

# Conjugated Linoleic Acid Suppresses Mammary Carcinogenesis and Proliferative Activity of the Mammary Gland in the Rat<sup>1</sup>

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## ABSTRACT

Conjugated linoleic acid (CLA) is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid. It is naturally occurring in meat and dairy products. We have previously reported (Ip, C., Chin, S. F., Scimeca, J. A., and Pariza, M. W. *Cancer Res.*, 51: 6118-6124, 1991) that 1% CLA in the diet suppressed mammary carcinogenesis in rats given a high dose (10 mg) of 7,12-dimethylbenz(a)anthracene. In the present study, dietary CLA between 0.05 and 0.5% was found to produce a dose-dependent inhibition in mammary tumor yield when fed chronically to rats treated with a lower dose (5 mg) of 7,12-dimethylbenz(a)anthracene. Short-term CLA feeding for 5 weeks, from weaning to the time of carcinogen administration at 50 days of age, also offered significant protection against subsequent tumor occurrence. This period corresponds to maturation of the mammary gland to the adult stage in the rat. The inhibitory response to short-term CLA exposure was observed with the use of 2 different carcinogens: 7,12-dimethylbenz(a)anthracene and methylnitrosourea. The fact that CLA was protective in the methylnitrosourea model suggests that it may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation. The proliferative activity of the mammary epithelium was assessed by the incorporation of bromodeoxyuridine. Immunohistochemical staining results showed that CLA reduced the labeling index of the lobuloalveolar compartment, but not that of the ductal compartment of the mammary tree. Since the lobuloalveolar structures are derived from the terminal end buds which are the sites of carcinogenic transformation, the above finding is consistent with the bioassay data of tumor inhibition. Thus, changes in gland development and morphogenesis may be a locus of action of CLA in modulating mammary carcinogenesis. CLA is a unique anticarcinogen because it is present in foods from animal sources. Furthermore, its efficacy in cancer protection is manifest at dietary concentrations which are close to the levels consumed by humans.

## INTRODUCTION

A voluminous amount of data is available in the literature linking increased consumption of fat and stimulation of mammary tumorigenesis in the animal model. This subject has been thoroughly discussed in several review articles (1, 2). The mechanisms that might account for the enhancing effect of fat have yet to be resolved. A high fat diet is also a calorie-dense diet. Thus, the relationship between dietary fat and mammary cancer could potentially be complicated by changes in energy intake. Any digestible fat at levels beyond that required for cellular homeostasis and structural integrity may serve as a source of excess calories, and it is this increased metabolizable energy that is conducive to the proliferation of cancer. There is, however, a specific effect of fat which relates to individual fatty acids. For example, linoleic acid has been consistently associated with enhancement of mammary cancer development in rodents (2). In contrast to linoleic acid, we have previously reported that isomers of linoleic acid, denoted as conjugated linoleic acid, are able to prevent mammary tumorigenesis induced by a carcinogen (3).

Linoleic acid is an essential polyunsaturated fatty acid of 18-carbon chain length with 2 double bonds in the 9 and 12 positions (both are in the *cis* configuration). CLA<sup>3</sup> is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid. The 2 double bonds in CLA are in positions 9 and 11, or 10 and 12, along the carbon chain, thus giving rise to the designation of a conjugated diene. Each of the double bonds can be in the *cis* or *trans* configuration. CLA, a normal isomerization metabolite of linoleic acid by rumen bacteria (4), is a naturally occurring substance in food. It was initially isolated and identified by Ha *et al.* (5) as an anticarcinogenic agent from grilled ground beef. These investigators subsequently showed that cheese is also a rich source of CLA (6). Rumen bacteria are unlikely to be the sole producer of CLA found in unprocessed food, since raw meat from nonruminants (*e.g.*, pork, chicken, and turkey) is known to contain measurable but lower amounts of this fatty acid (7). Cooking has been shown to increase the concentration of CLA in meat (6). However, the mechanism of linoleic acid conversion to CLA during cooking and food processing remains to be clarified.

