C for 24 h. The biologic indicator spore strips were aseptically placed in culture media [MesaLabs, Modified Tryptic Soy Broth (cat.no. TSP-BP16) or Crosstex Tryptic Soy Broth with BTB Indicator (cat. no. GMBTB)] and incubated at 35 °C for 7 d. A color change from purple to yellow indicated bacterial growth and a failure of the biologic indicator to achieve sterility. The color change of the chemical indicators used in both the autoclaved and dry heat-exposed trials was recorded.

Bacteria isolated from several trials were identified via 16S rRNA PCR of the hypervariable regions 3 to 9 followed by external Sanger sequencing (Azenta Life Sciences, Burlington, MA). Sanger sequencing results were trimmed and assembled (CLC Main Workbench 8, Qiagen, Hilden, Germany) using the default settings. Assembled 16S contigs were identified by uploading sequences into the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) using the 16S ribosomal RNA sequences (bacteria and archaea)' database, and the top BLAST score result was reported.

Pellet hardness testing. Our group previously described a method to measure feed pellet hardness.[36] Feed pellet hardness testing was measured using a Chatillon Digital Force Gauge (Model DFX II, Ametek, Largo, FL). The pound-force per inch squared required to break the pellets was measured on 90 randomly selected, uniformly sized pellets from each test group (trials 15 and 16). Pellet hardness testing was performed at room temperature (25 °C).

Vitamins analysis. Approximately 250 g of feed was ground in a sterile stainless-steel jar using a commercial blender, packaged in

Table 1. Summary of NIH-31: Dry heat exposure trials

Trial	Feed vendor (lot)	Temperature °C (°F)	Time (min)	Number cages exposed	Number cages tested	Chemical indicator color	Crosstex biologic indicator feed	Mesa-Labs biologic indicator feed	Beaker without feed biologic indicator(s)	Feed sterility (sterile/total)	Notes
1A	1 (A)	149 (300)	120	2	2	Dark green		Not sterile		0/2	
1B	1 (A)	160 (320)	60	2	2	Dark green		Not sterile		0/2	
2	2 (A)	140.5 (285)	120	30	9	Dark green		Not sterile		0/9	Temperature and time recommended by PCS. Thermocouples placed in all 9 test cages.
3	2 (A)	149 (300)	120	4	4	Dark green	Not sterile	Not sterile	Sterile (both)	0/4	
4	2 (A)	160 (320)	105	2	2	Dark green	Not sterile	Not sterile	Sterile (both)	0/2	
5	2 (A)	149 (300)	180	2	2	Black	Not sterile	Not sterile	Sterile (both)	0/2	
6	2 (A)	149 (300)	240	2	2	Black	Sterile	Not sterile	Sterile (both)	0/2	
7	2 (A)	149 (300)	240	2	2	Black	Sterile	Sterile	Sterile (both)	0/2	
8	2 (A)	149 (300)	240	2	2	Black	Sterile	Sterile	Sterile (both)	0/2	
9	2 (A)	160 (320)	120	2	2	Black	Not sterile	Not sterile	Sterile (both)	0/2	
10	2 (A)	160 (320)	180	2	2	Black	Sterile	Sterile	Sterile (both)	1/2	Only 1 of 2 feed samples were sterilized.
11	2 (A)	160 (320)	240	2	2	Black	Sterile	Sterile	Sterile (both)	2/2	
12	2 (A)	160 (320)	240	2	2	Black	Sterile		Sterile	2/2	
13	2 (A)	160 (320)	240	2	2	Black	Sterile		Sterile	2/2	
14	2 (A)	160 (320)	240	10	4	Black	Sterile		Sterile	4/4	Tested cages located at center of load.
15	2 (B)	160 (320)	240	10	4	Black	Sterile		Sterile	4/4	Tested cages located at center of load.
16	2 (B)	160 (320)	240	10	4	Black	Sterile		Sterile	4/4	Tested cages located at center of load.

