A METHOD FOR STEAM-STERILIZING SEMI-SOLID DIETS FOR GERM-FREE MICE RESEARCH

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SUMMARY

A semi-solid corn-meal diet sterilized in glass flasks in a hospital-type autoclave is described. The values of weight gain, feed efficiency, apparent digestibility, and cecal diameters were the same for germ-free mice fed either on this diet or on a soybean meal and alfalfa diet (L-485), widely used in germ-free research. The corn-meal diet has been successfully used for eight years.

INTRODUCTION

Germ-free (GF) and gnotobiotic animals are being used in increasing numbers to clarify specific biomedical problems related to the influence of the microbial flora on the metabolism of these animals. Supplies for GF animals are obviously sterilized, usually by heat or irradiation. Diet sterilization by heat requires a high vacuum autoclave that is not available in many places. In GF research, a supply cylinder has been extensively used as an accessory to sterilize diet, bedding, etc. Vieira *et al.* (1) reported on an adaptation of the supply cylinder to a hospital-type autoclave. For an expanding animal colony, however, this process of diet sterilization became inadequate.

The present paper informs on a covenient method for sterilization of a semi-solid corn-meal diet in a glass flask, using a hospital-type autoclave.

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MATERIAL AND METHODS

Animals

GF CFW mice were the progeny of a breeding nucleus supplied by Dr. Morris Pollard, LOBUND Laboratory, University of Notre Dame, USA. They were housed in plastic isolators (Standard Safety Co., Palatine, USA), according to established procedures (2). The conventional (CV) mice had been derived from the GF colony. They were maintained under otherwise similar conditions in the open animal room. The GF status of the isolators was maintained throughout the experiment, as determined by the method of Wagner (3).

Diets

Two diets were used (Table 1). The first was the soybean meal and alfalfa diet L-485 (4), except that the soy meal had 70% protein. The second diet (T-81) contained casein, corn meal, and corn oil as protein and energy sources. The diet ingredients were mixed, and the powder treated in three different ways: a) the powdered diets were mixed with two volumes of water. After thorough homogeneization, the resulting cake was spread over a tray, cut in 3 x 3 cm pieces, and dried in a ventilated oven at 60°C. Sterilization of the dried pieces, packed in plastic bags, was carried out by *gamma*-irradiation at 35 KGray. A ^{60}Co source was used. b) Another batch of the dried pieces was sterilized at 120°C for 60 minutes in a properly adapted supply cylinder, as described before (1). c) Powered diets were put in "square pak" flasks (American Sterilizer Co., Erie, USA) and two volumes of water were added. Then mixture was thoroughly homogeneized with a wooden stick. Sterilization was carried out at 120°C for 45 minutes. The "square pak" flask is shown diagramatically in Figure 1. A proper rubber closure allows the flow of steam during the process of sterilization, and is self-sealing on cooling. In many laboratories, these have been used for water sterilization.

Bedding

Bedding was prepared as follows. White toilet paper was throughly wetted and scrubbed in a metalic screen sieve, and the clumped paper dried in a ventilated oven at 60°C. Sterilization was carried out in a cloth bag using the supply cylinder.

Experimental Procedure

GF and CV 21-day-old male mice were used, the initial body weights ranging from 8.50 to 10.20 g. The animals were divided into two groups: six GF, and six CV. (The number of animals used in each group is indicated in the Tables). GF animals were housed in individual cages, inside plastic isolators, and these were cleaned weekly. Diets and water were supplied *ad libitum*. CV animals were maintained in the animal room and fed the same diet as their GF counterparts. The experiment lasted three weeks. The animals were weighed weekly.

TABLE 1

COMPOSITION OF THE DIETS (g/kg)

Ingredients	T-81	L-485[a]
70% soybean protein meal[b]	-	300
17% alfalfa meal	-	35
Corn meal	604	590
Casein	200	-
Corn oil	90	30
Sodium chloride	-	10
Dicalcium phosphate	-	10
Calcium carbonate	-	5
Mineral mixture[c]	50	-
Lysine	2	5
Methionine	-	5
Choline chloride	4	-
Cellulose	10	-
Butylated hidroxytoluene	-	0.125
Vitamin mixture	40[d]	10[e]
Trace mineral mixture[f]	-	0.25
% of protein (N x 6.25)	24.12	26.33
Energy content (kcal/kg)	4,034	4,010

a Kellog and Wostmann (1969) (4).

b Proteimax 70, kindly supplied by Sanbra S.A. (São Paulo, Brazil).

