CHAPTER 7
Study of the regulatory effects on colonic ACF lesions by dietary fish oil

7.1. ACF study

7.1.1. Aims

This study was designed to observe the effect of a range of fish oil diets on ACF formation in rat colon. Aberrant crypt foci (ACF) incidence and size were quantified following a 12 week regime of feeding and 2 injections of AOM at 15mg/kg.

It was hypothesised that the reduction in O^6medG levels as observed in the 7% METO group in the acute study would be translated into a reduction of ACF. In addition to testing this particular hypothesis, additional experimental diets were also tested which allowed us to explore whether the type of fish oil also affects ACF formation in the colon. These hypotheses were tested by completing the following aims.

The specific aims of this particular study were;

1. To observe any changes in weight, faecal and caecal pH and short chain fatty acid profiles.

2. To determine whether free fish oil protects against ACF formation.

3. To determine whether fish oil when encapsulated protects against ACF formation.

4. To determine whether the microencapsulant adds value to the effect of fish oil.

7.1.2. Experimental rationale

Following on from the acute dietary study, it was decided that the reduction of colonic O^6medG DNA damage observed in animals fed a diet of 7% encapsulated and 15% free tuna oil warranted further investigation for its consequent impact on oncogenesis.

Cumulative levels of O^6medG in the colon have been associated with eventual tumour yield [62, 65, 66] and therefore, it was decided that a longer term study would be useful in determining not only whether these fish oil diets are protective against the initiation of colorectal cancer but also whether O^6medG levels are indicative of cancer risk. As a long term study with cancer as the endpoint was not feasible at this point, an ACF study which would still produce quantifiable data but in a shorter time frame was utilised.

Aberrant crypt foci (ACF) lesions are now well recognised as putative precursors to adenomas in the development of colorectal cancer and numerous studies using animals have examined ACF as effective biomarkers of colorectal cancer [139, 204, 205]. It is plausible that ACF evolve from a variety of genetic alterations, though the precise trigger that causes an ACF to develop dysplasia remains uncertain. This is supported by the fact that genetic mutations found in advanced neoplasms have also been identified in ACF including K-ras [42], APC [39] and p53 [41] together with aberrant methylation of other genes also.

In humans, ACF can be identified using a high magnifying chromoscopic colonoscopy and dye spraying and incidence of ACF has been found to correlate well with colorectal cancer risk [206]. ACF incidence in animal models also correlates well with tumour incidence though it is less convincing. While numerous authors have demonstrated a close relationship between ACF number and tumour development, this has been disputed [207] and it has been suggested that the tumour histology in the AOM model is important and ACF may only correlate with the non-mucinous sub type of tumour found in the distal colon [208]. Nevertheless, when one takes into account ACF size the correlation to tumour incidence can be strengthened significantly [206, 209].

ACF lesions in rat colon were first described by R. Bird in 1987 [210] using a methylene blue stain. ACF are predominately characterised as being larger than adjacent normal crypts and as having thicker epithelial linings and slit like luminal openings. These characteristics can be easily identified in rat colon by their darker stain when immersed in methylene blue and the size of ACF can be determined by counting the number of these irregular crypts present within an ACF cluster.

As rats fed the 7% microencapsulated tuna oil had a lower level of O^6medG damage when compared to the 7% free tuna oil diet it was decided that these 2 diets would be reproduced and would be the focus of this ACF study. Both tuna oil diets were replicated identically to those used in the acute study (see 5.1.3). Similarly, a 20% sunflower oil diet was also used as a control group for comparisons sake. Acute O^6medG data for all 3 groups had been established previously and therefore, the additional ACF data would be of great use when trying to ascertain a relationship between levels of O^6medG damage and colonic ACF formation in the rat.

In addition to the sunflower oil control and the 2 tuna oil groups, a further 2 groups were tested that included menhaden oil at the same dose of 7% in free form and in encapsulated form. This alternative source of fish oil that is rich in EPA added an extra element to this experiment, allowing us to also establish whether not only if a diet including fish oil affects ACF incidence, but also if the type of fish oil used has an influence. Furthermore, the 2 types of fish oil being tested, that is tuna and menhaden oil, both have different EPA/DHA ratios. It was hypothesised that the high EPA menhaden oil may have greater protective effects on ACF incidence than the high DHA tuna oil.