Chemical indicator: Crosstex Dry Heat Indicator Strip (cat. no. DIS-100); bright green: unexposed; black/brown: exposed. Crosstex biologic indicator: *B. atropheaus* only (cat. no. BG-106) and Crosstex TSP media: (cat. no. GMBTB-100, lot no. 23038G). MesaLabs biologic indicator: Dual Species Spore Strips: *G. stearothermophilus* and *B. atropheaus* (cat. no. 5-5100T) and MesaLabs TSP media (cat. no. TSB-BP16, lot no. TSBP-044. PCS, process control services, LLC.

a sterile, plastic pouch, and shipped overnight on cold packs to a testing laboratory [NP Analytical Laboratories (NPAL), St. Louis, MO]. Vitamin analysis was performed by NPAL for vitamins A, B_1 (thiamine), B_6 (pyridoxine), B_{12} (cobalamin), and E. Two feed samples from each group were tested in trials 15 and 16.

Acrylamide analysis. Acrylamide was extracted from rodent feed by using a procedure that we had previously[21] adapted from one used for fried potato chips.[4] Acrylamide analysis was performed by HPLC–MS/MS on 2 feed samples from each of the tested trials (15 and 16), and all samples were assayed in triplicate. The limit of detection for acrylamide in feed was 5.0 parts per billion.

Statistical analysis. Statistical analysis was performed using a one-way ANOVA and Tukey multiple comparison test. All analyses were performed by using Prism 10.0.1 (GraphPad Software, San Diego, CA). Significance was set at a $P < 0.05$.

Results

Microbiological testing. The initial bacterial load of all lots of nonsterile, NIH-31 was 440 to 4,200 cfu/g, which is typical of our historical testing of the NIH-31 feed.

Table 1 summarizes the 16 trials of dry heat exposure to sterilize feed in this study. Trial 1 used 2 different dry heat exposure times and temperatures (1A, 149 °C [300 °F]/120 min; 1B, 160 °C [320 °F]/60 min). For trial 1, the tested exposure times and temperature combinations did not sterilize the feed or the biologic indicators. The dry heat-exposed chemical indicators should change from a light green (unexposed) color to a black/brown color if the proper temperature and time are achieved. In trial 1, the chemical indicators turned to a dark green color. In our opinion, this dark green color indicated that the proper time and temperature were not achieved. In contrast, the autoclaved and irradiated feeds tested in trial 1 were both sterile, the autoclaved biologic indicators achieved sterility, and the autoclaved chemical indicators turned black indicating proper temperature and time were achieved.

The thermocouples used in trial 2 confirmed that the desired temperature [127 °C (260 °F) for a minimum of 1 h] was achieved in both feed and bedding in the tested cages, as recommended by Process Control Solutions. However, neither the feed nor the biologic indicators in the 9 cages tested achieved sterility, and the chemical indicators turned a dark green color.

In trials 3 to 11, we used 2 different biologic indicator strips in each test cage (buried in feed) and in the glass beaker (no feed control). Process Control Solutions, the company that assisted with the thermocouple testing (trial 2) advised that *G. stearothermophilus* in the MesaLabs dual spore strip might not be suitable for dry heat sterilization. Both biologic indicators placed in the glass beaker achieved sterility in all trials (3 to 11). In trial 6 [149 °C (300 °F), 240 min], the Crosstex strip (single spore) in the tested feed samples was sterilized, but the MesaLabs dual spore strip in the tested feed was not. In this same trial (6), the feed was not sterilized in any tested cages. The bacteria that we isolated and identified from nonsterile (trial 1) and dry heat-exposed NIH-31 (trials 1, 6 to 10) are listed in Table 2. As expected, most isolates were spore-forming bacteria that often survive the manufacturing processes and are more likely to survive inadequate sterilization cycles.

We incrementally increased the dry heat sterilization temperature or time until the feed from all test cages were sterilized. This required an exposure of 160 °C (320 °F) for 4 h (240 min) (trial 11) (Table 1). We repeated these conditions for 2 cages (trials 12 and 13) and then for 10 cages (trials 14 to 16); the feed and biologic indicators were sterile in all trials. The chemical indicator strips in all test cages turned black/brown. In the final trials, 15 and 16, the irradiated and autoclaved feed was both sterile, all autoclaved biologic indicators achieved sterility, and all autoclaved chemical indicators turned black indicating proper temperature and time was achieved.