c AOAC (1965). Composition in mg/kg of diet: NaCl, 6965; KI, 39.5; KH_2PO_4, 1945; $MgSO_4.7H_2O$, 2865; $CaCO_3$, 1970; $FeSO_4.7H_20$, 1350; $MnSO_4.H_2O$, 200; $ZnSO_4.7H_20$, 27.4; $CuSO_4.5H_2O$, 23.9; $CoCl_2.6H_2O$, 1.15.

d Composition in mg/kg of diet: retinol acetate, 160; cholecalciferol, 1; vitamin E, 400; menadione, 20; riboflavin, 32; pantothenic acid, 160; niacin, 160; vitamin B_{12}, 012; thiamine chloride, 20; pyridoxine chloride, 20; folic acid, 8; p-aminobenzoic acid, 400; biotin, 16; *myo*-inositol, 400; sucrose to make 40,000.

e Composition in mg/kg of diet: retinol acetate, 27; cholecalciferol, 0.2; vitamin E, 789; menadione, 315; riboflavin, 105; pantothenic acid, 1,000; niacin, 193; choline chloride, 7,020; vitamin B_{12}, 14; thiamin chloride, 228; pyridoxine chloride, 70; folic acid, 35; *p*-aminobenzoic acid, 275; biotin, 28.

f Composition in mg/kg of diet: $MnSO_4.H_2O$, 132.3; $FeSO_4.7H_2O$, 66.1; $CuSO_4.5H_2O$, 5.4; $ZnSO_4.7H_2O$, 43.6; KI, 1.3; $COCl_2.6H_2O$, 1.3.

