Lipid absorption and intestinal tumour incidence in rats fed on varying levels of calcium and butterfat

BY A. R. BEHLING, S. M. KAUP, L. L. CHOQUETTE AND J. L. GREGER*

Department of Nutritional Sciences, University of Wisconsin, 1415 Linden Drive, Madison, WI 53706, USA

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The purpose of the 2 × 2 factorial study was to determine the effect of varying levels of dietary calcium (2·5 and 10 g/kg) and butterfat (50 and 200 g/kg) on lipid utilization and on development of colon tumours in animals initiated with 1,2-dimethylhydrazine dihydrochloride. Among rats fed on 200 g butterfat/kg, the fourfold increase in Ca intake induced more than a sevenfold increase in faecal excretion of total lipids and almost a fortyfold increase in faecal excretion of acid-extractable lipid. Among rats fed on 50 g butterfat/kg, the ingestion of supplemental Ca had a less dramatic effect and induced only a twofold increase in faecal excretion of total lipids and a threefold increase in acid-extractable lipid. The volume of intestinal adenocarcinomas was correlated with the excretion of acid-extractable lipid in faeces (R 0·369, $P < 0·02$). Caecal enzymic activity was not correlated with tumour incidence or size or faecal lipid excretion. Overall, the fourfold increase in Ca intakes decreased total lipid absorption significantly but by less than 6%.

Calcium: Lipid absorption: Butterfat: Rat

Ingestion of high-fat diets has been associated with increased tumourigenic events in both rodent and human colons (Rozhin *et al.* 1984; Willet & MacMahon, 1984; Nauss *et al.* 1987). Maize oil or beef tallow were the fats given in the animal studies. We could find no carcinogenesis studies in which butterfat, which has a higher proportion of saturated short- and medium-chain triglycerides than maize oil or even beef tallow, was studied.

Ingestion of high levels of calcium has been associated with a lower incidence of colon carcinogenesis in several epidemiological studies (Slattery *et al.* 1988; Wargovich, 1988). In experimental studies, ingestion or even infusion of high levels of Ca reduced colon tumourigenesis in several (Wargovich *et al.* 1983; Appleton *et al.* 1987; Pence & Buddingh, 1988) but not all studies (Kasprzak & Waalkes, 1986; Bull *et al.* 1987; Gregorie *et al.* 1989).

One mechanism that might explain differences in results is that Ca reduces the incidence of cancer through the formation of insoluble salts (soap) with bile acids and free fatty acids (Newmark *et al.* 1984). Thus ingestion of high levels of dietary Ca would be hypothesized to be more effective in reducing tumourigenesis if fat intake were high. Early investigators observed that ingestion of high levels of Ca reduced fat absorption (Drenick, 1961; Fleischman *et al.* 1966). However, the excretion of soaps in the faeces of animals fed on varying levels of Ca and fat has not been quantified in controlled carcinogenesis studies.

Another mechanism by which Ca and butterfat may affect intestinal tumourigenesis is by changing the activity of microbial flora (Reddy *et al.* 1977). Moreover, it might be hypothesized that the formation of Ca soaps would form more substrate for caecal bacteria.

The purposes of the present experiment were (1) to quantify fat complexes in the faeces of rats fed on various levels of Ca and butterfat, (2) to assess the relationship of caecal flora

* For reprints.

activity to the changes in available substrate and (3) to assess whether these changes in Ca and fat intake and fat excretion were related to the development of intestinal tumours in 1,2-dimethylhydrazine dihydrochloride (DMH)-initiated rats.

MATERIALS AND METHODS

Animals and diets

Weanling male rats (112) were assigned to one of four dietary treatments that differed in their level of calcium (2·5 or 10 g/kg) and butterfat (50 or 200 g/kg). The dietary treatments were: the basal low-fat (LF) and high-fat (HF) diets that provided 2·5 g Ca/kg and the Ca-supplemented low-fat diet (HCaLF) and high-fat diet (HCaHF) that provided 10 g Ca/kg.