Pellet hardness testing. We measured feed pellet hardness in nonsterile, irradiated, autoclaved, and dry heat sterilized NIH-31 feed from trials 15 and 16. The combined results from both trials are presented in Figure 1. As we demonstrated previously,[33] the autoclaved feed was significantly harder than the nonsterile or irradiated pelleted feed. However, the dry heat sterilized feed pellets were significantly less hard than those of any other test group.

Vitamin analysis. Vitamins were analyzed in nonsterile, irradiated, autoclaved, and dry heat-sterilized diets from trials 15 and 16. The results for these 2 trials were combined and are reported as mean ± SEM ($n = 4$/group) in Figure 2. For comparison, the figure also shows the National Research Council (NRC) recommended vitamin requirements for mice[25] and the calculated vitamin concentrations reported by the diet manufacturers. Vitamin A and thiamine (B_1) were both significantly lower in both the autoclaved and dry heat sterilized diets as compared with the nonsterile and irradiated diets. The vitamin A concentrations in the dry heat sterilized feed were

Table 2. Bacteria isolated from NIH-31 feed

Trial	Feed group	Time (min)	Bacterial isolate
1	Nonsterile		*Alkalihalobacillus clausii/rhizosphaerae* *Oceanobacillus caeni* *Weissella cibaria* *Weissella paramesenteroides*
	Dry heat (149 °C [300 °F])	120	*Alkalihalobacillus clausii/rhizosphaerae* *Bacillus haynesii/paralicheniformis/piscis/licheniformis* *Weizmannia acidiproducens*
	Dry heat (160 °C [300 °F])	60	*Bacillus haynesii/paralicheniformis/piscis/licheniformis*
6	Dry heat (149 °C [300 °F])	240	*Caldibacillus thermoamylovorans/hisashi*
7	Dry heat (149 °C [300 °F])	240	*Bacillus amyloliquifaciens* *Weizmannia coagulans*
8	Dry heat (149 °C [300 °F])	240	*Bacillus amyloliquifaciens/valensis*
9	Dry heat (160 °C [320 °F])	120	*Clostridium perfringens* *Clostridium puniceum* *Clostridium tertium* *Weizmannia coagulans*
10	Dry heat (160 °C [320 °F])	180	*Weizmannia coagulans*

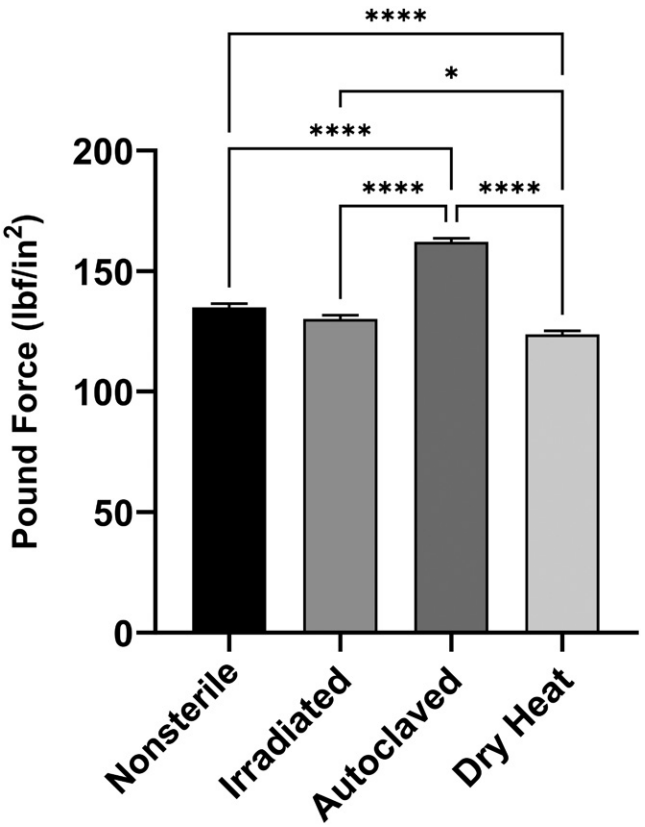

Figure 1. Feed pellet hardness. *, $P < 0.05$; ****, $P < 0.0001$.

