CHAPTER 5
Study of the effect of free and microencapsulated fish oil on the omega-3 PUFA content in tissue phospholipid.

5.1. Dietary intervention study

5.1.1. Aims

This chapter explores the physiological effects of both free and microencapsulated fish oil in an *in vivo* model. Diets containing either free tuna or microencapsulated tuna oil were compared to diets containing free or microencapsulated sunflower oil. These were fed to rats and a series of physiological endpoints were measured to determine what effects a diet containing the free tuna oil and the encapsulated tuna oil had in an animal.

It was hypothesised that the microencapsulation of tuna oil would result in greater omega-3 PUFA content in the phospholipid of the distal colon when compared to a diet of free tuna oil. A series of specific aims were carried out in order to test this hypothesis and specifically investigate any other potential effects caused by the microencapsulation of tuna oil.

The specific aims of this particular study include;

1. To determine any effect of diet on the weight gain of animals over the 4 week feeding period.

2. To establish both caecal and faecal short chain fatty acid profiles and pH values to determine any differential effects of diets on the fermentation properties in the colon.

3. To establish the long chain fatty acid (LCFA) profiles from a series of tissue types, including heart, liver, kidney, adipose, small intestine, proximal and distal colon from rats to determine any differences in lipid uptake.

4. To also measure the long chain fatty acid profile of plasma and faecal samples.

5.1.2. Experimental rationale

The primary objective of this study was to observe both the physical and physiological effects of the experimental diets on the animals. Samples from the caecum as well as faeces will be analysed for any changes caused by the diet, while a series of different tissues will also be analysed for the LCFA profile.

The LCFA analysis of tissues in particular is an important measure as the chemopreventative effects of fish oil have been linked to an increase in omega-3 PUFA content in tissue phospholipid. Essentially, a low omega-6/omega-3 ratio is more desirable in reducing the risk of CRC [25]. One of the primary reasons for this is the ability of omega-3 PUFAs to modify COX activity and contribute to the production of less potent 3 series eicosanoids that have anti-inflammatory and pro-apoptotic properties [136, 194]. Therefore, the LCFA analysis of tissues is an important endpoint providing useful information that will relate the dose of tuna oil in the diet and the level of omega-3 incorporation into different types of tissues.

This chapter also allows us to explore the effects of the newly formulated microencapsulated oil product. Following on from promising *in vitro* results (carried out by FSA) it was decided that the microencapsulated product would be tested in the rat-AOM animal model. This novel microencapsulated product has the ability to convert any chosen oil from its liquid form into a dry powdered product by enclosing microscopic oil droplets within an outer casing. As one of the main objectives of this thesis was to determine if fish oil regulated host responses in the colon to AOM, it was decided that the microencapsulated product containing fish oil also would also be prepared and tested. This would allow us to not only study the safety and the physiological effects of this product on rats but also allow us to explore its prospects as a chemopreventative agent.

Initial *in vitro* studies from FSA (unpublished data) demonstrated that more than 50% of the encapsulated oil reached the caecum and colon 9h after being ingested compared to trace amounts observed with the free oil comparison. These data suggest that this microencapsulated form of oil has the ability to bypass absorption in the small intestine and deliver its oil encapsulant directly to the colon. Following these preliminary results, it was hypothesised that a diet containing microencapsulated tuna oil would result in increased levels omega-3 PUFAs in the phospholipid of the distal colon when compared to a diet containing free tuna oil.

It was also hypothesised that the direct delivery of the tuna oil to the colon via the microencapsulated product oil may potentiate any possible regulatory effects on the acute homeostatic responses to AOM. A change in the modulation of any of these responses as a result of this product may either be the result of the hypothesised increase in colonic omega-3 or by other means. This specific hypothesis pertaining to the possible regulatory properties of the microencapsulated tuna oil products is addressed in chapter 6.

It was decided that the type of fish used would be tuna oil due to its high DHA content of 26.5%. Studies using fish oil high in DHA [145] and purified DHA [148] have shown protective effects against tumourigenesis and ACF and therefore, high DHA tuna oil was used in this dietary intervention study.

A 15% free tuna oil diet was used as the upper dose limit of fish oil based on previously published works that have shown protective effects at this level [131, 140, 142]. The fat content of this diet, like all remaining diets was brought up to 20% total fat with free sunflower oil to ensure a consistent fat level across all groups and also to ensure that essential fatty acid requirements were met for rats on a tuna oil diet.

A diet containing 7% microencapsulated tuna oil was also formulated. This diet was included based on the hypothesis that the ME product would deliver 50% more omega-3 PUFAs to the colon and as a result potentially increase the omega-3 content in the colonic phospholipid. Therefore, it was hoped that this

7% ME tuna oil diet would have comparable results to the higher 15% free tuna oil diet. A 7% free tuna oil diet was also included as was a 7% microencapsulated sunflower oil diet for comparisons sake.

Lower dose diets of 3.5% microencapsulated tuna oil, 3.5% microencapsulated sunflower oil and 3.5% free tuna oil were also fed to rats. Furthermore, microencapsulated diets containing 0.5, 1 and 2% ME tuna oil were also trialled. These lower dose diets were designed in order to determine whether a tuna oil diet would be effective at a lower dose when fed in this alternative form. Furthermore, there was concern that a high dose tuna oil diet may 'swamp' the colonic phospholipid and the preferential delivery effect from the ME product would not be observed. As a result animals were fed low dose ME tuna oil diets to overcome this potential problem.

An additional diet was also formulated that included 3.5% free tuna oil and 3.5% of the ME capsule product without the oil. This was included to determine whether any differential affects observed in either the phospholipid content or chemopreventative endpoints by microencapsulated diets was in fact due to the delivery of the oil and not simply the result of interplay between the capsule material and the tuna oil in the gut.