The basal low-fat diet was similar in formulation to the AIN-76 diet (American Institute of Nutrition, 1977) except that butterfat was the source of fat and the Ca level was half that recommended. All diets were supplemented with 10 g maize oil/kg to prevent essential fatty acid deficiency (Table 1). Diets were designed to ensure that the rats in each of the four treatments, on average, consumed equal amounts of energy and all nutrients, except lipid and Ca. The diets were formulated using mass-energy balance equations so that energy contents of low-fat and high-fat diets were 16·23 and 19·12 kJ/g diet respectively (Felder & Kosseau, 1978). At the beginning of the study, rats were assigned by weight to twenty-eight blocks; there was one animal from each treatment in each block. In each block, rats were given 85% (by weight) as much of the high-fat diets as consumed by rats in the block fed on low-fat diets *ad lib*. The rats fed on the high-fat diets ate all their dietary allotments. Accordingly, the average energy intake of all rats over the entire experimental period was 1020 (SE 8·4) kJ (244 (SE 2) kcal)/d.

Weanling male Sprague-Dawley rats (Harlan/Sprague-Dawley, Indianapolis, IN) were housed individually in stainless-steel wire-bottomed cages. The facilities met the standards of the American Association for Accreditation of Laboratory Animal Care. Deionized water was offered *ad lib*. Rats were weighed once weekly.

Beginning 2 weeks after rats were started on test diets, twenty rats per diet were injected subcutaneously (15 mg/kg body-weight) with DMH (Sigma Chemical Company, St Louis, MO) in saline (9 g sodium chloride/l) with 0·001 M-EDTA adjusted to pH 6·8. The remaining rats were injected subcutaneously with buffer only. Rats were injected weekly for 20 weeks. This protocol for intestinal tumour induction is consistent with that used by many other investigators (Goldin & Gorbach, 1981; Freeman, 1986; Pence & Buddingh, 1988).

Sample collection and analyses

Rats were fasted overnight, anaesthetized and killed by exsanguination 33–36 weeks after initiation of the study. The rats were killed on a staggered schedule (i.e. nine or ten rats killed/treatment per d with the 3 d separated by 1 week) so that samples could be processed efficiently. A few (n 8) animals that became very ill were killed before week 33. Number, location and size of all tumours were documented at autopsy with the help of a veterinary pathologist. All tumours were characterized histologically by defined standards (Ward, 1974).

Caecums of rats were tied off, excised and placed in an anaerobe chamber (Coy Laboratory Products Inc., Ann Arbor, MI) where the caecal contents were removed and diluted with 5–7 ml buffer (0·1 M-sodium phosphate buffer, saline, pH 7·4). The diluted caecal matter was transferred to sealed centrifuge tubes and centrifuged at 4° at 1500 ***g*** for 15–20 min. The resulting caecal suspension was analysed for β-glucuronidase (*EC* 3.2.1.31; Shiau & Chang, 1983) and nitroreductase (Wise *et al.* 1982) by standard methods in the anaerobe chamber. The activity of β-glucuronidase was expressed as units which

Table 1 *Composition of diets (g/kg) that contain low and high levels of butterfat with 2·5 g calcium/kg (diets LF and HF) or 10 g Ca/kg (diets HCaLF and HCaHF)*

	Low fat		High fat	
Component	LF	HCaLF	HF	HCaHF
Casein*	200·0	200·0	235·0	235·0
DL-Methionine*	3·0	3·0	3·5	3·5
Sucrose	200·0	200·0	235·0	235·0
Maize oil†	10·0	10·0	10·0	10·0
Butterfat, anhydrous*	50·0	50·0	200·0	200·0
Cellulose*	50·0	50·0	58·5	58·5
Vitamin mixture, AIN-76*	10·0	10·0	11·7	11·7
Mineral mixture, modified AIN-76‡	35·0	35·0	41·0	41·0
Choline bitartrate*	2·0	2·0	2·3	2·3
Calcium carbonate§	—	18·75	—	22·0
Maize starch	440·0	421·25	203·0	181·0

* Teklad Test diets (Madison, WI).

† Mazola corn oil (Best Foods, Englewood Cliffs, NJ).

‡ Supplies (mg/kg diet): sodium chloride 2590, calcium phosphate dibasic 8750, potassium citrate monohydrate 7700; potassium sulphate 1820, magnesium oxide 840, manganous carbonate 123, ferric citrate 140, zinc carbonate 28, cupric sulphate 10·5, potassium iodate 0·35, sodium selenite 0·35, chromium potassium sulphate 19·2; 9135 mg sodium orthophosphate monohydrogen/kg diet was added to the mineral mixture to replace an additional 8750 mg calcium phosphate dibasic (calcium source)/kg omitted from the standard formulation.