significantly lower than that of the autoclaved feed. Cobalamin (B_{12}) was also significantly lower in the autoclaved and dry heat-sterilized diets as compared with the nonsterile diet. The pyridoxine (B_6) concentration did not differ significantly in the nonsterile, autoclaved, or dry heat sterilized diets but was significantly lower in the irradiated diet as compared with the nonsterile diet, as has also been reported in irradiated human foods.[11,35] The only significant difference noted in vitamin E was between the irradiated and autoclaved samples. The difference in vitamin E was negligible, and we were unable to find any reports on vitamin E loss by irradiation.

Acrylamide analysis. Acrylamide was analyzed in nonsterile, irradiated, autoclaved, and dry heat exposed NIH-31 feed from Trials 15 and 16. All test groups used feed from the same lot. The acrylamide results for trials 15 and 16 are reported as mean ± SEM ($n = 4$/group) in Figure 3. The acrylamide concentration in the autoclaved feed was significantly higher than that of both the nonsterile (5.5-fold higher) and irradiated feeds (3.1-fold higher). The dry heat sterilized feed had significantly more acrylamide than did all other tested groups and was 10.5-fold higher than in the autoclaved group.

Discussion

Many animal research institutions are starting to use dry-heat sterilization of rodent cages both with and without feed due to the benefits mentioned earlier. We also found these benefits appealing and therefore studied the potential for using dry heat sterilization in our program. One evaluation criterion was the effect on feed quality. Feed quality is a potential extrinsic source of study variability that can be minimized if carefully evaluated. Two major contributors to potential dietary extrinsic variability are the concentrations and types of microbial or chemical contaminants present, especially after sterilization of natural ingredient diets.

At NIEHS, we feed sterilized diets to our rodent colonies by purchasing autoclavable or irradiated diets whenever possible. While the NIH-31 diet is formulated to be autoclaved, we have established a maximum concentration level of nonsterile, microbial contamination of 2×10^5 cfu/g.[20] We also screen our feed for total coliform concentration and the presence of *Salmonella* spp. Otherwise, we rarely identify specific bacteria present in the low number of nonsterile diets we use. The feed lots used in this study had a low initial bacterial burden (440 to 4,200 cfu/g), which is expected given that the heat used in the feed pelleting process kills a significant number of microbes, especially nonspore-forming bacteria.

Although one of our primary goals of this study was to establish dry heat parameters that resulted in feed sterility, we did identify bacteria isolated from our nonsterile feed and those that survived ineffective dry heat exposures (Table 2). Most isolates are common soil bacteria, which might be expected in a natural ingredient diet. One isolate from the nonsterile feed, *Weissella cibaria*, has been reported as an opportunistic human pathogen.[18] *Clostridium perfringens* was isolated from one sample of dry heat-exposed feed (trial 9). While *C. perfringens* is a well-known human and animal pathogen, we recently reported that *C. perfringens* is a common isolate from natural ingredient animal diets, but none of these feed isolates possessed the genes responsible for the major disease-causing toxins.[17] We also isolated *Clostridium tertium* from the same sample. This organism has been reported as a rare human pathogen.[29,38] We also isolated *Caldibacillus thermoamylovorans* from another dry-heat-exposed sample. This organism can cause milk spoilage and is a concern of the dairy industry.[10] The isolation of these bacteria from rodent feed justifies its sterilization before use.