In an earlier publication, we reported that 1% by weight of CLA in the diet maximally suppressed mammary carcinogenesis in rats given a 10-mg dose of DMBA (3). This was the first study demonstrating that chronic CLA feeding, from 2 weeks before DMBA administration until the end of the experiment, was effective in cancer prevention. The work reported in this paper was designed to: (a) evaluate the dose dependency of dietary levels of CLA in the range between 0.05 and 0.5% for mammary cancer inhibition in rats given a low dose of DMBA (5 mg); and (b) determine whether short-term CLA feeding from weaning (21 days of age) to the time of carcinogen administration (50 days of age) was able to offer protection against subsequent tumor development. This particular period corresponds to maturation of the rat mammary gland to the adult stage morphology (8). The effects of CLA on DMBA binding to mammary cell DNA as well as proliferative indices of the mammary epithelial component were also investigated to gain insight into whether alterations in these parameters might contribute to changes in cancer susceptibility.

## MATERIALS AND METHODS

**Mammary Tumor Induction by Carcinogen.** Pathogen-free weanling female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in a room with a 12-h light/12-h dark cycle. Mammary tumors were induced by the administration of a carcinogen at 50 days of age. In Experiment 1, all rats were given a p.o. intubation of 5 mg of DMBA (Sigma, St. Louis, MO) dissolved in 1 ml of corn oil. There were 50 rats/group. This sample size ensured adequate statistical power due to the reduced number of tumors produced per rat by the low dose of carcinogen. In Experiment 2, 2 different carcinogens were used. Rats were given either 10 mg of DMBA p.o. or 6 mg of MNU (Ash Stevens, Inc., Detroit, MI) i.p. There were 25 rats/group. All animals were palpated weekly to determine the appearance and location of tumors. Experiments 1 and 2 were terminated at 36 and 24 weeks, respectively, after carcinogen administration. At necropsy, the mammary glands were exposed for the detection of nonpalpable tumors. Only confirmed adenocarcinomas were reported in the results. Tumor incidences at

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<sup>3</sup> The abbreviations used are: CLA, conjugated linoleic acid; DMBA, 7,12-dimethylbenz(a)anthracene; MNU, methylnitrosourea.

the final time point were compared by  $\chi^2$  analysis, and the total tumor yield between groups was compared by frequency distribution analysis as described previously (9). The statistical analyses of intergroup differences in tumor incidence and number were corrected for multiple comparisons.

**Dietary Treatment and CLA Supplementation.** Rats were acclimatized immediately to the powdered AIN-76A diet (10) upon arrival. One modification was made in this standard formulation. A mixture of dextrose and corn starch (1:1 ratio) was substituted for sucrose as the carbohydrate source. In Experiment 1, different levels of CLA (0.05, 0.1, 0.25, and 0.5% by weight) were added to the basal diet. The CLA-containing diets were given to the animals starting at 2 weeks before DMBA administration and continuing for 36 weeks until the end of the experiment. In Experiment 2, a diet containing 1% CLA was given to the rats from weaning until 1 week past carcinogen administration (*i.e.*, for a total of 5 weeks). The animals were then returned to the basal diet without CLA for the duration of the experiment. For the DMBA binding and the mammary gland bromodeoxyuridine labeling studies, which will be described below, the CLA feeding protocol was similar to that of Experiment 2, *i.e.*, starting from weaning and continuing for a period of 5 weeks, after which the animals were sacrificed.

The method of CLA synthesis from 99+% pure linoleic acid has been detailed in our earlier publication (3). The CLA used in the present studies was custom ordered from Nu-Chek Prep, Inc. (Elysian, MN). Gas chromatography analysis of the CLA preparation showed the following composition: *c9,t11*- and *t9,c11*-CLA, 43.3%; *t10,c12*-CLA, 45.3%; *c9,c11*-CLA, 1.9%; *c10,c12*-CLA, 1.4%; *t9,t11*- and *t10,t12*-CLA, 2.6%; linoleic acid (unchanged parent compound), 4.4%; and remainder (unidentified), 1.4%. The chemical composition of CLA from Nu-Chek was very similar to that prepared at the Kraft General Foods Technology Center and which was used in our previous studies (3).

