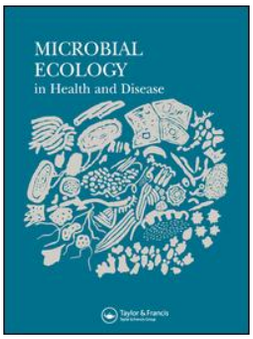

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Marine Oils in the Modulation of Colonic Flora and pH: Considerations for Colon Cancer

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BALB/c mice fed high fat diets containing coconut, safflower, or menhaden marine oil were evaluated for faecal flora and faecal pH. Animals fed marine oils showed significantly higher total aerobic Gram-negative rods at 10^9 CFU/gm, compared with those fed saturated or polyunsaturated fat diets with 10^6 CFU/gm. Marine oil fed mice exhibited a significantly lower $\log_{10}$ ratio of anaerobes:aerobes at 1·17, compared to the saturated and polyunsaturated fat fed mice at 3·49 and 2·87 respectively. Differences in flora composition were seen, with increased *E. coli* (10^9 CFU/gm) for the marine oils group compared to the saturated and polyunsaturated groups, each with 10^6 CFU/gm. Faecal samples from animals on marine oils exhibited a lower pH (6·1), compared to samples from animals on saturated or polyunsaturated fat with pH of 7·32 and 7·49 respectively. β-glucuronidase levels were also evaluated and showed no significant differences among the different diets.

KEY WORDS—Colonic flora; Marine oils; Dietary lipids; Faecal pH; β-glucuronidase; Colon cancer.

INTRODUCTION

Multi-national epidemiological studies and concordant laboratory data indicate that the incidence of colon cancer is proportional to the quantity of dietary lipids consumed.[5,29] Further, the gut microflora influenced by diet appear to play an important part in the aetiology of this disease.

Hill and associates[12,13] studied faecal flora and sterol excretions in comparative studies of 'Western' and vegetarian diets. Populations studied on 'Western' diets, where colon cancer rates are higher (USA, Britain), showed increased numbers of anaerobic gut bacteria, whereas populations on vegetarian diets, whose colon cancer rates are lower, had higher aerobic:anaerobic gut bacteria ratios. Qualitative differences also existed in the populations of the microflora of the two diet populations studied.

Data in support of dietary influences on the intestinal microflora have been reported from several sources.[7,17,21] In addition, data have also been evaluated in terms of populations at high and low risk for colon cancer with differences noted in the constituency of the intestinal microflora.[8]

Hill has suggested,[13] that a 'Western' diet favours the growth of bacterial flora in the gut which could promote the conversion of bile acids to carcinogen promotors. High dietary fat consuming populations, such as those on 'Western diets', have increased faecal bile acids.[10] This is associated with an increase in the faecal levels of the bacterial enzyme 7-α-dehydroxylase [28] which allows for the conversion of primary bile acids to secondary bile acids. The latter have been reported to promote colon cancer in rats.[19,22]

In vitro studies have shown the dehydroxylase enzyme to be inducible, and its activity markedly affected by the conditions of incubation (pH, glucose concentration, and other nutrients), suggesting that conditions in the intestine may be of critical importance in the microbial transformation of bile acids.[25]

Certain experimental carcinogens conjugated in the liver to glucuronides and excreted with bile, are believed to be activated in the colon by the bacterial enzyme β-glucuronidase.[28] Bacterial β-glucuronidase has a wide substrate specificity and can hydrolyse a large number of glucuronides.[10]

Substitution of a non-meat diet for a high meat diet has been shown to decrease faecal β-glucuronidase activity.[20] Other findings[11] also reflect higher β-glucuronidase activity levels in meat-based diets, compared to vegetarian diets.

Data from Blot *et al.*[3] show that Alaskan Eskimos

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have decreased mortality from colon cancer in comparison to U.S. Caucasians. Data on fats in the diets of Eskimos have shown these to be richly endowed with marine oils.[2,18,26] An analysis of the composition of these diets reflects high contents of polyunsaturated fatty acids, predominately of omega-3 class eicosapentanoic and dodeicosohexanoic acids.