The range of diets tested and their compositions can be seen in detail in section 5.1.3 in table 6.

5.1.3. Study design

28 Day old Sprague Dawley rats were caged in groups of 4 and housed in temperature and humidity controlled animal house. As with the previous time course experiment, animals were housed in cages with raised grid floors in order to prevent coprophagy and consumption of bedding materials. Rats were also acclimatised for a period of one week during which time they were fed a standard rat chow diet.

Rats were then assembled into groups of 12 and each group was assigned with an experimental diet. A control diet was formulated based on the standard control diet used previously. Diets were again based on a fat/carbohydrate/protein ratio of 20:50:20%. Experimental diets differed in the type of fat (sunflower or tuna oil) and the form (free or encapsulated) in which it was added to the diet. Additionally, animals fed diets containing microencapsulated oils were subjected to the additional carbohydrate/protein component that the capsule casing was prepared from.

Cornstarch levels also varied slightly between diets. As the final microencapsulated product only contained 25% oil, excess product was added to the diets to ensure the specified dose of microencapsulated oil and a total level of 20% fat was achieved. Therefore, to accommodate for the additional volume of ME product in the diet, levels of cornstarch were decreased accordingly.

Free tuna oil was tested at a variety of doses which included 3.5%, 7% and 15% tuna oil. Sunflower oil was then used in each diet to bring the total fat content up to 20% in line with the control. The same oils were used in the production of the encapsulated material. Microencapsulated tuna oil was tested at a variety of dosages in the diets including 0.5, 1, 2, 3.5 and 7%, while microencapsulated sunflower oil control groups were tested at 3.5 and 7%. Finally, a dietary group was designed that included 3.5% free tuna oil and 3.5% dry capsule product containing no oil. A comprehensive list of diets and the ingredient composition can be seen table 6

Table 6: Diet Composition for acute fish oil experiment (g/100g)

Ingredients	20% SO	3.5% MESO	7% MESO	0.5% METO	1% METO	2% METO	3.5% METO	7% METO	3.5%ME + TO	3.5% TO	7% TO	15% TO
Sunflower oil	20	16.5	13	19.5	19	18	16.5	13	16.5	16.5	13	5
Free Tuna oil									3.5	3.5	7	15
ME Sunflower oil		(3.5)	(7)									
ME Tuna oil				(0.5)	(1)	(2)	(3.5)	(7)				
ME Capsule									(3.5)			
Cornstarch	50	43	36	49	48	46	43	36	43	50	50	50
Cellulose	5	5	5	5	5	5	5	5	5	5	5	5
Casein	20	20	20	20	20	20	20	20	20	20	20	20
Minerals	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamins	1	1	1	1	1	1	1	1	1	1	1	1
Methionine	0.3	0.3	0.3	0.3	0.3	0.3	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	0.2	0.2	0.2	0.2	0.2	0.2
Total	100	100	100	100	100	100	100	100	100	100	100	100

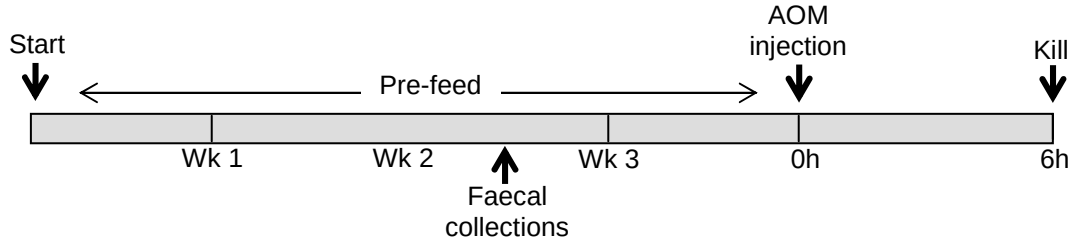
*( ) denotes ingredient added as element of microencapsulated product.

Diets were made as needed and were stored at -20°C. Rats were fed fresh diet on a daily basis were fed and watered *ad libitum* for a period of 4 weeks. Weights were recorded weekly.

Fresh faecal samples were collected from all rats after 3 weeks. Samples collected and were used to measure faecal pH levels, SCFA profiles and LCFA profiles.

After 4 weeks on the experimental diets, rats were first weighed and then given a single intraperitoneal injection of the colon specific carcinogen AOM at a dose of 10mg/kg body weight in the abdomen. All injections were given as close to 9am as possible to minimise any circadian variability.

Figure 36: Acute dietary study time line



Groups of rats were then killed by CO_2 asphyxiation and cervical dislocation 6h post AOM administration. During the final moments of CO_2 asphyxiation for

each animal, cardiac puncture was performed to retrieve a blood sample for analysis of plasma LCFA. Other tissues required for long chain fatty acid profiling were resected, placed in an eppendorf tube and immediately frozen in liquid nitrogen. Tissues collected included the heart, left liver lobe, left kidney, small intestine, colon and adipose taken from the abdomen. All tissues were stored at -80°C. Contents of the caecum were also removed, weighed and prepared for SCFA analysis.

5.1.4. Results

5.1.4.1. Rat Weights

There were no significant differences in weight with any of the experimental diets when compared to the 20% sunflower oil control diet. Animals fed the 20% sunflower oil control diet had a final mean weight of 298g ± 8.79 (SEM). Though animals fed the higher dose free fish oil diets did weigh slightly more than their sunflower fed counterparts at 316.41g ± 7.45 and 322.25g ± 7.74 (SEM) (15% and 7% tuna oil) all groups were within 10% of the control group's weight.

5.1.4.2. Caecal short chain fatty acid profile

The caecal contents from each rat were analysed for their short chain fatty acid profiles. Values for the more predominate fatty acids measured including acetate, propionate and butyrate and total SCFA are shown in table 7 and graphically displayed in figure 37. Acetate was present in the highest quantity, representing approximately half of the short chain fatty acid total in all samples. Levels of propionate were then closely followed by butyrate. Other fatty acids measured in trace amounts included isobutyric, isovaleric, valeric and caproic acid (data not shown) were not changed.