§ Mallinckrodt Inc. (Paris, KY).

were equivalent to μg phenolphthalein produced/h. Nitroreductase activity was expressed as units which were equivalent to nmol aminobenzoate produced/h. Protein contents of the caecal suspensions were determined by the method of Lowry *et al.* (1951).

Faeces were collected on days 26–28 from fifteen rats (ten DMH-treated and five placebo-treated rats) per treatment. Faecal lipids were extracted from freeze-dried faeces (Bligh & Dyer, 1959; Sukhija & Palmquist, 1988). Faeces were mixed with chloroform–methanol (2:1, v/v), then additional chloroform, methanol, chloroform again and water were added in the following ratio (1:2:1:1); thorough mixing followed each addition. The mixture was filtered, the fitrate was allowed to separate and the organic fraction was dried and weighed; this fraction was called the neutral lipid fraction. The filtered solids were mixed with hydrochloric acid (250 ml/l) in deionized water; then chloroform, methanol and finally water were added alternately as before and mixed. The second extraction mixture was filtered, the filtrate was allowed to separate and the organic fraction was dried and weighed; this fraction was called the acid-extractable lipid fraction. The total lipid was calculated to be the sum of the neutral- and acid-extractable lipid fractions.

Total lipids (sum of neutral- and acid-extractable fractions) were determined in low- (*n* 6 replicates) and high-fat diets (*n* 6) and found to be 7·5 (SE 0·3) and 22·2 (SE 0·5)%, respectively. Known quantities of oleic acid (an example of a long-chain fatty acid), trilauric acid (an example of a triacylglycerol) and butyric acid (an example of a short-chain fatty acid) were added to faeces (three replicates for each addition) and the faeces were treated as described previously. Recoveries of these compounds were found to be (%): oleic acid 88 (SE 8), trilauric acid 100 (SE 6), butyric acid 6 (SE 2). Thus, this methodology is very good for recovery of triacylglycerols and long-chain fatty acids but will not recover water-soluble, unesterified short-chain fatty acids.

Percentage digestibility was calculated by the formula: ((dried feed intake − dried faecal weight) ÷ dried feed intake) × 100. Feed efficiency ratios were calculated by the formula: (weight gain (g) ÷ energy intake) × 100.

Statistical analysis

The effects of dietary treatments and of DMH were evaluated within the framework of general linear models for analysis of variance (SAS Institute Inc., 1985). The effect of the treatments on faecal losses of lipids and faecal enzyme activity were factored into effects due to blocks, DMH,variations in levels of dietary Ca and variations in levels of dietary fat. The effect of the treatments on tumour incidence was analysed using the chi-square test of homogeneity; the effect of the treatments on tumour number and size was analysed using Kruskal–Wallis (non-parametric) tests.

RESULTS

Rats fed on the high- (200 g/kg) butterfat diet with moderate amounts (2·5 mg Ca/g diet) of Ca were larger ($P < 0·05$) than rats fed on the other diets throughout the study except at weeks 1 and 10, even though average energy intakes of rats in the four treatment groups were the same (Fig. 1). This difference in body weights of rats reflects differences in feed efficiency (Table 2). Rats fed on the 200 g butterfat/kg diet utilized feed slightly ($P < 0·05$) more efficiently than rats fed on 50 g butterfat/kg. However, increases in either the Ca or butterfat content of the diet were associated with decreased ($P < 0·0001$) diet digestibility.

Increased Ca intake not only reduced the total diet digestibility but also reduced ($P < 0·001$) the apparent absorption of fat (Table 3). Thus rats, whether treated with DMH or the placebo, lost at least sevenfold more lipid in their faeces when fed on 200 g butterfat/kg with high levels of Ca (HCaHF) than when fed on the moderate amount of Ca with 50 or 200 g butterfat/kg diets (LF or HF).