Recent studies[23] on rats placed on high Menhaden fish oil diets and subjected to azoxymethane-induced colon carcinogenesis showed no tumour promoting effect in the large intestine compared to a high corn oil diet. Similar evidence has been obtained with transplantable colon tumours, where fewer, smaller and less frequently metastasising tumours were seen in mice on marine oils.[4]

Recent studies have looked at the proneness to colon cancer in relationships to faecal pH. Populations with higher colon cancer mortality rates were seen to exhibit higher faecal pH values.[27] Another study by MacDonald *et al.* has shown that faecal pH is elevated in colon cancer patients compared to control populations.[16] MacDonald has suggested that pH changes in the gut may be important to critical enzyme functions suspected in colon carcinogenesis.

Recent studies with wheat bran, a dietary component believed to decrease risk to colon cancer, have shown this to decrease intestinal pH.[14] The same study suggests that dietary effects on gut pH may be important in colon carcinogenesis, and that wheat bran may afford protection by this mechanism.

Consideration is therefore given to the possible modulation of the intestinal microflora and pH by diets rich in marine oils.

MATERIALS AND METHODS

A comparative study was undertaken using BALB/c male weanling mice from Charles River Breeding Laboratories (Wilmington, MA). Six mice were assigned to each diet group. The semi-synthetic diets were formulated in accord with Control of Diets in Laboratory Animal Experimentation[6] and have been used in previous studies in our laboratory.[4] These were composed of 20 g coconut oil for the saturated fat diet, 20 g safflower oil for the polyunsaturated diet, and 20 g Menhaden oil obtained from Zapata Haynie Corporation (Readville, VA) for the marine oil diet. Approximately 4·88 kcal of energy was obtained per gram of each diet, with 46·5 per cent of the total kcal derived from dietary fat. The mice were fed their particular diet for 30 d prior to onset of studies and held in a separate stainless steel cage in an animal holding room, under controlled environmental conditions. All animals had free access to food and water.

Freshly passed stools weighing from 8·8 to 60 mg were diluted 1:10 in 10 ml of normal saline. Serial dilutions corresponding to concentrations of 10^{-3}, 10^{-5} and 10^{-7} were prepared, from which inocula corresponding to 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} were seeded onto their respective agar plates.

Phenylethyl alcohol (PEA) blood agar plates (Gibco Biologicals) were used in enumerating streptococci and staphylococci. The agar was stabbed to favour the growth of Gram-positive anaerobes. Colonies on PEA were identified on the basis of Gram morphology and haemolytic factors. Catalase and coagulase tests were used in the identification and differentiation of staphylococci and streptococci. Streptococci were also evaluated based on their haemolytic activities. Bile aesculin was used to distinguish Group D streptococci, and mannitol salt broth (BBL Corp.) in distinguishing enterococci from non-enterococci.

Anaerobic flora was enumerated on Kanamycin-Vancomycin (KV) agar (Gibco Biologicals). MacConkey agar prepared from commercial media (Gibco Biologicals) was used for isolation and differentiation of *Enterobacteriacae*. Colonies growing on MacConkey agar were evaluated for their Gram and colonial morphology, and the ability to ferment lactose. Species identifications on the isolated *Enterobacteriacae* using the API 20E System (Analytab Products) were performed by the Dept. of Microbiology, University Hospital, Boston, MA.

Lactobacilli MRS agar modified with the addition of tomato juice was used to identify lactobacilli. Lactobacillus MRS agar was prepared from lactobacillus MRS broth (Gibco Biologicals), to which 15 g of agar and 10 g of sodium acetate were added per litre, and the pH adjusted to 5·4 to 5·5.

PEA, KV, and MRS plates were incubated under anaerobic conditions using the GAS-PAK System from BBL Corporation Cockeysville, MD. These plates were incubated for 48 h prior to examination. These cultural methods have previously yielded in our laboratory anaerobe recoveries of about 90 per cent with respect to microscopic count. MacConkey plates were incubated for 24 h aerobically prior to examination. All plates were incubated at 37°C. Scoring the colonies was attempted from plates with 30 to 300 viable colonies.