7.1.3. Study design

6 Groups of 15 male Sprague-Dawley rats at 5 weeks of age were housed in cages of 5. Rats were caged on a wire grid to minimise coprophagy and consumption of bedding materials and were kept in a temperature and humidity controlled animal house facility.

The composition of the 20% SO control diet remained unchanged when compared to the previous acute dietary experiment (see 5.1.3). As this ACF study had the purpose of further investigating the effects of the 7% METO diet trialled in the previous acute study, the composition of this diet in particular was replicated and its free tuna oil comparison was generated accordingly. In addition to the tuna and sunflower oil diets, it was decided that diets containing menhaden fish oil would also be made at a dose of 7% and tested in its free oil form (MO) and also in an encapsulated form (MEMO).

As a result 6 diets were used and were as follows; 20% sunflower oil control (20% SO), 7% microencapsulated sunflower oil control (7% MESO), 7% free tuna oil (7% TO), 7% free menhaden oil (7% MO), 7% microencapsulated tuna oil (7% METO) and 7% microencapsulated menhaden oil (7% MEMO). The compositions of these diets are listed below in table 20.

Table 20: Diet Compositions for ACF study (g/100g)

	Dietary group					
Ingredients	20% SO	7% TO	7% MO	7% MESO	7% METO	7% MEMO
Sunflower oil	20	13	13	13	13	13
Free Tuna oil		7				
Free Menhaden oil			7			
ME Sunflower oil				(7)		
ME Tuna oil					(7)	
ME Menhaden oil						(7)
Cornstarch	50	50	50	36	36	36
Cellulose	5	5	5	5	5	5
Casein	20	20	20	20	20	20
Minerals	3.5	3.5	3.5	3.5	3.5	3.5
Vitamins	1	1	1	1	1	1
Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.2	0.2	0.2	0.2	0.2	0.2
Total	100	100	100	100	100	100

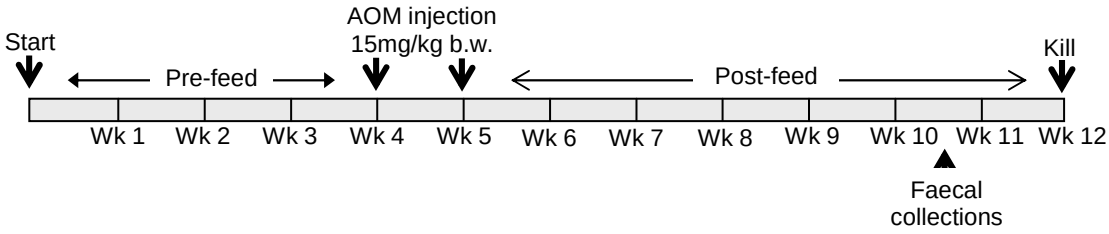
*( ) denotes ingredient added as element of microencapsulated product.

All oils used in diets were sourced by Food Sciences Australia. Menhaden oil (EPA 19.2%, DHA 13%) was purchased from Lysi, Capitol Ingredients, and tuna oil (HiDHA, EPA 5.3%, DHA 26.5%), keeping in conjunction with previous experiments, was sourced from NuMega. All microencapsulated products were made at FSA (Werribee), vacuum packaged, refrigerated and sent along with

corresponding free oils to Flinders University. When making the fish oil diets, all encapsulated products and free fish oils were kept refrigerated and were flushed with nitrogen after use to prevent oxidation of fatty acids. All diets were made as required and fresh diet and water were provided *ad libitum* on a daily basis.

Following a 4 week pre-feeding period all rats were weighed and two consecutive intraperitoneal injections of the AOM at a dose of 15mg/kg body weight in the abdomen. This dose has been shown to be sufficient in causing colorectal tumourigenesis [211]. The general condition of animals and their weights were closely monitored for signs of toxicity. All animals were then left to feed on their respective diets for a further 8 weeks. Faecal collections were taken for pH and SCFA analysis during the final week of feeding.

Figure 62: ACF study time line



All rats were killed by CO_2 asphyxiation and immediately following their death the colon of each animal was resected, cleaned and opened out on to Hybond-C protein paper (Amersham Biosciences). Colons were then fixed in buffered formalin overnight and placed in 70% ethanol before being stained in methylene blue (see 2.6.6.) and analysed for ACF. Stained colons were blindly examined under a dissecting microscope from distal to proximal end and the number, size and position of each ACF were recorded.