Rats injected with DMH excreted less neutral-extractable ($P < 0·01$) and acid-extractable ($P < 0·005$) lipid than rats injected with buffer only. Elevation of dietary Ca content increased ($P < 0·0001$) excretion of both neutral- and acid-extractable lipids in faeces. Elevation of dietary fat increased excretion of both neutral- and acid-extractable lipids in faeces only when the high level of Ca was given. Rats fed on diet HCaHF excreted almost fortyfold more acid-extractable lipid daily but only two- to threefold more neutral-extractable lipid daily than rats fed on diet HF. This dramatic increase in acid-extractable lipid excretion by rats fed on diet HCaHF caused large differences in the relative composition of faecal lipid. Rats fed on diet LF excreted predominantly (86–87%) neutral-extractable lipid; rats fed on diet HCaHF excreted predominantly (67–68%) acid-extractable lipid.

This change in the distribution of faecal lipids from the neutral- to acid-extractable fractions corresponded with the higher tumour incidence in animals fed on diet HCaHF (Table 4). Chi-square analyses indicated that dietary Ca ($P < 0·173$) and dietary fat ($P < 0·069$) tended to affect tumour incidence. The dietary treatments did not significantly affect the number of tumours per animal or tumour volume. Tumour volume per animal was correlated with the amount of acid-extractable lipid excreted daily (Pearson correlation factor, R 0·369, $P < 0·02$) and with the percentage of faecal lipid in the acid-extractable fraction (Pearson correlation factor, R 0·381, $P < 0·02$). All intestinal tumours were classified as adenocarcinomas by the veterinary pathologist.

Caecal microbial activity and mass did not correlate with any of the measures of tumour development or lipid composition of faeces (Table 5). Rats injected with DMH had larger

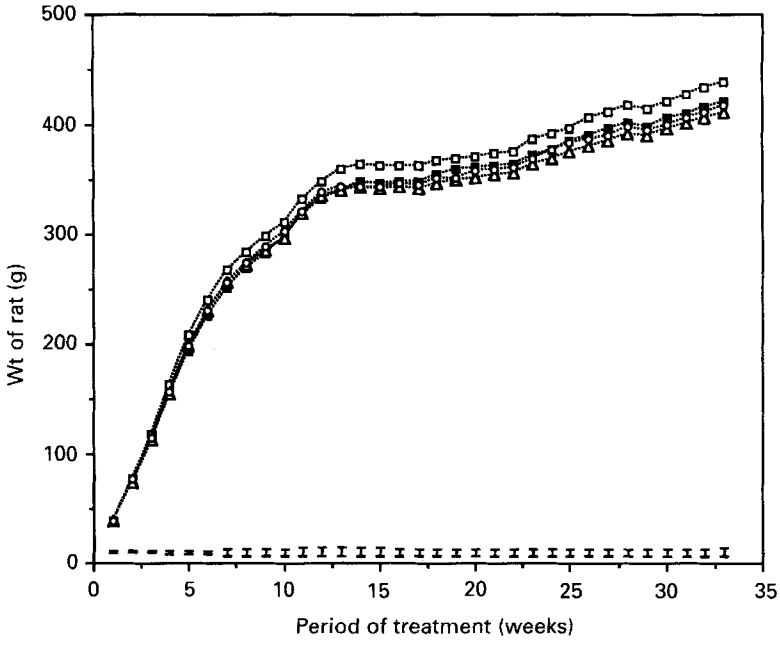

Fig. 1. Rats fed on high levels of butterfat (diet HF; □---□) were significantly larger ($P < 0.05$) than rats fed on low levels of butterfat (diet LF; ○---○), or either level of fat with the high level of calcium (diet HCaLF; △---△ or diet HCaHF; ■---■) at all time-points except weeks 1 and 10. Values are means, with their standard errors represented by vertical bars at the bottom of the figure, for twenty-eight rats; 1,2-dimethylhydrazine dihydrochloride- and placebo-treated rats combined.

Table 2. *Feed utilization of rats fed on two levels of butterfat (diets LF and HCaLF* v. *HF and HCaHF) and two levels of calcium (diets LF and HF* v. *HCaLF and HCaHF) and injected with 1,2-dimethylhydrazine dihydrochloride (DMH) or placebo solutions**

(Mean values for twenty rats for DMH treatment and eight rats for placebo treatment)

Carcinogen	Diet	Feed conversion efficiency (g wt gain/100 kJ)	Diet digestibility† (%)
DMH	LF	11·7	94·3
	HF	12·2	93·0
	HCaLF	11·7	91·8
	HCaHF	12·0	89·0
SEM		0·08	0·13
Placebo	LF	12·3	94·2
	HF	12·8	93·3
	HCaLF	12·1	91·5
	HCaHF	12·5	87·5
SEM		0·13	0·21
Statistical effects as determined by analyses of variance			
DMH	—	$P < 0.0005$	NS
Diet Ca	—	NS	$P < 0.0001$
Diet fat	—	$P < 0.05$	$P < 0.0001$

NS, not significant.