Free fish oil diets alone had no impact on any of the SCFA measured when compared to the sunflower oil control. Similarly, low dose microencapsulated diets (0.5 – 3.5%) regardless of the oil used also resulted in no significant changes to the SCFA profiles.

However, changes were noted in the SCFA profile of animals fed diets containing the highest dose of 7% ME product with either sunflower or tuna as the oil encapsulant. The 7% microencapsulated sunflower oil diet had significantly higher total caecal SCFA when compared to the 20% free sunflower oil control. While both the microencapsulated sunflower oil and tuna oil diets fed at 7% had significantly higher propionate levels at 23.6 ± 1.0 (SEM) and

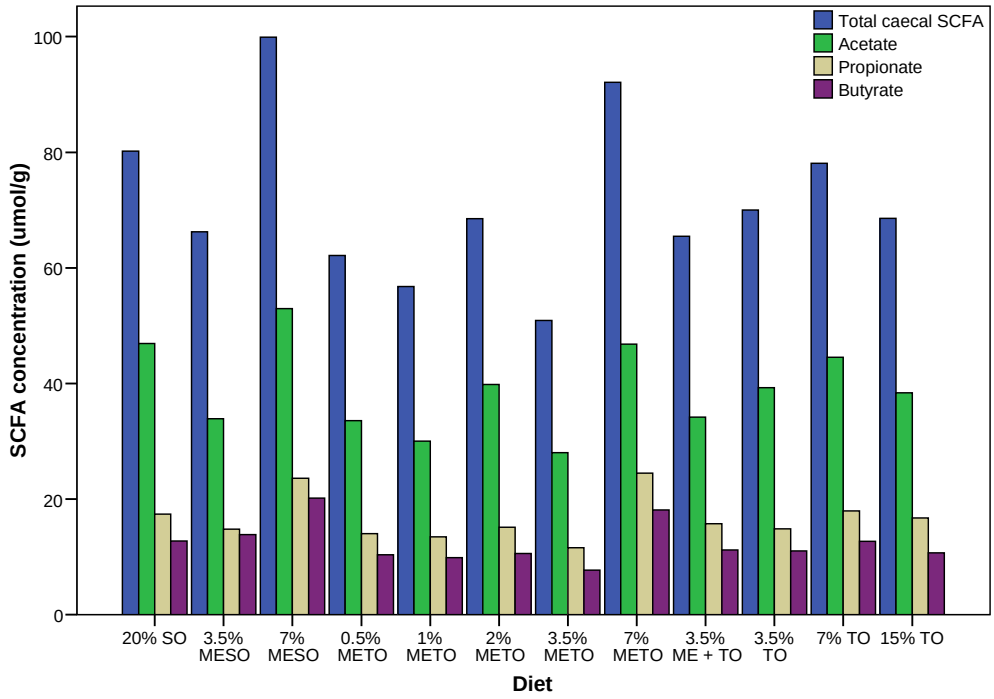
24.4 ± 1.2 (SEM) and butyrate levels at 20.1 ± 1.1 (SEM) and 18.0 ± 1.1 (SEM) (figure 38), when compared to the 20% SO control and their free oil equivalents.

Table 7: Caecal SCFA data

Diet	Acetate	Propionate	Butyrate	Total SCFA
20% SO	46.8 ± 2.0	17.4 ± 0.8	12.7 ± 0.7	80.1 ± 3.5
3.5% MESO	33.9 ± 2.3	14.8 ± 1.1	13.8 ± 1.2	66.2 ± 4.3
7% MESO	52.9 ± 2.6	23.6 ± 1.0[ab]	20.1 ± 1.1[ab]	99.9 ± 4.2[a]
0.5% METO	33.5 ± 1.9	14.0 ± 0.7	10.3 ± 0.7	62.1 ± 3.2
1% METO	30.0 ± 1.5	13.4 ± 1.1	9.8 ± 0.7	56.7 ± 3.3
2% METO	39.8 ± 2.6	15.1 ± 1.0	10.6 ± 1.0	68.4 ± 4.3
3.5% METO	28.0 ± 2.2	11.6 ± 0.8	7.6 ± 0.5	50.9 ± 3.7
7% METO	46.8 ± 1.6	24.4 ± 1.2[ab]	18.0 ± 1.1[ab]	92.0 ± 3.0
3.5% ME+TO	34.1 ± 2.9	15.7 ± 1.2	11.2 ± 1.0	65.4 ± 4.8
3.5% TO	39.2 ± 2.8	14.8 ± 1.0	11.0 ± 1.2	69.9 ± 4.2
7% TO	44.5 ± 2.2	17.9 ± 0.7	12.6 ± 0.8	78.0 ± 3.4
15% TO	38.3 ± 2.0	16.7 ± 1.0	10.7 ± 0.8	68.5 ± 3.7