Faecal pH evaluations

BALB/c male weanling mice were grouped, maintained and fed in a manner similar to that previously described for the gut flora survey. Freshly passed stools were weighed and emulsified in a 1:10 dilution with normal saline. A Beckman pHi 40 meter was used to measure pH, and all readings were checked against buffer solutions of pH 4·0, and 7·0. Stool pH was calculated by converting the pH of the saline solution and pH of the emulsified stool to hydrogen ion concentration. The difference from these values was then adjusted for the 1:10 dilution, and converted back to pH by taking the $\log_{10}$ value.

Assay of β-glucuronidase activity

Four animals from each diet group were evaluated. Freshly passed stools were weighed and dissolved in 3 ml of diluting solution, which had been boiled for 60 min and allowed to cool prior to stool addition. The diluting solution was prepared from 0·2 g gelatin, 50 ml distilled H_2O, and 50 ml salts solution. Salts solution was prepared from 0·2 g $CaCl_2$ (anhydrous), 0·2 g $MgSO_4$:$7H_2O$, 1·0 g K_2HPO_4, 1·0 g KH_2PO_4, 10 g $NaHCO_3$ and 2·0 g NaCl per litre. Cysteine-HCl, 0·05 g, was added to each tube as a reducing agent. Resazurin solution, 10 per cent, was added as a reduction indicator. Stools were suspended by agitation with a glass rod, and centrifuged for 30 min at 100 g at 4°C. The resulting supernatant was decanted and centrifuged for 30 min at 15,000 g at 4°C in a Sorvall RC-5 refrigerated centrifuge, using an SS-34 rotor. The bacterial pellet was washed three times by centrifugation as above, and re-suspended in 3 ml of normal saline. The resuspended pellet was sonicated in an ice bath for 5 min (20 sec bursts followed by 20 sec rest periods). Sonication was performed by a Sonifier Cell Disruptor, model W 185 from Ultrasonics Inc. (Plainview, NY) using a large tip with a power output of 65–70 watts.

The assay for β-glucuronidase activity was carried out according to the Fishman method.[9] Phenolpthalein release was measured after 30 min at 550 nm. 0·1 ml of sample was used in this assay. Protein determinations were made using the Lowry method.[15] Data were expressed as units of β-glucuronidase activity per mg protein.

β-glucuronidase dependence on pH

E. coli β-glucuronidase (Sigma), was used in evaluating pH dependence. 1000 unit vials were diluted in 1 ml of pH 7·0 phosphate buffer resulting in 100 units/0·1 ml. The activity was evaluated based on the Fishman method.[9] Phenolpthalein release was measured as before.

RESULTS

In evaluating the faecal aerobic Gram-negative rods (*Enterobacteriacae*) obtained from MacConkey agar, it was noted that the total population of Gram-negative rods (Figure 1) were significantly greater in the marine oil diet group (10^9 CFU/g) than in the polyunsaturated fat diet (10^6 CFU/g), or the saturated fat diet group at 10^6 CFU/g. In the marine oil group, greater numbers of *Escherichia coli* (10^9 CFU/g) were noted, compared to 10^6 CFU/g for each of the polyunsaturated and saturated fat groups. Of the mice fed a saturated fat diet, *E. coli* comprised only 39·1 per cent of the total Gram-negative rod population, whereas a much higher percentage of *E. coli* was observed in the polyunsaturated fat and marine oil groups (82·6 per cent and 77·8 per cent respectively).

Comparison of the *Klebsiella sp.* for each of the three diet groups reflected significantly greater numbers in the marine oil diet (10^8 CFU/g) versus 10^5 CFU/g for the saturated fat diet. *Klebsiella sp.* were not isolated from the polyunsaturated fat diet group.

No significant differences were observed in either lactobacilli or *Bacteroides sp.* among the three diet groups evaluated, with approximately 10^9 CFU/g and 10^9 to 10^{10} CFU/g of faeces respectively for all three diet groups.