Feed pellet hardness is not routinely tested as part of our feed quality evaluation, but this feature can be an important aspect of feed manufacturing and sterilization processes.[32] Pellet hardness can be affected by several factors including the pellet's size and shape, moisture content, concentration of various ingredients (especially binding agents), pelleting time, and temperature.[32] Steam autoclaving increases pellet hardness, most likely by caramelization of carbohydrates.[21,32] Increased pellet hardness can reduce feed intake in mice, thereby reducing weight gain and reproductive performance.[20] Before this study, no data were available on the effect on dry heat exposure on rodent feed pellet hardness, and we wanted to determine whether the higher temperatures used with dry heat influenced pellet hardness as compared with steam autoclave sterilization. As previously reported,[13] dry heat exposures of feed in our study did not result in feed clumping that often occurs after steam sterilization. Our pellet hardness data demonstrate that the feed exposed to dry heat was significantly less hard than the autoclaved feed (Figure 1). Moreover, the nonsterile and irradiated pellets were both harder than the feed exposed to dry heat. Sterilization by dry heat occurs primarily by dehydration and oxidation of the exposed product,[28] and the dry heat could dehydrate the feed such that the feed pellets break more easily.

All of the contacted US animal research facilities using dry to sterilize cages or cages with feed used biologic indicators to assess sterility. Our results indicate that the spore strip biologic indicators that we tested were not reliable indicators of feed sterility. The published standard entitled, "Standardization of Health Care Products," from the International Organization for Standardization, states, "It is important to note that biologic indicators are not intended to indicate that the products in the