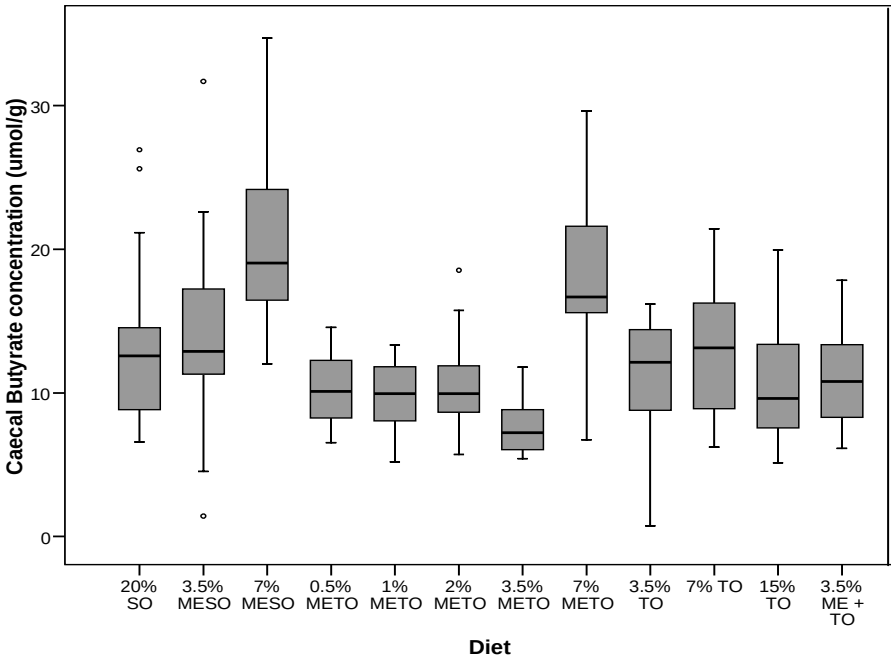
All data expressed as means ± SEM for 144 rats (n=12).
[a]p< 0.05 represents means significantly different from 20% SO control group by independent T-test.
[b]p< 0.01 signifies mean significantly different from corresponding free oil group.

Figure 37: Caecal SCFA levels



All data expressed as means ± SEM for 144 rats (n=12).

Figure 38: Caecal Butyrate levels



All data expressed as means ± SEM for 144 rats (n=12). [a] signifies means significantly higher than 20% SO control group (P< 0.05)

5.1.4.3. Faecal short chain fatty acid profile

The short chain fatty acid profiles were considerably lower when measured in faecal stool samples. Faecal SCFA levels across all dietary groups were approximately halved when compared to levels measured from the caecum (table 8 and figure 39).

Animals fed the 7% microencapsulated sunflower oil diet had significantly higher levels of acetate, propionate, butyrate and total SCFA when compared to the 20% free sunflower oil control. Faecal butyrate levels in particular were notably higher in this group when compared to all other animals (figure 40). This however, was not extended to the 7% microencapsulated tuna oil group as was observed in the caecal data.

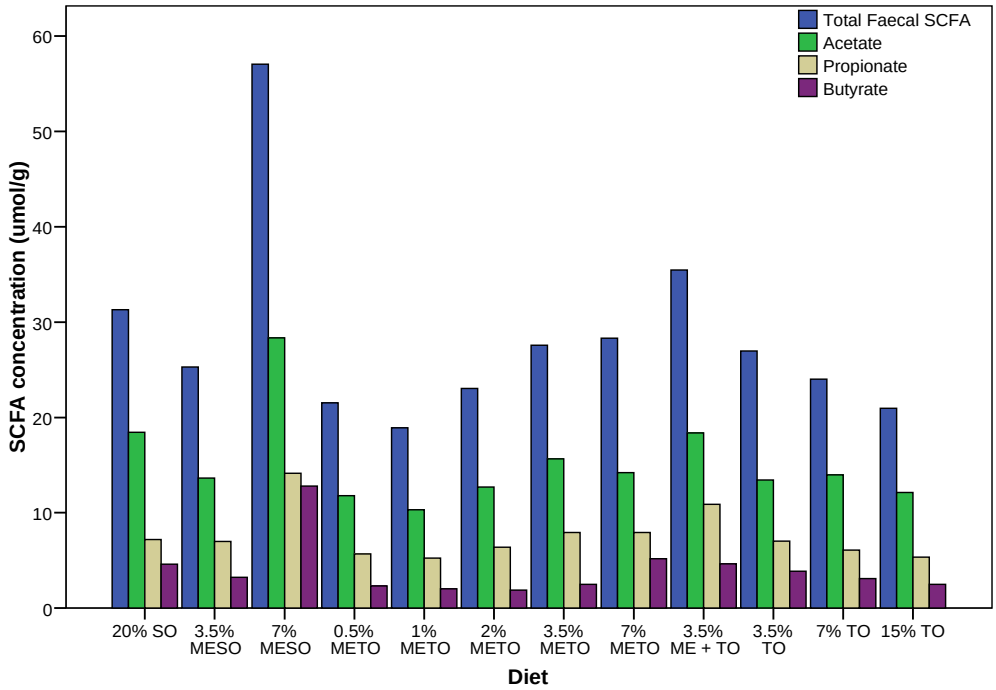
Levels of other SCFA including isobutyric, isovaleric, valeric and caproic acid were also measured in trace amounts, however, no significant differences or trends were noted between dietary groups (data not shown).

Table 8: Faecal SCFA data

Diet	Acetate	Propionate	Butyrate	Total SCFA
20% SO	18.4 ± 2.0	7.1 ± 0.7	4.6 ± 0.5	31.3 ± 3.2
3.5% MESO	13.6 ± 0.8	6.9 ± 0.4	3.2 ± 0.4	25.2 ± 1.7
7% MESO	28.3 ± 2.6[a]	14.1 ± 1.1[a]	12.8 ± 1.5[a]	57.0 ± 4.9[a]
0.5% METO	11.8 ± 1.4	5.6 ± 0.7	2.3 ± 0.3	21.5 ± 2.6
1% METO	10.3 ± 1.4	5.2 ± 0.7	2.0 ± 0.2	18.9 ± 2.3
2% METO	12.7 ± 1.5	6.3 ± 0.5	1.9 ± 0.2	23.0 ± 2.6
3.5% METO	15.6 ± 0.9	7.9 ± 0.6	2.5 ± 0.3	27.5 ± 1.7
7% METO	14.2 ± 1.4	7.9 ± 0.9	5.1 ± 0.8	28.2 ± 2.9
3.5% ME+TO	18.3 ± 1.8	10.8 ± 1.0	4.6 ± 0.5	35.4 ± 3 .4
3.5% TO	13.4 ± 1.2	7.0 ± 0.7	3.8 ± 0.5	26.9 ± 2.7
7% TO	13.9 ± 1.2	6.0 ± 0.6	3.0 ± 0.5	24.2 ± 2.3
15% TO	12.1 ± 1.2	5.3 ± 0.6	2.4 ± 0.5	20.9 ± 1.9