**DMBA Binding to Mammary Cell DNA.** Rats were fed either the basal or 1% CLA diet from weaning. At 50 days of age, they were given 10 mg of  $^3\text{H}$ -labeled DMBA (1 mCi/rat; Amersham) *i.g.* and were sacrificed 1, 2, 4, or 7 days later. There were 4 rats/time point. Mammary glands were excised and immediately dropped in liquid nitrogen. Frozen mammary tissue was pulverized and mammary epithelial aggregates were dissociated from adipocytes and stromal materials by collagenase digestion. The methodologies involved in mammary cell DNA isolation, purification, quantitation, and the determination of bound DMBA by liquid scintillation counting have been described in detail in a previous publication by Ip and Daniel (11).

**Bromodeoxyuridine Labeling of the Mammary Gland.** Rats were fed either the basal or 1% CLA diet from weaning ( $n = 15/\text{group}$ ). For the last 3 days of the 5-week CLA feeding period, rats were given 5 *i.p.* injections of bromodeoxyuridine at 12-h intervals. A total of 58.6  $\mu\text{mol}$  were administered per rat, and the proportion of labeled cells was detected by immunohistochemical staining using the procedure described by Eldridge *et al.* (12). Rats were euthanized 12 h after the final bromodeoxyuridine injection. The thoracic mammary glands were rapidly excised and fixed in methacarn. Cells that incorporated bromodeoxyuridine were identified by brown to black granules over the nuclei. One thousand consecutive ductal nuclei were counted, and the number that stained positive was noted. The same procedure was followed for the lobuloalveolar nuclei. The level of positive labeling in each mammary compartment was expressed as a percentage.

## RESULTS

**Dose Response of Dietary CLA in Inhibition of Mammary Tumorigenesis.** In this experiment, rats were fed a diet containing 0.05, 0.1, 0.25, or 0.5% of CLA starting 2 weeks before DMBA and continuing for 9 months. With a 5-mg dose of DMBA, tumors take a longer time to develop and usually begin to level off by 8–9 months after carcinogen administration. The time course of cumulative tumor yield in the control and CLA-treated groups is shown in Fig. 1. The complete tumor data and their statistical analysis are summarized in Table 1. Two types of analysis were done with the data. (a) The entire data set for the different levels of CLA was analyzed as a whole to look for a dose-dependent effect. (b) Each dietary level of CLA was compared to the control in order to find out the particular level of CLA at which inhibition of tumorigenesis first became statistically significant.

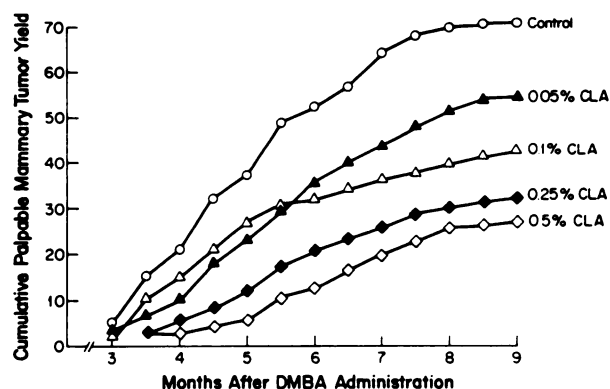


Fig. 1. Cumulative appearance of palpable mammary tumors as a function of time after DMBA administration in rats fed different levels of CLA.

Table 1. Mammary cancer prevention by supplementation of different levels of CLA in the diet<sup>a</sup>

% dietary CLA supplementation	Tumor incidence <sup>b</sup> (%)	Total no. of mammary tumors <sup>c</sup>	Final body wt (g)
None	28/50 (56)	74	367 ± 10
0.05	29/50 (58)	58	371 ± 9
0.1	21/50 (42)	47 <sup>d</sup>	370 ± 10
0.25	17/50 (34) <sup>d</sup>	37 <sup>d</sup>	374 ± 11
0.5	18/50 (36) <sup>d</sup>	31 <sup>d</sup>	369 ± 9

<sup>a</sup> Rats were given 5 mg of DMBA *i.g.* CLA was present in the diet starting at 2 weeks before DMBA and continuing for 36 weeks until the end of the experiment.

<sup>b</sup> Change in tumor incidence response to 0.05 to 0.5% CLA analyzed by logistic regression,  $P < 0.05$ .

<sup>c</sup> Change in mammary tumor yield response to 0.05 to 0.5% CLA analyzed by polynomial regression ( $P < 0.02$ ).