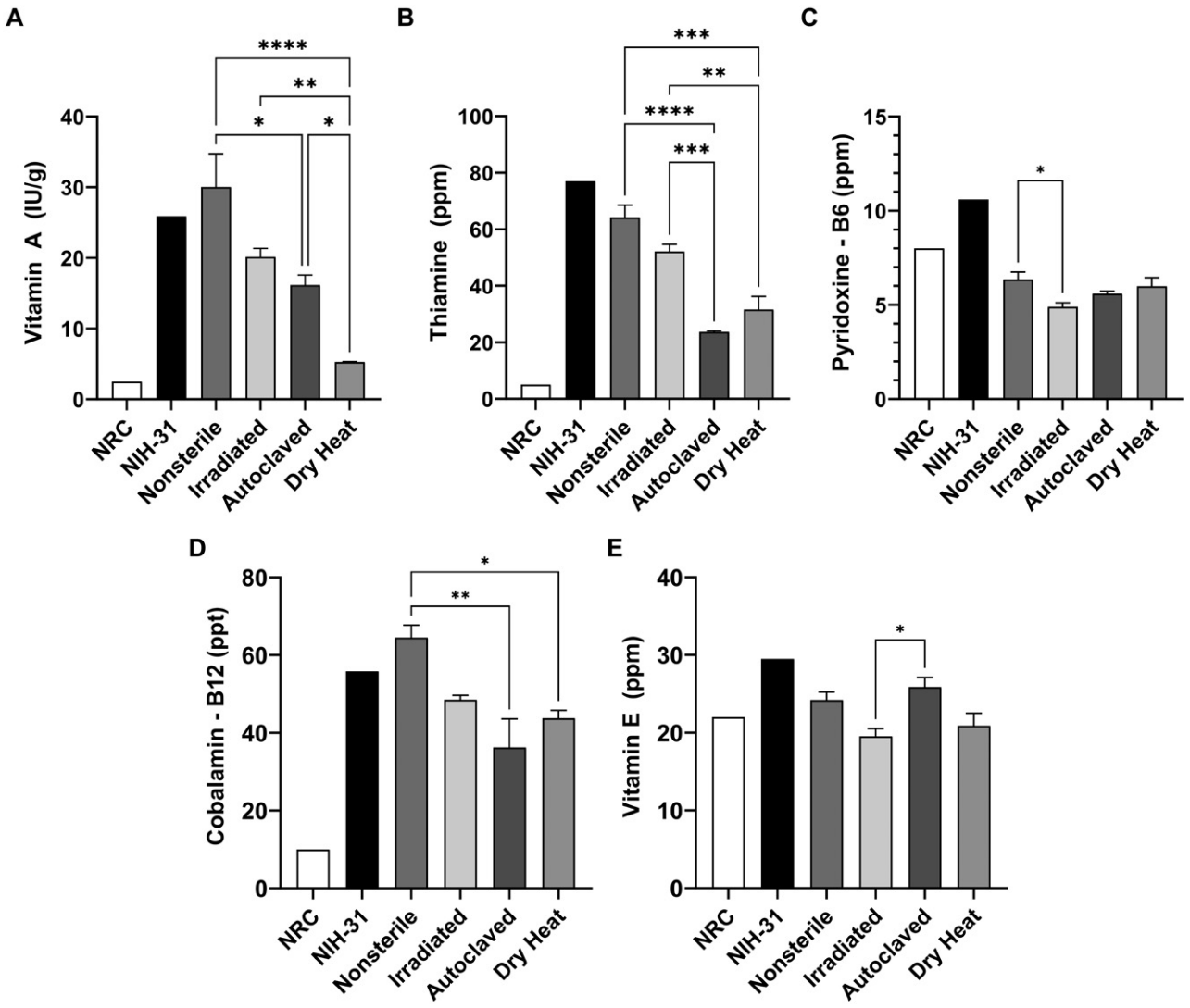

Figure 2. Vitamin analysis of NIH-31 feed (trials 15 and 16). (A) Vitamin A (IU/g), (B) thiamin (B_1; ppm), (C) pyridoxine (B_6; ppm), (D) cobalamin (B_{12}; ppt), and (E) vitamin E (ppm). NRC, National Research Council recommended feed concentration for mice; NIH-31, feed manufacturer's published concentration. The test groups nonsterile, irradiated, autoclaved (121.1 °C [250 °F]), and dry heat (160 °C [320 °F]) are shown. Values are reported as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$; ****, $P < 0.0001$.

load being sterilized are sterile. Biologic indicators are used to test the effectiveness of a given sterilization process and the equipment used, by assessing microbial lethality according to the concept of sterility assurance level."[1] Our results support this statement and expose a dilemma regarding the reliability of biologic indicators for the validation of the sterilization of products using various methods. As stated earlier, dry heat sterilizes via the conduction of heat from the outer surface to the inner layers until the entire product reaches the proper temperature for the required time.[30] In our dry heat trials in which the BIs achieved sterility but the feed did not, we speculate that the dry heat was able to reach the BI and adequately penetrate its 2- to 3-mm thickness to achieve sterility within the exposure time, whereas the dry heat could not sufficiently penetrate the entire feed pellet during the exposure period. Because of this problem, programs that use dry heat for sterilization should initially or periodically test the sterility of their product, rather than just the BI, when validating sterilization.

Heating feed can have a significant effect on nutrient composition, especially protein availability and vitamin concentrations.[33] We chose to measure vitamin A, thiamine (B_1), cobalamin (B_{12}), pyridoxine (B_6), and vitamin E as indicators of this heat-induced effect. Our results demonstrate a significant loss of vitamin A and thiamine (B_1) in both the autoclaved and dry-heat-sterilized diets. These data seem to contradict a previous report on dry heat sterilization of rodent feed.[13] However, the previous report did not provide the dry heat sterilization parameters or the method used to validate sterilization and cannot be directly compared with our results. Although this vitamin loss in both the autoclaved and dry heat-sterilized diets is significant as compared with nonsterile feed, the concentrations in this "autoclavable" diet formulation were above the NRC-recommended mouse concentrations after autoclaving. Our measured concentration of pyridoxine (B_6) was below the NRC recommended concentration (8.0 ppm) in all test groups, and the concentration of vitamin E in the irradiated, autoclaved, and dry heat sterilized diets was below NRC recommended concentration (22.0 ppm). At NIEHS, we have used the NIH-31 diet as our standard rodent diet for over 25 y and have not seen any issues related to a deficiency of these vitamins.

A primary goal of this study was to evaluate acrylamide production as a result of dry heat sterilization. We hypothesized that the higher temperatures required for dry heat sterilization of feed would significantly increase acrylamide production.