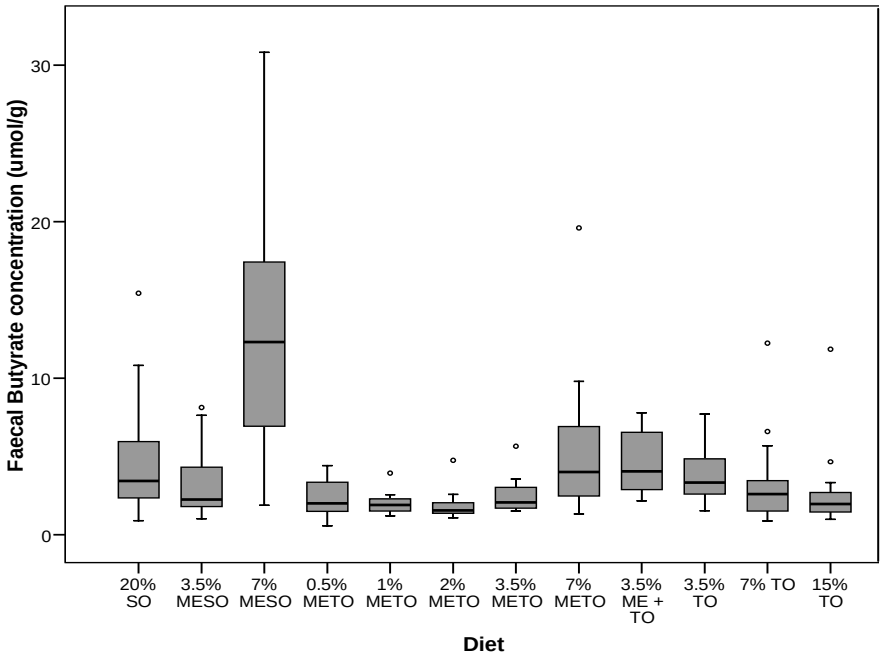
All data expressed as means ± SEM for 144 rats (n=12).
[a] signifies means significantly higher than 20% SO control group (P< 0.05).

Figure 39: Faecal SCFA levels



All data expressed as means ± SEM for 144 rats (n=12).

Figure 40: Faecal Butyrate levels



All data expressed as means ± SEM for 144 rats (n=12). [a] signifies means significantly higher than 20% SO control group (P< 0.05)

5.1.4.4. Faecal pH levels

The pH of faecal samples were measured immediately after collection and are shown below in table 9. The free sunflower oil and tuna oil groups appeared to have more neutral stools with pH values of 7 and above, however no clear trend was observed with regard to the microencapsulated product and it could not be related to a lowering of faecal pH in a dose-dependant manner.

Table 9: Faecal pH

Diet	pH value
20% SO	7.10 ± 0.03
3.5% MESO	6.68 ± 0.01
7% MESO	6.88 ± 0.01
0.5% METO	6.85 ± 0.02
1% METO	6.76 ± 0.00
2% METO	6.48 ± 0.03
3.5% METO	6.46 ± 0.01
7% METO	6.96 ± 0.04
3.5% ME+TO	6.63 ± 0.02
3.5% TO	6.78 ± 0.03
7% TO	7.09 ± 0.03
15% TO	7.22 ± 0.02

All data expressed as means ± SEM for 144 rats (n=12).

5.1.4.5. Long chain fatty acid phospholipid profiles

The incorporation of fatty acids into the phospholipid membrane of tissues was measured using GLC following the lipid extraction, separation and methylation assay as described in 2.6.5.

Following the 4 week feeding period, it was found that diet did alter the long chain fatty acid profile of the phospholipid membrane of tissues measured. A consistent dose-dependant trend was observed across all tissues with regards to the dose of tuna oil in the diet and the incorporation of omega-3 fatty acids into the phospholipid.

Delivery of oil by way of the microencapsulated product however, resulted in no significant changes in the uptake of omega-3 PUFAs when compared to their free oil equivalents in any of the tissues measured or for any of the doses tested.

Heart tissue had the greatest ratio change of fatty acids out of all samples measured (table 10). The incorporation of omega-3 PUFAs into the heart phospholipid came at the expense of saturated and more predominately the omega-6 PUFAs, while the monounsaturated level remained relatively unchanged. The LCFA profile of the heart tissue went from a saturated: omega-6: omega-3 ratio of 43.3:48.3:4.0 on the 20% sunflower oil diet to 37.9:28.5:28.7 when fed a 15% tuna oil diet. This effectively changed the omega-6: omega-3 ratio from 12.07 ± 0.4 (SEM) when fed a 20% sunflower oil diet to 0.99 ± 0.1 (SEM) when fed a diet of 15% tuna oil.

When comparing results of the animals fed free oil and encapsulated oil diets it was shown that diets containing 7% tuna oil did cause a significant change to the heart phospholipid profile of animals overall. Though a significantly reduced omega-6:omega-3 ratio of 1.24 ± 0.6 (SEM) and 1.39 ± 0.5 (SEM) was measured in animals fed the 7% free oil and 7% microencapsulated oil diets respectively, it was evident that the microencapsulated product did not result in an increased omega-3 content in the heart phospholipid.