In comparing the total Gram-positive flora isolated on PEA plates for the three diet groups, no significant variation was noted with approximately 10^9 to 10^{10} CFU/g isolated. Differences, though, were noted at species level (Table 1). Approximately 10^9 CFU/g of non-enterococci were noted in the marine oil group whereas in the saturated fat group, 10^7 CFU/g occurred. In the polyunsaturated fat diet 10^8 CFU/g were noted. This contributed to a higher overall number of α-haemolytic streptococci for the marine oil group, 10^{10} CFU/g, compared to 10^8 CFU/g for the saturated fat diet group. The polyunsaturated fat diet group was in an intermediate position with 10^9 CFU/g. Greater numbers of coagulase negative staphylococci were isolated from the marine oil group with 10^9 CFU/g compared to 10^6 CFU/g and 10^8 CFU/g for the saturated and polyunsaturated fat diet groups, respectively.

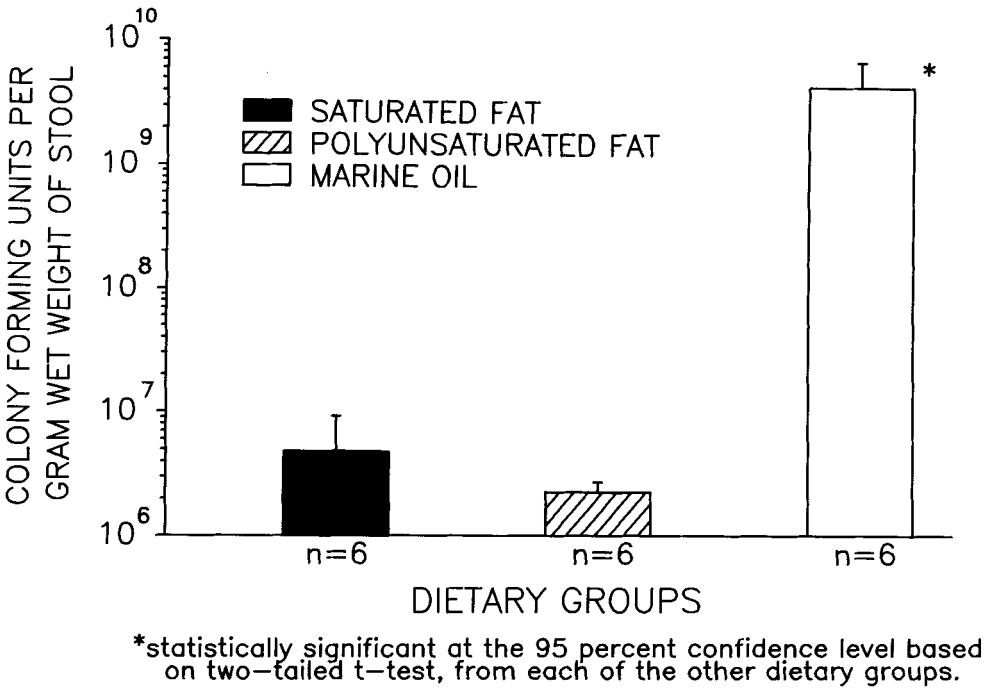

Figure 1. Comparison of total faecal aerobic Gram-negative rods

Table 1. Comparisons of Gram-positive bacteria in three diet groups (expressed as CFU/gm of wet weight of stool)

	α-haemolytic Non Group D Streptococci	Enterococci	Non-Enterococci	α-haemolytic Streptococci Totals
Diet group				
Saturated	10^8	10^7	10^7	10^8
Polyunsaturated	10^9	10^8	10^8	10^9
Marine oil	10^9	10^8	10^9	10^{10}
	γ-haemolytic Streptococci	All Streptococci	Coagulase negative Staphylococci	
Saturated	10^8	10^7	10^6	
Polyunsaturated	10^9	10^8	10^8	
Marine oil	10^9	10^9	10^8	

($\bar{x}$ of totals of all groups; n = 6; Log_{10} Mean).