ACF counts were expressed as total ACF and also as large ACF (3 or more crypts) and small ACF (less than 3 crypts). Data were also divided into proximal and distal counts. Tukeys ANOVA was used to analyse the relationship between the free oil groups and the microencapsulated fish oil groups, while independent T-tests were used to analyse the effect of the microencapsulation of fish oil against free fish oil.

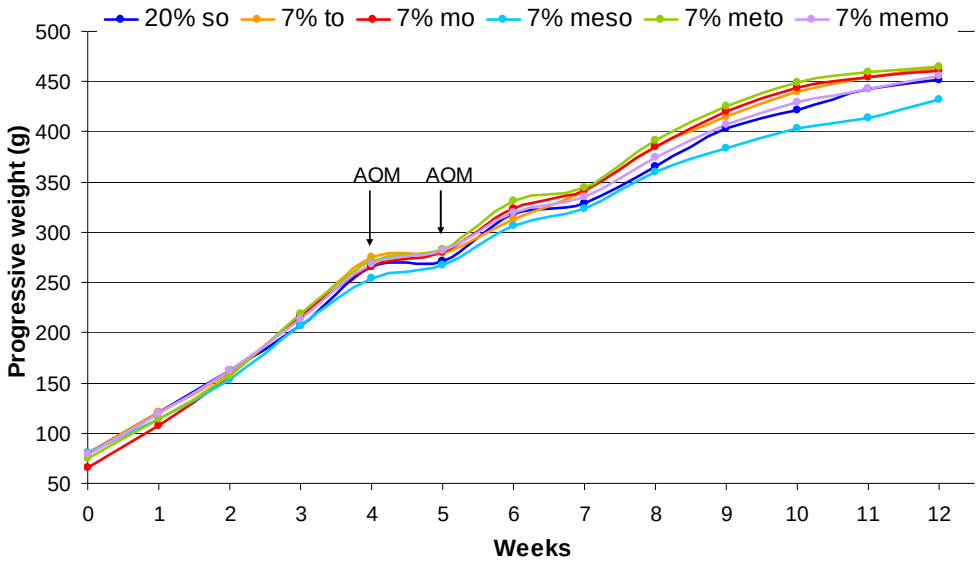
7.1.4. Results

7.1.4.1. Progressive rat weights

4 week old Sprague-Dawley rats arrived with a mean weight of approximately 76g. All rats steadily gained weight throughout the 4 week pre-feed period as shown in figure 60. The week following the administration of the first AOM injection, the weight of rats plateaued regardless of the dietary group. Though the majority of rats did not gain any significant weight at this time, no rat lost weight at any point during the experiment.

During the final 5 weeks of the experiment, rats continued to increase in weight though the rate of weight gain slowed considerably. The final mean weight of the 20% SO control rats was 451.87g ± 8.1 (SEM), the heaviest rats at the time of kill belonged to the 7% METO group at 465.07g ± 6.2 (SEM), while the lightest group, 7% MESO, recorded a weight of 432.33g ± 8.7 (SEM). At all times during the experiment the weights between groups did not differ by more than 10%, while the group weights at the time of AOM injections and at the time of kill were not statistically different from each other.

Figure 63: Progressive rat weights (g)



Weight gain over 12 week ACF study. All data expressed as means ± SEM for 90 rats (n=15).

7.1.4.2. Dietary effect on caecal and faecal contents

The pH of the caecal contents at the time of the kill averaged at 6.77 ± 0.04 (SEM) for all 3 free oil groups. A trend towards a lower caecal pH in all 3 encapsulated groups was observed, though only the 2 fish oils groups, 7% METO and 7% MEMO, reached significance with p=0.01 and p=0.001 respectively (see table 21).

The faeces of rats were analysed for pH levels and SFCA profiles. PH levels followed a similar pattern to that observed with the caecal content. The faecal pH in the free oil groups were slightly lower than those measured in the caecal content initially, however a greater drop in pH was also recorded in these groups, with the 7% MESO, 7% METO and 7% MEMO reaching significantly lower levels when compared to the 20% SO control and also their free oil equivalents ($p<0.0001$, $p<0.0001$ and $p=0.015$ respectively).