The 3.5% tuna oil diets also resulted in a significant increase of omega-3 PUFAs into the heart membrane of animals, reaching a omega-6:omega-3 ratio of 1.7 ± 0.4 (SEM), 1.8 ± 0.7 (SEM) and 1.8 ± 0.9 (SEM) for the two diets comprised of 3.5% free tuna oil and the 3.5% microencapsulated tuna oil, but again levels in all 3 groups were comparable.

While heart tissue appeared to incorporate the highest levels of DHA into the phospholipid membrane (figure 41), it was noted that this omega-3 PUFA was readily incorporated and was close to saturation levels when delivered at a dose of 15% tuna oil in the diet. This was supported by the fact that the 7% tuna oil diets were reaching approximately 22 – 25% omega-3 incorporation while the double dose of 15% tuna oil was only delivering approximately a further 3% on top of this to achieve a total omega-3 phospholipid content of 28%. While DHA appeared to be at saturation levels in animals fed a diet high in tuna oil, EPA levels in the heart phospholipid were minimal.

Incorporation of omega-3 PUFAs into liver phospholipid was also significant when animals were fed a diet of tuna oil. Like the heart tissue, no clear trend was observed with regard to the monounsaturated fatty acids, but the saturated:omega6:omega3 ratio of 44.7:48.9:2.5 in animals fed the 20% sunflower oil diet was significantly changed to 43.8:33.2:18.3 in animals fed a 15% tuna oil diet (table 11). That equates to a omega-6:omega-3 ratio change of 19.57 ± 0.5 (SEM) to 1.6 ± 0.6 (SEM). The LCFA of liver phospholipid was also comparable to the results observed from heart tissue in that the minimal addition of 0.5% tuna oil to the diet resulted in a doubling of DHA levels into the phospholipid membrane (figure 42).

Total omega-3 incorporation into kidney tissue was not as high as observed in the heart and liver, peaking at a mean of 12.7% ± 0.3 (SEM) in animals fed the 15% tuna oil diet. However, the EPA: DHA ratio was notably higher in the kidney (see table 12 and figure 43).

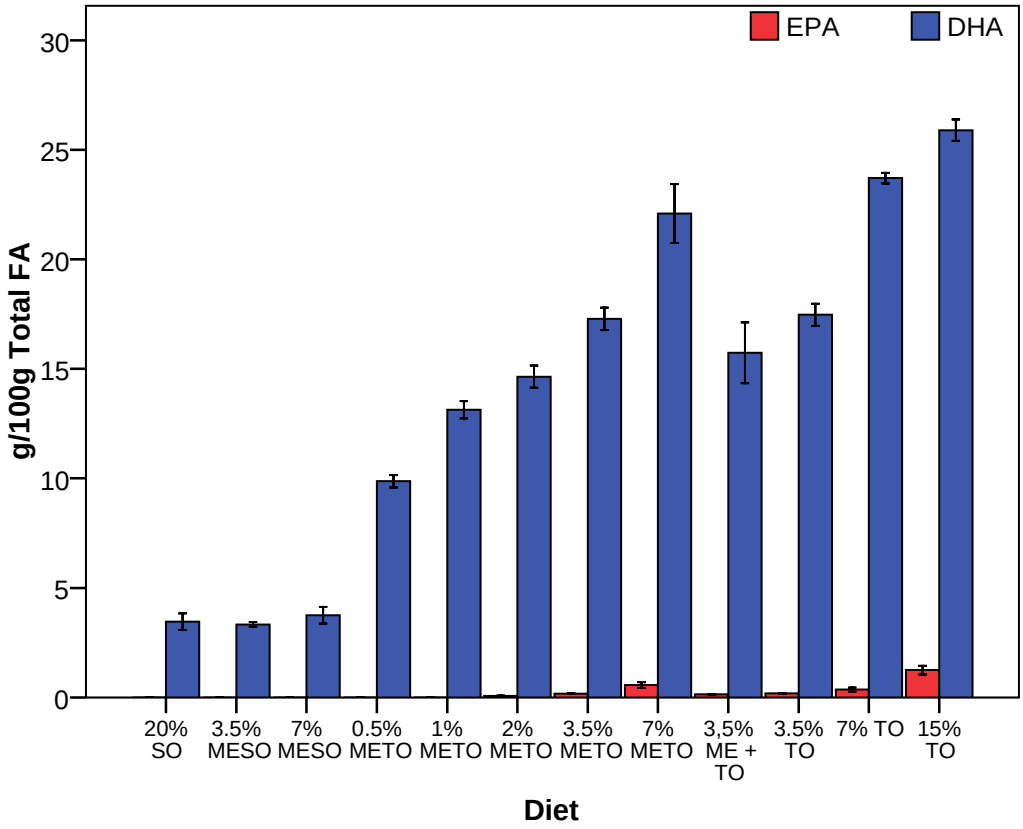
Heart

Table 10: Fatty acid Profile from phospholipid of heart in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	43.3 ± 1.0	4.1 ± 0.0	48.3 ± 0.5	4.0 ± 0.4
3.5% MESO	45.9 ± 0.5	4.9 ± 0.1	45.4 ± 0.5	3.6 ± 0.0
7% MESO	41.4 ± 0.6	4.4 ± 0.2	49.7 ± 0.6	4.4 ± 0.3
0.5% METO	44.7 ± 0.3	4.7 ± 0.0	39.8 ± 0.2[b]	10.5 ± 0.2[b]
1% METO	37.0 ± 0.1[b]	5.1 ± 0.1[a]	41.7 ± 0.2[b]	16.0 ± 0.3[b]
2% METO	35.5 ± 0.3[b]	4.9 ± 0.1	41.3 ± 0.4[b]	18.1 ± 0.6[b]
3.5% METO	35.1 ± 0.2[b]	4.5 ± 0.0	38.9 ± 0.6[b]	21.4 ± 0.6[b]
7% METO	38.7 ± 2.0[b]	4.2 ± 0.2	32.0 ± 0.6[b]	22.9 ± 1.7[b]
3.5% ME+TO	37.6 ± 1.0[b]	4.7 ± 0.1	37.4 ± 0.8[b]	20.1 ± 1.3[b]
3.5% TO	43.5 ± 0.5	4.1 ± 0.1	32.9 ± 0.7[b]	19.3 ± 0.5[b]
7% TO	38.2 ± 0.8[b]	4.7 ± 0.3	31.5 ± 1.1[b]	25.4 ± 0.2[b]
15% TO	37.9 ± 0.4[b]	4.7 ± 0.1	28.5 ± 0.4[b]	28.7 ± 0.3[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 41: DHA and EPA levels in Heart phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