Significant differences existed among the three diets in the total number of aerobes, whereas no differences existed in the total number of anaerobes. 10^9 CFU/g of total aerobes were seen in the marine oil group, whereas 10^7 and 10^6 CFU/g, respectively, occurred in the animals on the saturated and

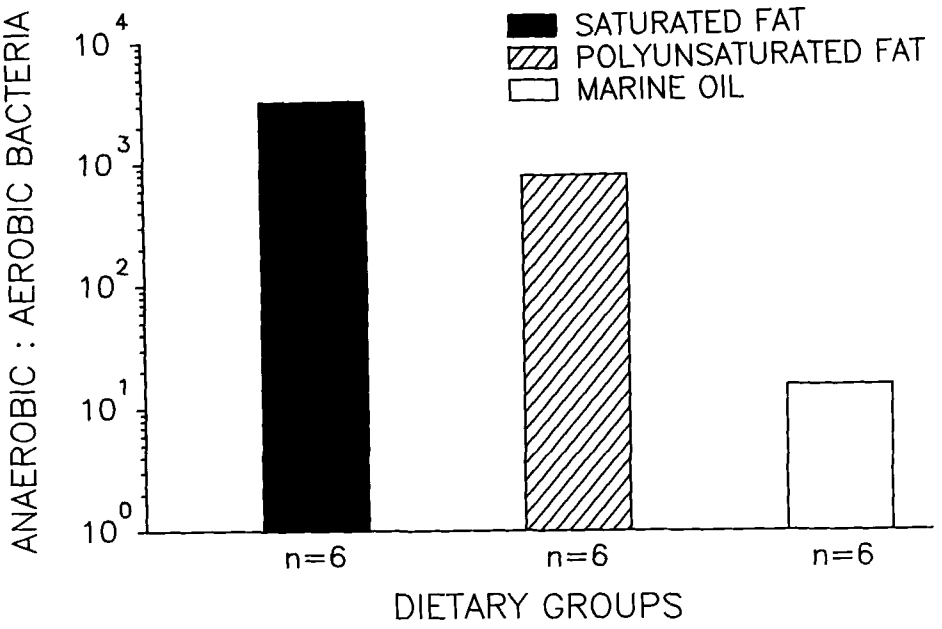

Figure 2. Comparison of ratios of total faecal anaerobic to aerobic bacteria

polyunsaturated fat diets. Approximately 10^{10} CFU/g of total anaerobes were seen in each of the diets.

The mean $\log_{10}$ (Figure 2) for the anaerobe: aerobe ratio was 3·49 for the saturated fat diet, indicating a greater anaerobic flora presence in the gut of animals on such a diet. This ratio differed substantially from the animals on the polyunsaturated fat diet where the mean $\log_{10}$ of the ratio was 2·87. An even greater difference was seen with the marine oil fed animals where the ratio was 1·17. Results on the pH of stools from each of the three diet groups reflect a lower stool pH in the marine oil fed animals (6·10) compared to the saturated and polyunsaturated fat fed animals (Figure 3), with stool pH of 7·32, and 7·49, respectively.

In evaluating the levels of β-glucuronidase in the three diets (Figure 4), no significant differences were noted. Levels for the saturated and polyunsaturated fat diets were 10^1 and 10^2 Fishman units/mg protein respectively, compared to 10^2 units/mg protein for the marine oil diet. Limitations though may exist in this interpretation of the data since β-glucuronidase activity assayed at 30 min may not reflect differences in activity which could become apparent at time intervals beyond 30 min. Evaluation of the pH dependence of bacterial β-glucuronidase demonstrated the activity of this enzyme to be greater at pH 6·5 and above (Figure 5).

DISCUSSION

These studies have focused on the effects of dietary marine oils in modifying the intestinal flora and have indicated that marine oils exhibit a different modifying influence on the colonic environment than saturated or polyunsaturated fats. The most notable differences exhibited with the marine oil diet consisted of increases in gut *Enterobacteriacae*, and the lowering of faecal pH.