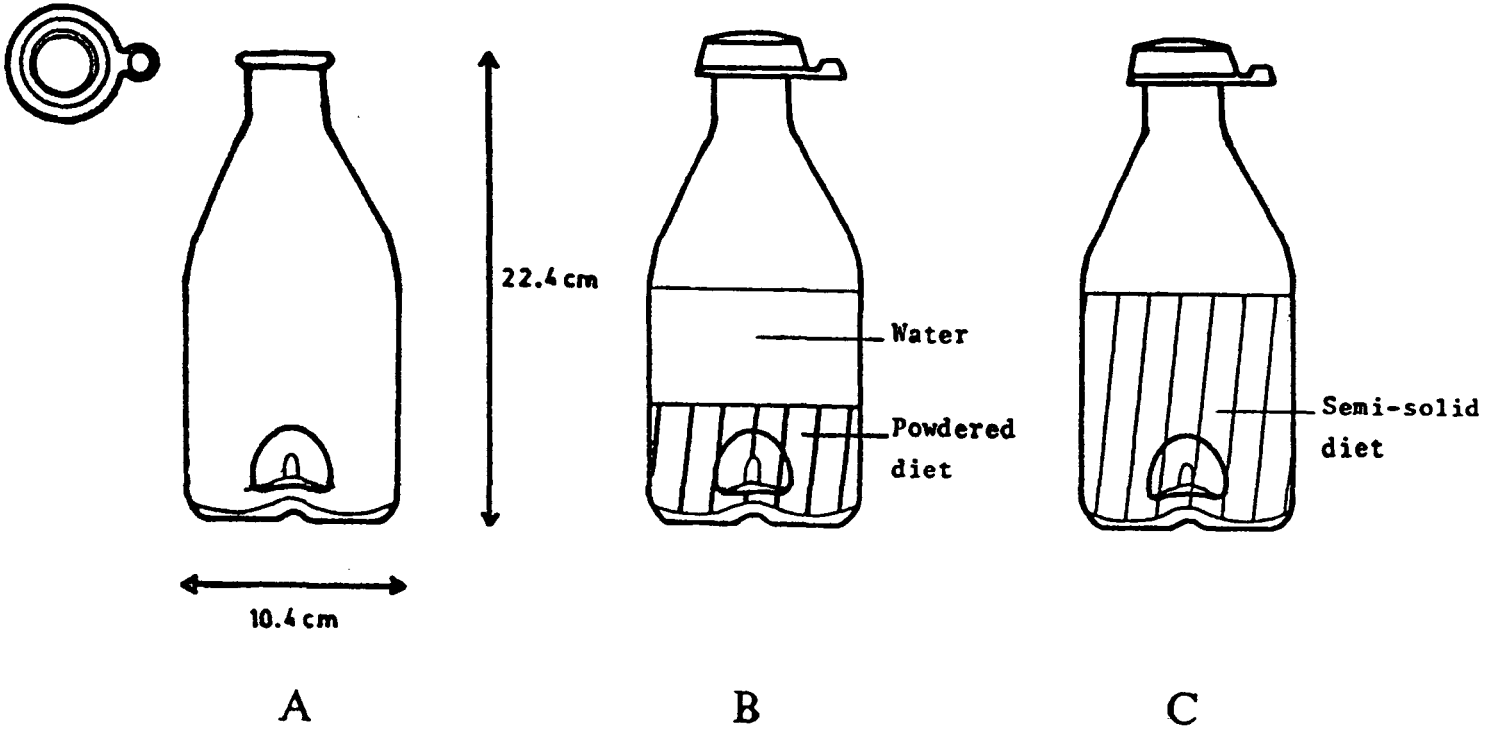

FIGURE 1

"Square pak" flasks used for semi-solid diet sterilization: empty (A); loaded with powdered diet and water before mixing (B); loaded with semi-solid diet after mixing and sterilization (C)

After three weeks, all animals were killed under ether anesthesia. The cecum was removed, washed with saline, and then spread out over a graph paper in order to measure its length and width. Feces, bedding, and food residues of each animal were pooled, and feces and food residues were separated from bedding, dried at 105°C, and weighed. The nitrogen content of food, feces, and spoiled food was determined by the Kjeldahl method. Since the moisture content was not constant, the amount of food eaten or wasted was calculated from the nitrogen determination, and was expressed as dry weight. Feed efficiency (FE) and apparent digestibility of nitrogen (Dapp) were calculated as follows:

$$FE = \frac{\text{Weight gain (g)}}{\text{Food intake (g)}}$$

$$DAPP = \frac{\text{Nitrogen intake (g) - Fecal nitrogen (g)}}{\text{Nitrogen intake (g)}}$$

Analysis of variance was performed using an ANOVA program. Data were compared and tested by the least significant difference.

RESULTS AND DISCUSSION

Table 2 shows weight gain, feed efficiency, and apparent digestibility of CV and GF mice fed the two diets, sterilized by three different ways. The values of these parameters were the same for diets T-81 and L-485 when sterilization was carried out by heat in the cylinder. When sterilization was carried out by *gamma*-irradiation, a lower weight gain and a lower feed efficiency were found in GF mice fed on diet T-81. This effect could be explained by the possible presence of any radiolytic products (6) available in the diets, which is more active in GF animals because of the lacking of a metabolizing microflora. The weight gain of CV mice fed on T-81 was significantly higher ($p < 0.05$) than that obtained with L-485, when the diets were sterilized by heat, while the weight gain and the feed efficiency of GF mice fed on T-81 were significantly higher when sterilization in "square pak" flasks was used.

When pelleted diet was used (sterilization by *gamma*-irradiation or heat in supply cylinder) diet T-81 presented a higher apparent protein digestibility than diet L-485. This was probably due to the higher digestibility of casein proteins as compared to those of soy or alfalfa. Nevertheless, when T-81 was sterilized in "square pak" flasks, the apparent protein digestibility values revealed no difference. This may be attributed to the heat effect on proteins, more pronounced when purified ingredients have a higher water content. The overall results, as depicted in Table 2, led to the conclusion that T-81 diet is, as recommended, as the widely used L-485 diet which is sterilized in a high vacuum autoclave.

Table 3 indicates that the cecal dimensions were not affected by the kind of diet in all sterilization procedures.

In most gnotobiology laboratories, diet and bedding are sterilized either

TABLE 2

WEIGHT GAIN, FEED EFFICIENCY (FE), AND APPARENT DIGESTIBILITY (DAPP) OF GERM-FREE (GF) AND CONVENTIONAL (CV) MICE FED EITHER ON L-485 OR T-81 (TABLE 1) DIETS STERILIZED BY THREE DIFFERENT PROCEDURES