* For details of treatments and dietary regimens, see p. 506 and Table 1.

† ((Diet intake − g faeces) ÷ diet intake) × 100; faecal collection during week 4 of study.

Table 3. *Faecal lipid excretion of rats after being fed on two levels of butterfat (diet LF and HCaLF v. HF and HCaHF) and two levels of calcium (diets LF and HF v. HCaLF and HCaHF) and injected with 1,2-dimethylhydrazine dihydrochloride (DMH) or placebo solutions**

(Mean values for ten rats for DMH treatment and five rats for placebo treatment)

		Total lipid		Faecal neutral lipid		Faecal acid-extractable lipid	
Carcinogen	Diet	Apparent absorption (%)	Faecal losses (mg/d)	(mg/d)	% of total lipid	(mg/d)	% of total lipid
DMH	LF	97·3	27	23	86	4	14
	HF	99·1	30	26	86	4	14
	HCaLF	93·9	57	42	72	15	28
	HCaHF	93·1	223	70	32	153	68
SEM		0·55	13·9	4·3	3·7	10·5	3·7
Placebo	LF	96·8	35	30	87	5	13
	HF	99·0	38	32	86	6	14
	HCaLF	93·9	66	47	72	19	28
	HCaHF	90·8	350	114	33	236	67
SEM		0·78	31·9	8·5	5·1	23·7	15·1
Statistical effects as determined by analysis of variance							
DMH	—	NS	$P < 0·005$	$P < 0·01$	NS	$P < 0·005$	NS
Diet Ca	—	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$
Diet fat	—	NS	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$	$P < 0·0001$

NS, not significant.

* For details of treatments and dietary regimens, see p. 506 and Table 1.

Table 4. *Intestinal tumour development among 1,2-dimethylhydrazine dihydrochloride (DMH)-treated rats fed on two levels of butterfat (diets LF and HCaLF v. HF and HCaHF) and two levels of calcium (diets LF and HF v. HCaLF and HCaHF)**

Diet	n†	No. of tumour-bearing animals (% of total)	No. of tumours per tumour-bearing animal	Tumour volume per tumour-bearing animal (log 10 mm^3)
LF	20	40	2·6	2·83
HF	19	53	2·0	2·54
HCaLF	19	47	1·6	2·77
HCaHF	20	75	1·5	3·02
SEM	—	—	0·3	0·14

* For details of treatments and dietary regimens, see p. 506 and Table 1.

† Only DMH-treated rats included; placebo-treated rats had no tumours. All tumours in DMH-treated rats were identified as adenocarcinomas by the pathologist.

($P < 0·0005$) caecal mass and lower β-glucuronidase ($P < 0·0001$) and nitroreductase ($P < 0·005$) activity than rats injected with the buffer solution only. In general, the dietary treatments did not have as dramatic an effect on caecal mass and microbial activity as they did on faecal lipid excretion. Rats given 200 rather than 50 g butterfat/kg had reduced caecal mass ($P < 0·05$) and nitroreductase activity ($P < 0·0001$). Elevation of dietary Ca decreased ($P < 0·05$) caecal protein concentrations and this resulted in a increase ($P < 0·0001$) in nitroreductase activity when expressed as U/mg protein.

Table 5. *Caecal flora of rats fed on two levels of butterfat (diets LF and HCaLF* v. *HF and HCaHF) and two levels of calcium (diets LF and HF* v. *HCaLF and HCaHF) and injected with 1,2-dimethylhydrazine dihydrochloride (DMH) or placebo solutions**