Table 21: pH levels in caecal and faecal samples

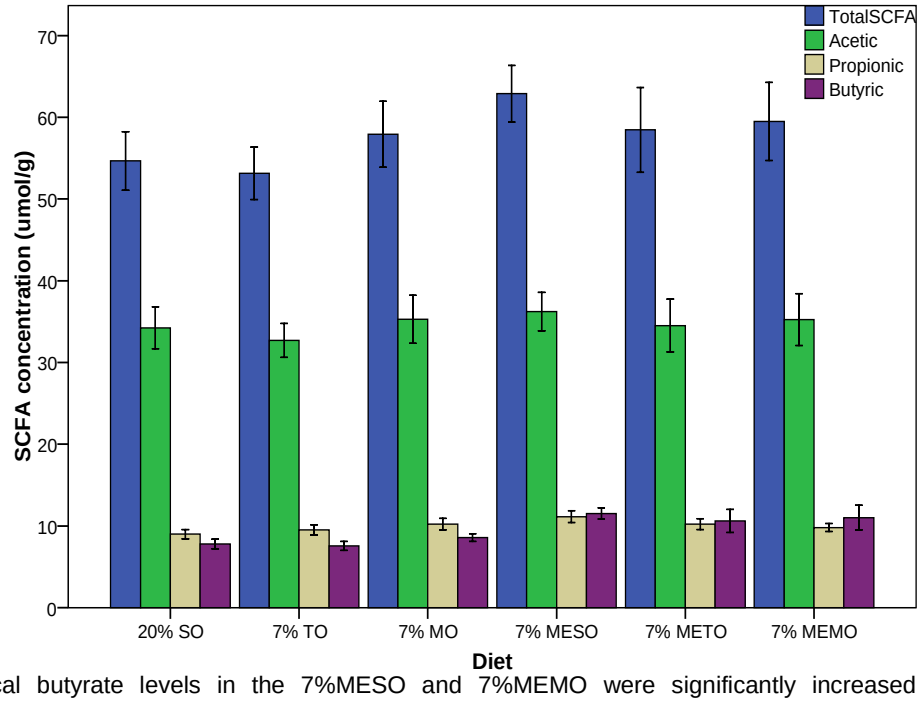
Diet	Caecal pH	Faecal pH
20% SO	6.77 ± 0.04	6.56 ± 0.07
7% TO	6.77 ± 0.05	6.57 ± 0.09
7% MO	6.79 ± 0.03	6.40 ± 0.08
7% MESO	6.62 ± 0.09	6.01 ± 0.07[a]
7% METO	6.50 ± 0.09[a]	5.82 ± 0.05[a]
7% MEMO	6.48 ± 0.06[a]	6.04 ± 0.11[a]

All data expressed as means ± SEM for 90 rats (n=15). [a]p< 0.05 represents means significantly different from 20% SO control group by independent T-test.

Short chain fatty analysis of the caecal contents showed no significant changes between the more predominate acids including acetic and propionic (figure 61) and the lesser acids including isobutyric, isovaleric, valeric and caproic acid (data not shown). The total level of caecal SCFA was also unaffected by diet. No trends or significant differences were observed with regard to the faecal SCFA profiles (figure 62).

An increase in the caecal butyrate levels in the 3 encapsulated groups was noted, with 7% MESO and 7% MEMO reaching significantly higher levels then the 20% SO control (see table 22). A positive correlation between the caecal pH and butyrate levels was observed ($p<0.05$).