Enterobacteriacae were present in the marine oil group in significantly greater numbers than that found in the saturated or polyunsaturated fat diets. *Enterobacteriacae* made up a greater proportion of the intestinal flora in the marine oil diet group, as is reflected in the ratios of total aerobes to total anaerobes for this group. The increase noted in the *Enterobacteriacae* within the marine oil group was due mainly to an increase in *E. coli*, and to a lesser extent *Klebsiella sp.*

These findings parallel those of Hill *et al.*[12] who showed that the ratio of faecal anaerobes to aerobes was significantly higher in 'Western diet' populations, and in whom the consumption of saturated

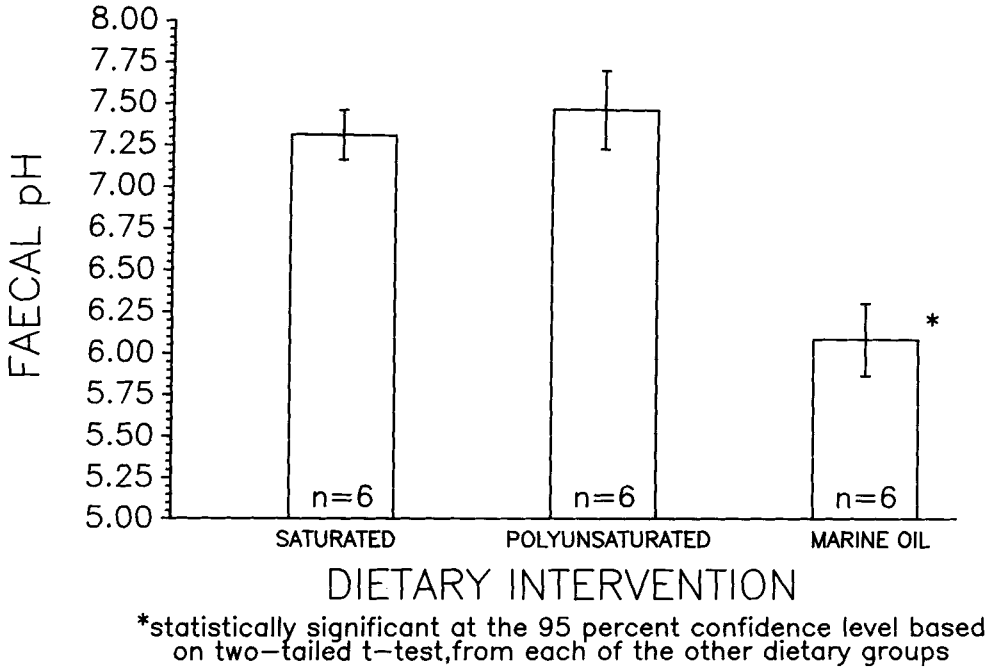

Figure 3. Effect of diet upon faecal pH in BALB/C mice

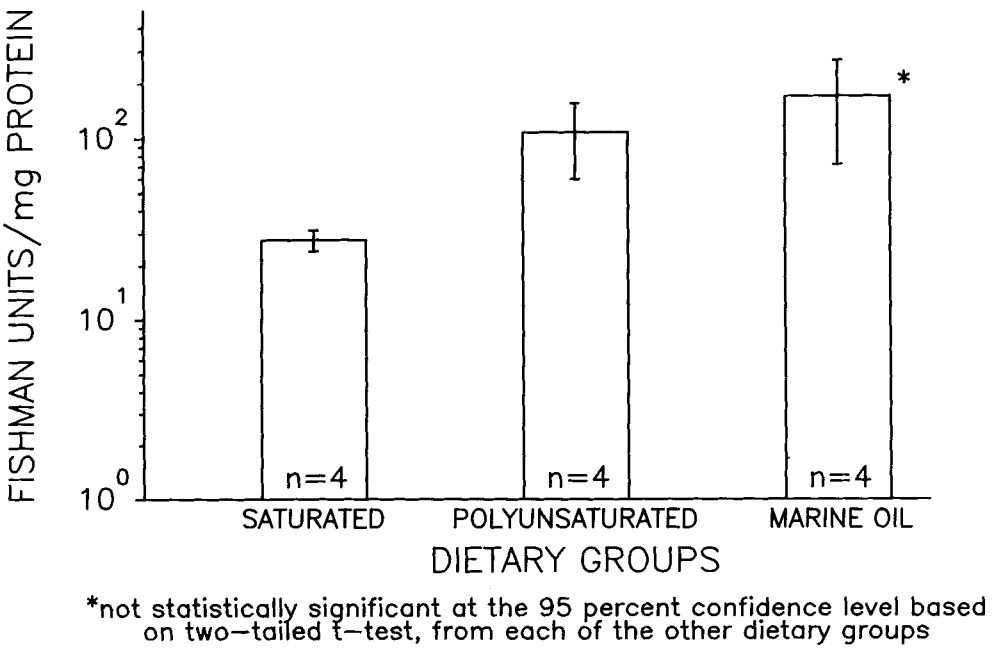

Figure 4. Faecal β-glucuronidase activity for the evaluated dietary interventions