Procedure used in sterilization	Group	Diet (No. of animals)	Weight gain (g ± SE)	FE (g ± SE)	Dapp (% ± SE)
gamma-	GF	T-81 (5)	6.34 ± 0.99[a]	0.07 ± 0.009[a]	97.52 ± 0.20[a]
		L-485 (5)	9.40 ± 0.59[b]	0.11 ± 0.004[b]	71.23 ± 6.42[b]
irradiation	CV	T-81 (9)	11.16 ± 0.99	0.13 ± 0.007[b]	96.35 ± 0.67[ab]
		L-485 (10)	11.68 ± 0.54[b]	0.13 ± 0.006[bc]	89.97 ± 0.63[b]
Heat in a supply	GF	T-81 (7)	11.23 ± 0.72[b]	0.13 ± 0.004[b]	94.88 ± 1.18[a]
		L-485 (6)	11.95 ± 0.74	0.14 ± 0.004	86.68 ± 1.64
cylinder	CV	T-81 (6)	12.90 ± 0.58[a]	0.14 ± 0.004[abc]	90.92 ± 0.77[abc]
		L-485 (6)	9.62 ± 0.41[c]	0.11 ± 0.004[b]	82.10 ± 1.99[c]
Heat in "square pak"	GF	T-81 (7)	13.57 ± 0.48[c]	0.16 ± 0.004[c]	87.24 ± 2.19[b]
		L-485 (8)	12.18 ± 0.53	0.16 ± 0.007	86.87 ± 1.57
flasks	CV	T-81 (6)	12.68 ± 0.45[a]	0.15 ± 0.004[c]	89.21 ± 0.82[c]
		L-485 (7)	10.77 ± 0.34[bc]	0.14 ± 0.004[c]	85.50 ± 1.70[bc]

a Significant differences ($p < 0.05$) between T-81 and L-485 diets in the same group.

b,c Significant differences ($p < 0.05$) between sterilization procedures in group with the same microbiological status (GF or CV) and fed the same diet (T-81 or L-485).

TABLE 3

WET WEIGHT, LENGTH AND WIDTH OF THE CECUM FROM GERM-FREE (GF) AND CONVENTIONAL (CV) MICE FED EITHER ON L-485 OR ON T-81 DIETS (TABLE 1) STERILIZED BY THREE DIFFERENT PROCEDURES

Procedure used in sterilization	Group	Diet (No. of animals)	Wet weight (mg ± SE)	Length (cm ± SE)	Width (cm ± SE)
gamma-irradiation	GF	T-81 (5)	100.00 ± 18.71	2.18 ± 0.098	1.10 ± 0.054
		L-485 (5)	94.00 ± 5.48	2.12 ± 0.067	1.10 ± 0.063
	CV	T-81 (9)	68.33 ± 13.29	1.18 ± 0.030	0.77 ± 0.040
		L-485 (10)	60.00 ± 8.94	1.42 ± 0.047	0.77 ± 0.035
Heat in a supply cylinder	GF	T-81 (7)	98.57 ± 19.52	2.16 ± 0.075	1.11 ± 0.041
		L-485 (7)	101.67 ± 7.53	2.15 ± 0.030	1.10 ± 0.072
	CV	T-81 (6)	50.00 ± 6.32	1.22 ± 0.028	0.73 ± 0.020
		L-485 (6)	66.67 ± 8.16	1.33 ± 0.049	0.68 ± 0.069
Heat in "square pak" flasks	GF	T-81 (7)	91.67 ± 7.53	1.93 ± 0.053	0.95 ± 0.072
		L-485 (8)	94.00 ± 5.48	2.18 ± 0.053	1.10 ± 0.049
	CV	T-81 (6)	75.00 ± 8.37	1.30 ± 0.024	0.70 ± 0.037
		L-485 (7)	70.00 ± 8.16	1.26 ± 0.060	0.68 ± 0.034

by steam in a high vacuum autoclave or by *gamma*-irradiation. High vacuum autoclave or *gamma*-irradiation facilities are not available in many places.

In this research work, the diet was sterilized in a semi-solid state in glass flasks. Removal of the diet from the flasks was easily done with a spatula. T-81 diet had a better consistency than L-485 processed this way. It has been shown that damage to vitamins (7) and protein quality (8) by autoclaving become less severe with increasing moisture content. Growth and survival rates were higher for animals fed on a semi-solid diet than on dry diets (9).

Diet sterilization, as described in this work makes possible the maintainance of research with GF and specific pathogen-free (SPF) animals in places where high vacuum autoclave, autoclavable diets, or *gamma*-irradiation facilities are not available. This semi-solid, heat sterilized diet has been successfully used for eight years and many generatons of GF rats, and mice were raised without any noticeable sign.

RESUMEN

UN METODO DE ESTERILIZACION POR VAPOR DE DIETAS SEMISOLIDAS PARA INVESTIGACION EN RATONES LIBRES DE GERMENES

Se describe una ración de maíz semisólida, esterilizada en frascos de vidrio, utilizando un autoclave de tipo hospitalario. Los valores de ganancia de peso, eficacia alimentaria, digestibilidad aparente y diámetro del intestino ciego fueron los mismos para ratones libres de gérmenes, alimentados con esta ración, o con la ración de soya e alfalfa (L-485), ampliamente usada en gnotobiología. La ración de maíz ha sido utilizada con éxito durante un período de ocho años.

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