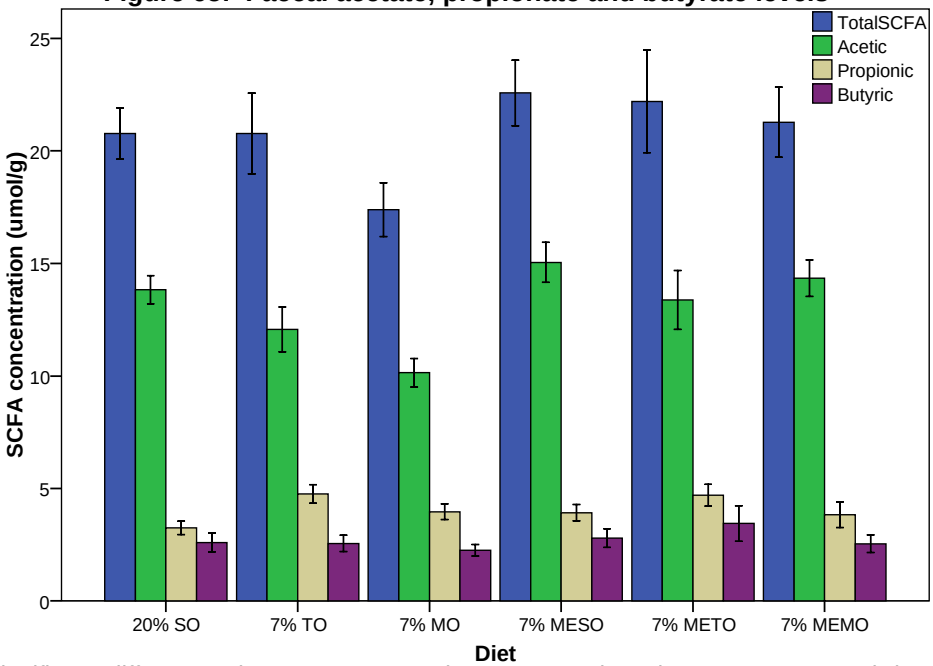
Figure 64: Caecal acetate, propionate and butyrate levels



Caecal butyrate levels in the 7%MESO and 7%MEMO were significantly increased when compared to the 20% SO control, $p<0.05$ (ANOVA, Tukey). All data expressed as means ± SEM for 90 rats (n=15).

Faecal butyrate levels were significantly lower across all groups when compared to levels measured in the caecum. Levels in the 7% MESO and 7% METO were slightly higher than the free oil groups, but this increase was marginal and not statistical significant.

Figure 65: Faecal acetate, propionate and butyrate levels



No significant differences between groups when compared to the 20% SO control (ANOVA, Tukey). All data expressed as means ± SEM for 90 rats (n=15).

Table 22: Butyrate levels in caecal and faecal samples

Diet	Caecal butyrate	Faecal butyrate
20% SO	7.79 ± 0.61	2.59 ± 0.42
7% TO	7.56 ± 0.55	2.55 ± 0.35
7% MO	8.57 ± 0.48	2.25 ± 0.25
7% MESO	11.52 ± 0.69[a]	2.78 ± 0.41
7% METO	10.62 ± 1.41	3.44 ± 0.78
7% MEMO	11.01 ± 1.50[a]	2.54 ± 0.39

All data expressed as means ± SEM for 90 rats (n=15).
[a]$p < 0.05$ represents means significantly different from 20% SO control group by independent T-test.

7.1.4.3. Dietary effect on ACF formation

Few ACF were observed in the proximal colon, with the mean total proximal count across all dietary groups falling below 1. Therefore, statistical analyses were performed on total colonic counts regardless of the site. Table 23 shows results for ACF distribution and size according to the dietary treatment, while figure 63 displays the total ACF count.

The total number of ACF measured appeared to be reduced in all 4 fish oil groups whether microencapsulated or not when compared to their equivalent sunflower oil controls. However, a significant reduction in ACF was only seen with the free fish oil diets, 7% tuna oil and 7% menhaden oil with p values of 0.02 and 0.032 respectively when compared to the 20% sunflower oil control.

In addition, the 7% free tuna oil diet significantly reduced large ACF (3 crypts or greater) when compared to the 20% sunflower oil control. This trend was supported by ANOVA ($p=0.07$) and reached significance when analysed by an independent t-test ($p=0.007$).

ACF counts in the encapsulated fish oil groups were lower than their respective 20% encapsulated sunflower oil control. Yet, this reduction was not significant. When the total ACF count for each of the individual encapsulated oil diets were compared to their free oil comparisons no significant differences were found.