fats is typically high. This compared to the vegetarian diet populations studied by Hill *et al.*,[12] whose diets are rich in polyunsaturated fats and in whom lower faecal anaerobe to aerobe ratios are exhibited. In this study differences similar to those observed by Hill *et al.*[12] were seen between the saturated and

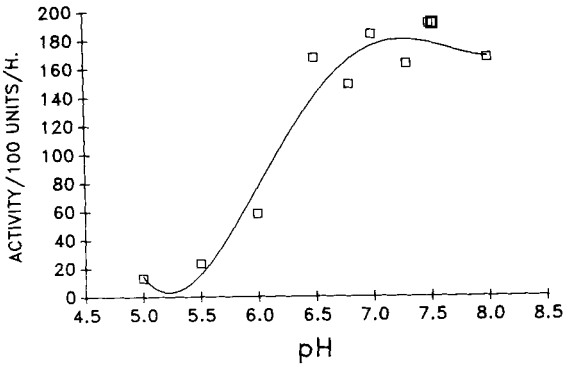

Figure 5. β-glucuronidase dependence on pH

polyunsaturated fat diet groups. However, the marine oil diet was seen to have an anaerobe:aerobe ratio which was significantly lower than that seen of the polyunsaturated fat diet. This is mainly due to the significant increase of *Enterobacteriacae* in the marine oil diet.

Shifts in the composition and metabolic activity of colonic flora are important when risks to colon cancer are considered. *Enterobacteriacae* isolated by Hill *et al.*[12] from both 'Western' and vegetarian diet groups were devoid of 7-α-dehydroxylase activity while a greater percentage of anaerobe strains were capable of 7-α-dehydroxylation.

Differences seen among the diet groups in faecal pH may also be important in evaluating 7-α-dehydroxylase activity. Animals on marine oil diets were seen to have a lower faecal pH when compared to those animals on the saturated or polyunsaturated fat diets. Studies by Aries and Hill[1] have shown that 7-α-dehydroxylase activity is optimal between pH of 7·0–8·0, while this activity is absent in pH of 6·5 or less. The pH dependence of this enzyme's activity may be important when consideration is made of the faecal pH previously discussed for the three diet groups.

Walker *et al.*,[27] in comparing the faecal pH of rural and urban S. African blacks, S. African Indians, and urban whites, noted a lower pH in the former groups. Lower pH values were seen to be associated with decreased colon cancer mortality rates.

No significant differences in β-glucuronidase levels in the faeces of mice on saturated, polyunsaturated, or marine oil diets were obtained. Rothman and Broitman[24] in comparing rats on low (5 per cent) and high (20 per cent) saturated fat diets, and subjected to DMH for colonic tumour induction, noted no increase in the levels of β-glucuronidase in the former diet.

Rather than having an effect on the enzyme levels *per se*, marine oils may modulate the activities of bacterial β-glucuronidase by their effect on gut luminal pH. To better understand the effects of pH on β-glucuronidase activity, the pH dependency of this enzyme was evaluated. Higher activity rates for β-glucuronidase were seen in the pH range of 6·5–8·0. Based on these activity differences, higher rates of β-glucuronidase activity would be expected in saturated and polyunsaturated fat fed animals versus animals on marine oils which exhibited lower faecal pH.

In summary, dietary marine oils in addition to inhibiting tumour growth, may also reduce colon cancer risk by favouring the growth of *Enterobacteriacae* and by decreasing faecal pH. The importance of these changes to two bacterial enzymes associated with enhanced colon cancer risk were considered. The findings suggest that dietary marine oils may act to decrease the activity of these bacterial enzymes by their ability to modulate colonic flora and colonic pH.

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