<sup>d</sup>  $P < 0.05$  compared to the corresponding control value.

A dose-dependent inhibition of mammary carcinogenesis by CLA was observed in the range between 0.05 and 0.5%. The progressive decreases in tumor incidence ( $P < 0.05$ ; Table 1, Footnote b) and total tumor yield ( $P < 0.02$ ; Table 1, Footnote c) as a function of increasing dietary CLA levels were analyzed by logistic regression and polynomial regression, respectively. Intergroup comparisons showed that as little as 0.1% CLA was sufficient to cause a significant reduction in the total number of tumors ( $P < 0.05$ ; Table 1, Footnote d). Thus, this study convincingly demonstrated that the administration of CLA via the dietary route is an effective way of achieving cancer prevention.

In our previous publication with the DMBA model (3), we reported that dietary CLA at 1.5% failed to produce any change in growth rate, food intake, or organ weight (liver, spleen, kidney, and uterus). Also discussed in the paper was an independent pathology examination study in which rats were fed 1.5% CLA for 36 weeks, although they were not treated with DMBA. There was no evidence of histomorphological abnormality observed in any one of 15 different tissues. The current experiment used lower levels of CLA, and again we did not detect any change in growth of the animals (data not shown). This is mentioned to affirm that reproducible results were obtained with different batches of CLA. The growth curves from all 5 groups would have been superimposable if they were plotted out graphically. Their body weights at necropsy are included in Table 1 to corroborate that CLA is a safe and effective anticarcinogen which can be consumed chronically without any apparent adverse effect on the host.

**Mammary Cancer Prevention by Short Term Feeding of CLA.** The period from weaning to about 50 days of age in the rat (the time of carcinogen administration) corresponds to maturation of the mammary gland to the adult stage with the number of terminal end buds decreasing gradually and differentiating to alveolar buds and lobules (8). It should be noted that carcinogenic initiation of the rodent mammary model occurs primarily in the epithelium of the terminal end

buds. We wanted to see whether limiting CLA feeding to this particular time frame would offer any protection against subsequent tumorigenesis. Two different carcinogens were used in this experiment for mammary tumor induction: DMBA, which requires metabolic activation, and MNU, which is a direct alkylating agent. Rats were fed 1% CLA from weaning to 1 week past carcinogen treatment, they were then returned to the basal diet without CLA until sacrifice. Our results in Table 2 showed that CLA exposure during this narrow window of mammary gland morphogenesis was able to significantly reduce total mammary tumor yield by 39 and 34% in the DMBA and MNU models, respectively. Based on the results of the first experiment, the addition of 1% CLA would be expected to produce a greater suppressive effect. However, it should be taken into consideration that the administration of a high dose of carcinogen for mammary tumor induction in this experiment (see Table 2 for doses of DMBA and MNU), coupled with the shorter duration of CLA feeding, probably accounted for the attenuated inhibitory response. But more importantly, the fact that CLA is protective in the MNU model suggests that it may have a direct modulating effect on susceptibility of the target organ to neoplastic transformation.

**Effect of CLA Feeding on Bromodeoxyuridine Labeling of the Mammary Gland.** To follow up on the above observations, we carried out an experiment to evaluate the effect of CLA on proliferative activity of the mammary gland. CLA feeding was started from weaning and continued until about 55 days of age. Essentially the protocol was identical to the previous one with the exception that no carcinogen was used in this experiment. Multiple injections of bromodeoxyuridine, as described in "Materials and Methods," enable a higher proportion of cells to be labeled by this biochemical marker and thereby increases the sensitivity of the assay. The results in Table 3 show that CLA reduced the labeling index of the lobuloalveolar compartment ( $P < 0.05$ ), but not that of the ductal compartment of the mammary tree. The significance of this finding will be discussed below.

**Effect of CLA on DMBA Binding to Mammary Cell DNA.** It is possible that the lower proliferative activity of the mammary gland following CLA feeding may not fully explain the increased resistance of the target organ to carcinogenesis. In order to determine whether the inhibitory response in the DMBA model to CLA feeding prior to carcinogen administration might be related to changes in DMBA activation, total DMBA binding to mammary epithelial cell DNA was measured at different times after a single dose of radioactive DMBA. The results in Table 4 indicate conclusively that total DMBA binding was not affected by CLA. Although these data do not rule out the possibility that differences may exist in the formation of specific DMBA-DNA adducts, our past experience has suggested that total DMBA binding is a fairly reliable indicator of changes in DMBA activation and therefore of adduct formation.