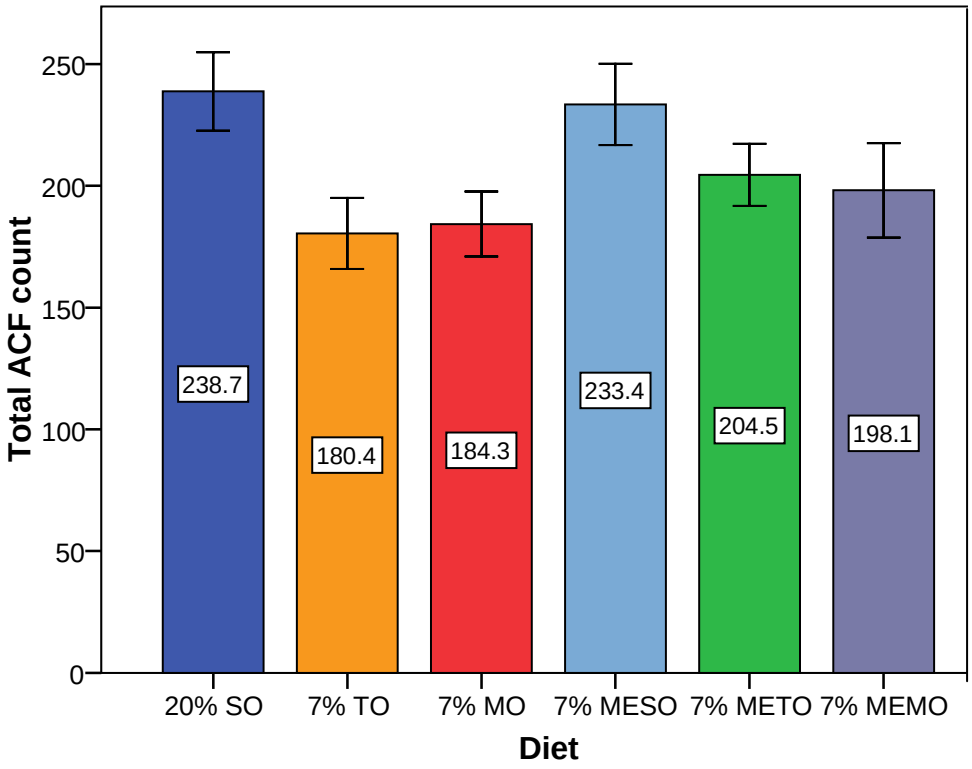
The type of fish oil was not important with regard the total ACF number. Both tuna oil and menhaden oil given either freely or encapsulated resulted in similar reductions in ACF counts.

Table 23: Analysis of Colonic ACF Counts

Diet	ACF (1-3)	ACF (>3)	Proximal ACF	Distal ACF	Total ACF
20% SO	216.4 ± 13.5	15.6 ± 2.0	0.07 ± 0.0	238.6 ± 16.1	238.7 ± 16.0
7% TO	171.6 ± 14.0[a]	8.7 ± 1.1[a,b]	0.33 ± 0.1	180.0 ± 14.6	180.4 ± 14.5[a]
7% MO	167.5 ± 12.6[a]	16.7 ± 2.8	0.07 ± 0.6	184.2 ± 13.3	184.2 ± 13.3[a]
7% MESO	219.9 ± 15.4	13.4 ± 2.8	0.07 ± 0.6	233.3 ± 16.6	233.4 ± 16.6
7% METO	189.1 ± 12.8	15.4 ± 2.1	0.73 ± 0.5	203.8 ± 12.8	204.5 ± 12.7
7% MEMO	180.4 ± 15.1	10.5 ± 1.9	0.43 ± 0.2	197.7 ± 19.4	198.1 ± 19.3

All data expressed as means ± SEM for 90 rats (n=15).
[a]p< 0.05 represents means significantly different from 20% SO control group by Tukeys one way ANOVA.
[b]p< 0.01 signifies mean significantly different from corresponding ME group by independent T-test.

Figure 66: Total ACF Count



All data expressed as means ± SEM for 90 rats (n=15). [a]=p<0.05, compared to 20% SO control group by Tukeys one way ANOVA.

7.1.5. Discussion

The safety of a fish oil diet is supported by this study as no significant weight loss or any other detrimental effects were observed in any animal.

The effects of the encapsulation of fish oil on faecal and caecal fermentation measures were comparable to those observed in the acute dietary study. Caecal and faecal pH levels dropped significantly in animals fed the encapsulated oil product as would be expected given the presence of the carbohydrate component in the capsule [212]. This effect was associated with an increase in the production of butyrate. Butyrate levels were increased in all 3 encapsulated oil groups when compared to the free oil groups.

Though this effect was reproduced from the acute dietary study, the levels of all SCFAs were generally lower across all samples. This variation may have been due to the fact that samples were collected in each experiment following different periods of feeding. Collections were taken after a period of 11 weeks on the diet as opposed to 3 weeks in the acute study. Thus adaptation over time might explain the difference or alternatively, this overall decrease in SCFA production as seen in the ACF study may simply be the result of experimental variation.