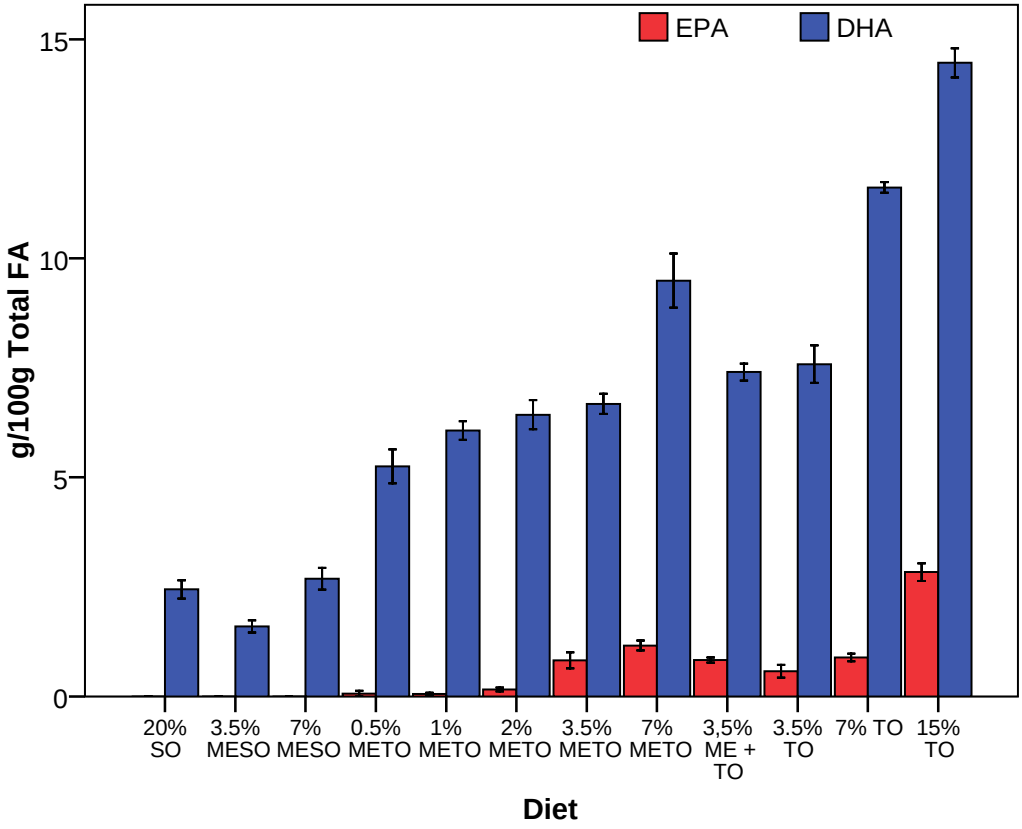
Liver

Table 11: Fatty acid Profile from phospholipid of liver in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	44.7 ± 0.8	3.7 ± 0.1	48.9 ± 0.8	2.5 ± 0.2
3.5% MESO	55.6 ± 0.8	0.9 ± 0.1	41.1 ± 0.8	2.3 ± 0.1
7% MESO	44.0 ± 0.5	3.4 ± 0.1	49.6 ± 0.7	2.8 ± 0.2
0.5% METO	53.1 ± 0.5	0.8 ± 0.0	39.7 ± 0.9	6.2 ± 0.4
1% METO	39.2 ± 0.5	0.0 ± 0.0	51.4 ± 0.6	9.2 ± 0.2[b]
2% METO	38.8 ± 1.0	1.0 ± 1.0	50.1 ± 0.5	9.8 ± 0.3[b]
3.5% METO	41.3 ± 0.4	0.0 ± 0.0	46.8 ± 0.4	11.7 ± 0. b[b]
7% METO	41.4 ± 1.0	6.8 ± 2.9	38.9 ± 3.5[b]	12.7 ± 1.6[b]
3.5% ME+TO	40.8 ± 7.3	9.1 ± 7.1	39.6 ± 5.3	10.3 ± 1 .8[b]
3.5% TO	51.2 ± 0.9	2.2 ± 0.8	35.3 ± 0.9[b]	11.1 ± 0.5[b]
7% TO	44.6 ± 1.2	3.5 ± 0.1	39.0 ± 0.8[a]	12.7 ± 0.2[b]
15% TO	43.8 ± 0.2	4.5 ± 0.4	33.2 ± 0.8[b]	18.3 ± 0.2[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 42: DHA and EPA levels in Liver phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