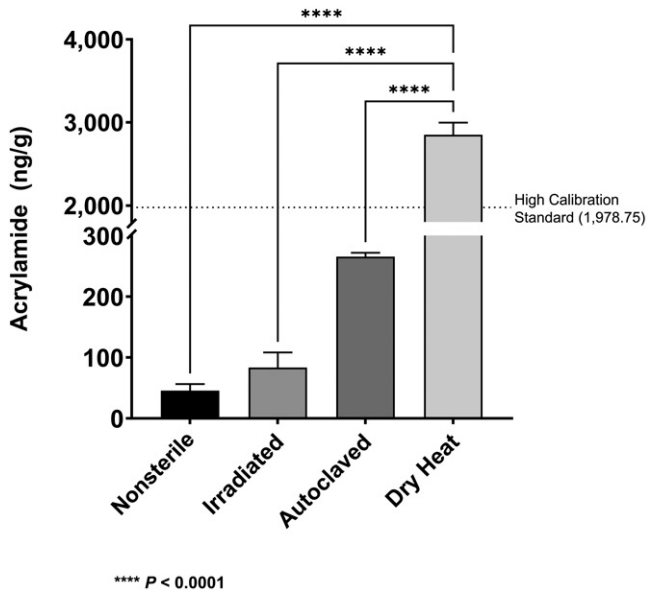

Figure 3. Acrylamide analysis of NIH-31 feed (ng/g). ****, $P < 0.0001$.

However, water promotes acrylamide production via the Maillard reaction,[31] and dry heat sterilizes in part by dehydration,[28] such that dry heat could actually reduce acrylamide production. We found significantly more acrylamide in feed after dry heat exposure (2,527 ± 124 ng/g) as compared with nonsterile (43 ± 12), irradiated (78 ± 23), and autoclaved (242 ± 11) feeds. The acrylamide concentration in the autoclaved feed was significantly higher than that of the nonsterile and irradiated feed, as previously shown.[21] We did not evaluate the in vivo effects of feeding these sterilized diets to mice; however, our previous study demonstrated that feeding autoclaved diets with increasing amounts of acrylamide resulted in a concomitant increase in the number of hepatic DNA adducts, which are precursors to neoplastic transformation.[21]

Based on our results, we conclude that dry heat is not an appropriate method for sterilization of a pelleted, natural ingredient animal feed within our operations. This is due in part to the production of high concentrations of acrylamide and the vitamin loss that occurs when using the parameters necessary to sterilize the feed. A report on the roasting process of a "ready-to-eat" cereal for human consumption demonstrated that acrylamide production can be reduced by using lower temperatures and longer roasting times to achieve the desired moisture content of the finished product.[16] Lower dry heat temperatures with longer exposure times have been validated for the sterilization of medical products, and animal feed sterilization using lower dry heat temperatures and longer exposure times could theoretically result in lower acrylamide production. Our study tested the sterility of small quantities of feed (approximately 385 g) from individual cages, with a maximum of 10 cages per trial. While this low number of cages may work for small facilities, NIEHS is a single centralized facility that requires the processing of large quantities of feed, bedding, water, and cages in a short period of time. We bulk autoclave feed (25-lb bags) and bedding (2.2 ft^3/bag) but not cages or water. Because of the slow transfer of dry heat, we do not believe that we could sterilize sufficient amounts of feed or bedding within a reasonable time frame.

External or extrinsic factors can affect research outcomes, especially in animal studies, and some relevant factors may not be readily apparent. The ability to measure small changes with more precision is constantly improving and even minor effects of extrinsic factors may alter study outcomes. Animal feed is one of many sources of extrinsic variability that are often overlooked.[20] Researchers may assume that feeding a high-quality diet from a reputable feed manufacturer will not cause variability in their study, but microbial and chemical contaminants can both alter research outcomes. All potential chemical contaminants cannot be eliminated from natural ingredient diets fed to research animals, but we can nonetheless try to maintain consistency in feed over time. Microbial contamination can be controlled by sterilization of feed before use, but even this intervention can be a source of variability. In the end, we must pick our poison and decide what we can control and what we must accept. However, being able to measure and define the poison will make for better research.

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Conflict of Interest

The author(s) have no conflict(s) of interest to declare. Our study did not require an ethical board approval because it did not contain human or animal trials.

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Data Availability

Data requests should be directed to david.kurtz@nih.gov.

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