This study suggests that fish oil, in its free form, protects against ACF formation when induced by AOM. Both tuna oil and menhaden oil significantly reduced the total number of ACF in the colon when compared to a sunflower oil diet.

Free tuna oil also significantly reduced the number of large ACF in the colon, thereby suggesting a slightly stronger protective property over menhaden oil, perhaps due to the differential EPA: DHA ratio. The analysis of LCFA profile from the colonic mucosa of animals from both dietary groups would be useful in the further investigation of this result. This analysis may provide additional information concerning the differential effects of diet on the DHA: EPA ratio in the phospholipid of tissues and its possible effect on the regulation of COX-2 and other important enzymes present in the cellular membrane.

Though free fish oils appeared to be protective against ACF formation, this protection was not so obvious when fish oil was encapsulated. While encapsulation of both tuna and menhaden oil did result in lower total ACF numbers when compared to their respective encapsulated control, this trend was not significant. In addition, no significant differences were observed between the respective free oil controls and the encapsulated oil for any oil tested. This implies that not only did encapsulating fish oil not protect against ACF, but it also added no value to the effect of free fish oil.

It was previously implied that a reduction of O^6medG may infer possible protection against the development of colorectal cancer. This ACF study enabled us to explore the specific hypothesis that a reduction in colonic O^6medG levels, as previously observed in animals fed 7% encapsulated tuna oil, would be translated into a reduction in ACF incidence. However as a significant reduction of either total ACF or large ACF was not observed in this group, this hypothesis was not directly supported.

In theory, it was considered that the reduction of acute colonic O^6medG DNA damage leads to an overall reduction of mutations and hence, the incidence of the preneoplastic ACF lesions would also be reduced. Though other publications have correlated O^6medG with tumour incidence as outlined in 7.1.2, ACF data from this study does not support this concept. This is reinforced by the fact that a significant reduction of ACF was observed in the 7% free tuna oil group, and yet this diet resulted in no significant changes to O^6medG formation.

It is possible that the potential interference with the metabolism of the AOM by omega-3 (as discussed in 6.2) may have influenced both the O^6medG and ACF result. Therefore, the lack of any correlation has to be interpreted cautiously. However, if a particular diet was to inhibit the metabolism of AOM, it can be presumed that the level of interference may be the same within groups fed the same diet. Therefore, ultimately the amount of activated carcinogen may still be consistent within the same dietary group regardless whether they were tested for acute or advance endpoints. Therefore theoretically, this should not affect the association of both the O^6medG and ACF data.

A more likely explanation may be the fact that this study used ACF as an endpoint and did not measure cancer as an endpoint. Though ACF have been supported as a biomarker for eventual tumour formation, doubts as to whether they represent a true biomarker of cancer formation are still held by some. It is therefore possible that the use of ACF as an endpoint is a limitation of this study and determining any type of correlation between O^6medG and ACF in this instance may be inappropriate. Hence, a longer term study measuring incidences of cancer in animals fed these diets would be of great value. Such data would assist in not only investigating fish oil as a chemopreventative agent but also in exploring the longer term consequences on colorectal oncogenesis from O^6medG regulation.

Finally, a second limitation of this study that may interfere with analysis of the results is the difference in the study designs between the two studies. The analysis of O^6medG load in the short term study was performed at a single time point 6h following the administration of a single injection of AOM at a dose of 10mg/kg. ACF formation however, was recorded some weeks after a double dose regime of AOM at 15mg/kg. The inconsistency between the two study designs may have impacted the result and therefore it may be inappropriate in this instance to make an assumption with regard to the correlation between the O^6medG load and ACF formation. In future studies, a more appropriate design may include an equivalent regime of carcinogen administration for both the measurement of O^6medG load and ACF and also a measurement of the cumulative O^6medG load over time rather than at a single time point.

In summary, though an association between the formation of O^6medG and ACF incidence was not established, the specific aims of this study were fulfilled. The physiological effects of all diets were observed and changes to SCFA profiles and pH levels by the encapsulated material were noted. A diet high in free fish oil was found to be protective against ACF formation, regardless of the type of the fish oil used. And lastly, fish oil when encapsulated, does not protect against ACF formation or enhance any of the protective effects of a high dose fish oil diet.