Table 2 Mammary cancer prevention by short term feeding of CLA<sup>a</sup>

Carcinogen <sup>b</sup>	% dietary CLA supplementation	Tumor incidence (%)	Total no. of mammary tumors
DMBA	None	20/25 (80)	62
	1	13/25 (52) <sup>c</sup>	38 <sup>c</sup>
MNU	None	22/25 (88)	76
	1	15/25 (60) <sup>c</sup>	50 <sup>c</sup>

<sup>a</sup> CLA was added to the diet starting from weaning until 1 week past carcinogen for a total period of 5 weeks.

<sup>b</sup> Rats were given 10 mg of DMBA i.g. or 6 mg of MNU i.p. at 50 days of age.

<sup>c</sup>  $P < 0.05$  compared to the corresponding control value.

Table 3 Effect of CLA feeding on proliferative activity of ductal and lobuloalveolar mammary epithelial cells<sup>a</sup>

Dietary CLA supplementation	No.	% mammary epithelial component <sup>b</sup>	
		Ductal	Lobuloalveolar
None	15	25.7 ± 3.1	32.4 ± 2.6
1%	15	21.9 ± 2.5	24.8 ± 2.9 <sup>c</sup>

<sup>a</sup> CLA was added to the diet starting from weaning and continuing for the next 5 weeks before excision of the mammary gland.

<sup>b</sup> Results are expressed as percent of cells (mean ± SE) labeled by bromodeoxyuridine.

<sup>c</sup>  $P < 0.05$ .

Table 4 Effect of CLA feeding on total DMBA binding to mammary cell DNA<sup>a</sup>

Dietary CLA supplementation (%)	Time course of total DMBA binding <sup>b</sup> (pmol/mg DNA)			
	Day 1	Day 2	Day 4	Day 7
None	33 ± 5.2	40 ± 6.9	31 ± 5.0	22 ± 3.8
1%	31 ± 6.1	38 ± 4.7	36 ± 3.6	25 ± 3.4

<sup>a</sup> Rats were fed either the basal or 1% CLA diet from weaning. At 50 days of age, they were given 10 mg of <sup>3</sup>H-labeled DMBA and were sacrificed at 1, 2, 4, or 7 days later.

<sup>b</sup> Measured by total tritium binding to DNA after <sup>3</sup>H-labeled DMBA administration. Results are expressed as mean ± SE ( $n = 4$ /group).

## DISCUSSION

Of the large number of naturally occurring substances that have been demonstrated to have anticarcinogenic activity in experimental animal models, all but a handful of them are of plant origin (13). CLA is unique because it is present preferentially in food from animal sources. CLA is closely related to linoleic acid, but differs from linoleic acid in the position and configuration of the double bonds. Unlike the stimulatory effect of linoleic acid on mammary carcinogenesis (14), CLA inhibits tumor development. As shown in Table 1, as little as 0.1% of CLA in the diet is sufficient to produce a significant reduction in mammary tumor yield in rats given a low dose of DMBA. The low dose carcinogen protocol was intended to increase the sensitivity of the animal bioassay so that the efficacy of CLA could be titrated more precisely. A 350-g rat fed a 0.1% CLA diet will consume about 0.015 g CLA/day. In a direct extrapolation to a 70-kg person, this is equivalent to a daily CLA intake of 3 g, an amount slightly higher than the estimated consumption of approximately 1 g/person/day in the United States (6). In the much quoted nurses' study by Willet *et al.* (15), dietary fat was found not to be a risk factor for breast cancer. Although the range of fat intake in this cohort was not as broad as that observed in international ecology studies, which generally report a positive correlation between breast cancer mortality and fat intake (16), it is tempting to speculate that the presence of CLA in the Western diet may play some role in moderating the impact of high fat consumption on breast cancer risk. Preliminary experiments from our laboratory indeed suggest that CLA could negate the stimulatory effect of fat on mammary carcinogenesis in the rat DMBA model.<sup>4</sup> Thus, the cancer protective efficacy of CLA needs to be further characterized in order to fully delineate its contribution to health maintenance and disease control.