Carcinogen	Diet	*n*†	Caecal mass (g/kg body-wt)	Protein (mg/g contents)	β-Glucuronidase‡ (units/mg protein)	Nitroreductase§ (units/mg protein)
DMH	LF	19	6·3	17·9	65·1	35·5
	HF	16	5·3	16·6	47·4	25·6
	HCaLF	20	7·0	11·9	53·1	41·5
	HCaHF	19	6·2	14·6	44·1	39·5
SEM			0·23	0·88	3·41	1·40
Placebo	LF	8	5·2	15·1	98·7	38·8
	HF	8	3·7	14·6	90·6	30·6
	HCaLF	6	4·5	11·4	91·1	53·0
	HCaHF	8	4·0	10·5	85·8	46·4
SEM			0·38	1·38	5·53	2·27
		Statistical effects as determined by analysis of variance				
DMH	—	—	$P < 0·005$	NS	$P < 0·0001$	$P < 0·005$
Diet Ca	—	—	NS	$P < 0·05$	NS	$P < 0·0001$
Diet fat	—	—	$P < 0·05$	NS	NS	$P < 0·01$

NS, not significant.
* For details of treatments and dietary regimens, see p. 506 and Table 1.
† Caecal enzyme activity not measured in rats killed before week 33.
‡ Units expressed as μg phenolphthalein produced/h.
§ Units expressed as nmol aminobenzoate produced/h.

DISCUSSION

Among rats in the present study fed on a low-fat diet, a fourfold increase in Ca intake increased faecal excretion of total lipids by about twofold and increased faecal excretion of acid-extractable lipids by more than threefold without a change in intestinal tumour incidence. However, among rats given the high-fat diet the same increase in Ca intake induced more than a sevenfold increase in faecal excretion of total lipids, almost a fortyfold increase in faecal excretion of acid-extractable lipid, and tended to increase the incidence of intestinal adenocarcinomas. No similar additive effect of dietary Ca and fat was observed on caecal flora.

These findings are consistent with the observations of Gregoire *et al.* (1989). They found that subjects who previously had surgery for colorectal adenocarcinoma had elevated concentrations of deoxycholic acid in the aqueous phase of their faeces when they were given supplemental Ca, but this was associated with no change in colon mucosal proliferation. Bull *et al.* (1987) reported that the addition of Ca (10 g/kg) to a high- (300 g/kg) fat diet but not a low- (50 g/kg) fat diet increased the incidence of bowel tumour in rats treated with azoxymethane.

Together these findings with our findings suggest that the importance of Ca as a protective agent in colon tumourigenesis needs to be re-evaluated. Previously, Wargovich *et al.* (1983) have noted that supplemental Ca decreased the proliferative effects of fatty acids and bile acids on colon epithelium. At least two other groups of investigators also noted decreased intestinal tumour incidence among rats orally administered supplemental Ca (Appleton *et al.* 1987; Pence & Buddingh, 1988).

A number of potentially important variations in the protocols of these studies could be responsible for the divergent results. One difference is the form of Ca give in the various studies. Calcium carbonate was given in the present study. Calcium gluconate and lactate

(Wargovich *et al.* 1983; Appleton *et al.* 1987; Pence & Buddingh, 1988) and calcium phosphate (Bull *et al.* 1987) were given in other studies. The form of Ca salt given will affect faecal pH. Behling & Greger (1988) found that rats fed on calcium carbonate had a higher faecal pH than rats fed on yoghurt or calcium phosphate but lower pH than rats fed on an amino acid chelate of Ca. These changes in pH could effect gut flora and the solubility of faecal bile acids and soaps.

A second difference in these studies is the type of dietary fat. Investigators gave maize oil (Pence & Buddingh, 1988) or beef tallow (Bull *et al.* 1987), or instilled deoxycholic acid on colonic epithelium (Wargovich *et al.* 1983). In the present study, butterfat was used because Ca is found most often with butterfat in dairy products.

In a previous study, rats initiated with DMH and fed on 200 g butterfat with 10 g Ca/kg for 7 weeks had elevated caecal nitroreductase and β-glucuronidase activity and lowered diet digestibility (Behling *et al.* 1990). This suggested that during early promotion the availability of substrate (i.e. undigested dry matter) was correlated with caecal enzyme activity. However, the caecal flora of rats at the end of this present study were not correlated with the incidence or size of intestinal tumours or with faecal losses of dry matter and lipid after 26–28 d.

In general, a fourfold change in Ca intake had dramatic effects on excretion of acid-extractable lipid in faeces. However, the overall effect on percentage absorption of lipid, although statistically significant, was small, i.e. less than 6%.

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