Scanty information is available in the literature on the anticarcinogenic activity of CLA *in vivo*. To date, only 3 papers have been published regarding CLA supplementation in animal tumor models. The 2 papers from Ha *et al.* (5, 17) reported successful tumor inhibition in skin and forestomach of mice treated with a carcinogen. In both cases, CLA was delivered acutely in single doses prior to either DMBA (for initiation of skin papillomas) or benzo(a)pyrene (for induction of forestomach tumors) administration. Our previous study with the rat DMBA mammary tumor model was the first to show that dietary CLA is effective in cancer protection (3). Experiments will be

<sup>4</sup> C. Ip, and J. A. Scimeca, unpublished observations.

under way to contrast the action of CLA in the initiation and post-initiation phases of mammary carcinogenesis.

In the present study, we failed to detect changes in total DMBA binding to mammary epithelial cell DNA following CLA feeding. Although definitive conclusion about DMBA activation still needs to be confirmed by the determination of specific DMBA-DNA adducts, we feel that this might not be a promising avenue to pursue future mechanistic investigation. Total DMBA binding was measured at 4 different time points over a span of 7 days (Table 4). No difference was noticeable between the control and CLA-fed animals during this period, suggesting that the kinetics of DMBA metabolism were not affected. Urinary DMBA levels were similarly unchanged (data not shown). By day 7 after DMBA, there was virtually no more radioactive DMBA being excreted in the urine. The lack of an effect on DMBA removal would be consistent with our previous finding that CLA has no effect on glutathione-S-transferase or UDP-glucuronyl transferase activities (3). Based on these observations, it seems that neither Phase I nor Phase II detoxification enzymes may be the targets for the action of CLA in events associated with modulation of DMBA-induced mammary carcinogenesis.

As shown in Table 3, CLA reduced the proliferative activity of the lobuloalveolar structures of the mammary gland, but not of the ducts. This difference, however, was small and needs to be confirmed. Since the determination was made at a single time point, it is unclear whether this outcome is due to a change in the time course of mammary gland maturation or a quantitative shift in gland composition. Multiple time point analysis on both kinetic and morphological parameters would be necessary in order to address this question. Similar studies should also be carried out with and without carcinogen administration. In the absence of any information correlating the effect of CLA on proliferation and differentiation during maturation of the mammary gland, it is not possible to make a definitive conclusion regarding the impact of CLA on susceptibility of the target organ to carcinogenesis. Nonetheless, suffice it to note that the lobuloalveolar structures are derived from the terminal end buds which are the sites of carcinogenic transformation. The observation that the proliferative activity of the lobuloalveolar units is reduced by CLA feeding is congruent with the bioassay results of tumor inhibition. What is so tantalizing about this finding is that exposure to CLA during mammary gland morphogenesis in the adolescent period (30 to 60 days of age in a rat) may be able to provide lasting protection against subsequent cancer risk. In humans, early onset of puberty increases, but early pregnancy decreases, the risk of breast cancer (18). Both factors highlight the importance of developmental changes of the mammary gland during the period of adolescence in determining the risk of breast cancer. Further investigation will be focused on examining the role of CLA in influencing mammary gland morphogenesis and proliferation.

The subject of CLA and cancer prevention is in its infancy. In the previous paper as well as the present one, we have identified several areas that are critical in expanding the horizon of CLA research. CLA is by far the most powerful naturally occurring fatty acid known to

modulate tumorigenesis. The fact that a fatty acid can produce such a striking effect at near nutritional levels of intake is fascinating. However, foods that are high in CLA are also high in fat content. Consequently, it is difficult to evaluate from epidemiology data the impact of CLA alone in the context of a high fat intake in humans. Much emphasis has been placed on total fat consumption and breast cancer risk, perhaps justifiably so. On the other hand, the equivocal and often negative results from case-control and cohort studies have hinted at the complexity of this issue (16). The challenge of future research will be to define the potential benefit of CLA in our diet, to characterize its anticancer activity, to elucidate its mechanism of action at the sub-cellular level, and to design new strategies for enriching foods with CLA if this approach is deemed appropriate.

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