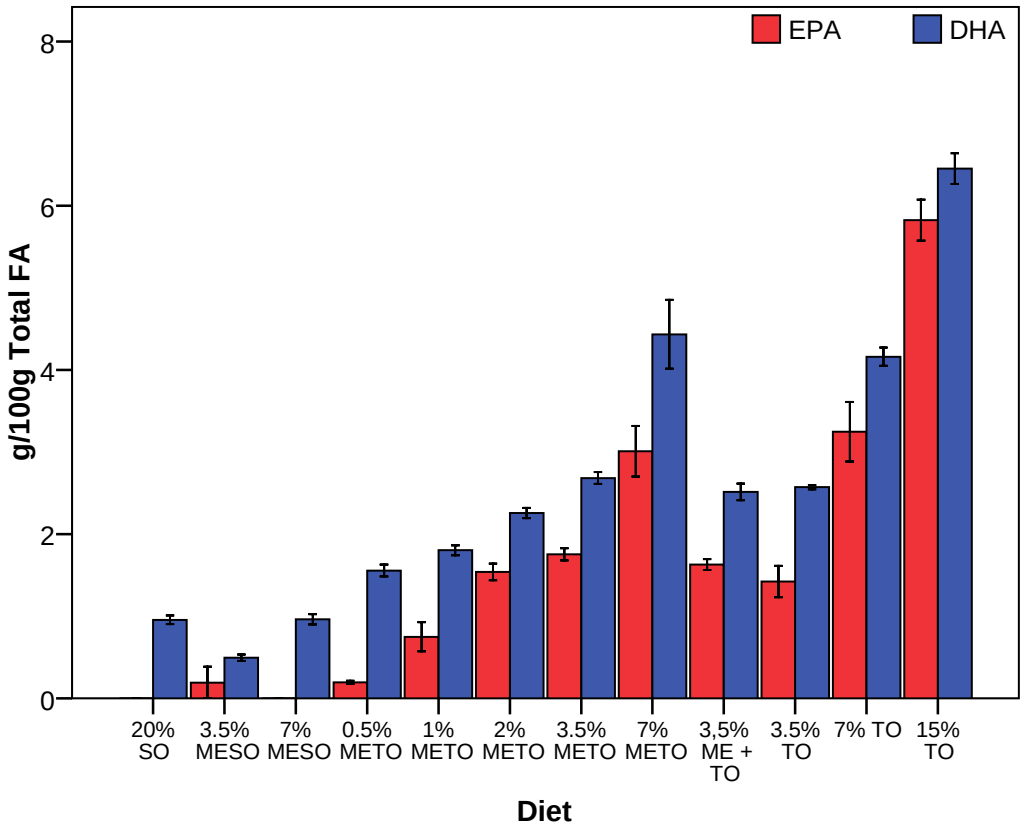
Kidney

Table 12: Fatty acid Profile from phospholipid of kidney in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	42.3 ± 0.5	7.5 ± 0.0	49.0 ± 0.5	1.1 ± 0.0
3.5% MESO	46.0 ± 0.5	8.2 ± 0.1	44.8 ± 0.6	0.7 ± 0.2
7% MESO	44.8 ± 3.4	8.3 ± 0.8	45.5 ± 4.3	1.2 ± 0.0
0.5% METO	44.5 ± 0.4	10.6 ± 0.1	42.5 ± 0.3	2.2 ± 0. 1
1% METO	37.0 ± 4.7	12.2 ± 0.7	46.8 ± 3.8	3.8 ± 0.2
2% METO	33.1 ± 1.7	12.0 ± 0.4	46.6 ± 5.0	8.1 ± 2.9[b]
3.5% METO	33.1 ± 2.5	13.3 ± 0.8	46.4 ± 3.7	7.0 ± 0. 5[b]
7% METO	38.1 ± 2.6	12.0 ± 4.1	41.8 ± 2.2	7.9 ± 0.1[b]
3.5% ME+TO	31.6 ± 0.4[a]	11.4 ± 0.1	50.9 ± 0.1	5.9 ± 0.8[b]
3.5% TO	45.4 ± 0.3	9.6 ± 0.0	40.2 ± 0.2	4.5 ± 0.1
7% TO	42.4 ± 0.8	7.2 ± 0.1	42.5 ± 0.6	7.8 ± 0.4[b]
15% TO	43.7 ± 0.1	7.1 ± 0.1	36.3 ± 0.5[a]	12.7 ± 0.3[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 43: DHA and EPA levels in Kidney phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

The incorporation of omega-3 PUFA into adipose tissue had greater variation within groups and though levels still corresponded to the relative doses of tuna oil in the diets, a significant change in either saturated, monounsaturated or either the omega-6 and omega-3 PUFAs was not observed (table 13 and figure 44).

The long chain fatty acid analysis of the phospholipid membrane of the digestive tract was of particular interest. Small intestine (table 14 and figure 45), proximal colon (table 15 and figure 46) and distal colon (table 16 and figure 47) were all analysed and an obvious dose-response pattern with regard to both total omega-3 and the DHA and EPA fatty acids were observed in all three tissues.

Levels of omega-3 measured in the phospholipid membrane decreased as one moved down the length of the digestive tract. The small intestine recorded the highest levels of total omega-3 incorporation at percentages of 17.7 ± 0.9 (SEM), 10.7 ± 0.2 (SEM) and 9.6 ± 0.2 (SEM) for the free 15%,7% and 3.5% tuna oil diets respectively. This was followed by the proximal colon with figures of 16.4 ± 0.3 (SEM), 10.7 ± 0.1 (SEM) and 4.1 ± 0.3 (SEM), and then the distal colon which measured 15.8 ± 0.7, 8.7 ± 0.3 and 3.5 ± 0.0 (SEM). Only the 15% tuna oil diets in all 3 tissue types reached significance with regard to total omega-3 incorporation into the phospholipid.

All three tissues had higher DHA: EPA ratios, with EPA being detected at a dose of 3.5% tuna oil in the diet and above. When comparing the groups containing equivalent doses of either free or microencapsulated oil, all samples had very similar levels of DHA and EPA. The long chain fatty acid profile of the phospholipid membrane of the small intestine, proximal and distal colon of animals fed the ME product were not different to animals fed a diet containing the equivalent dose of free oil.

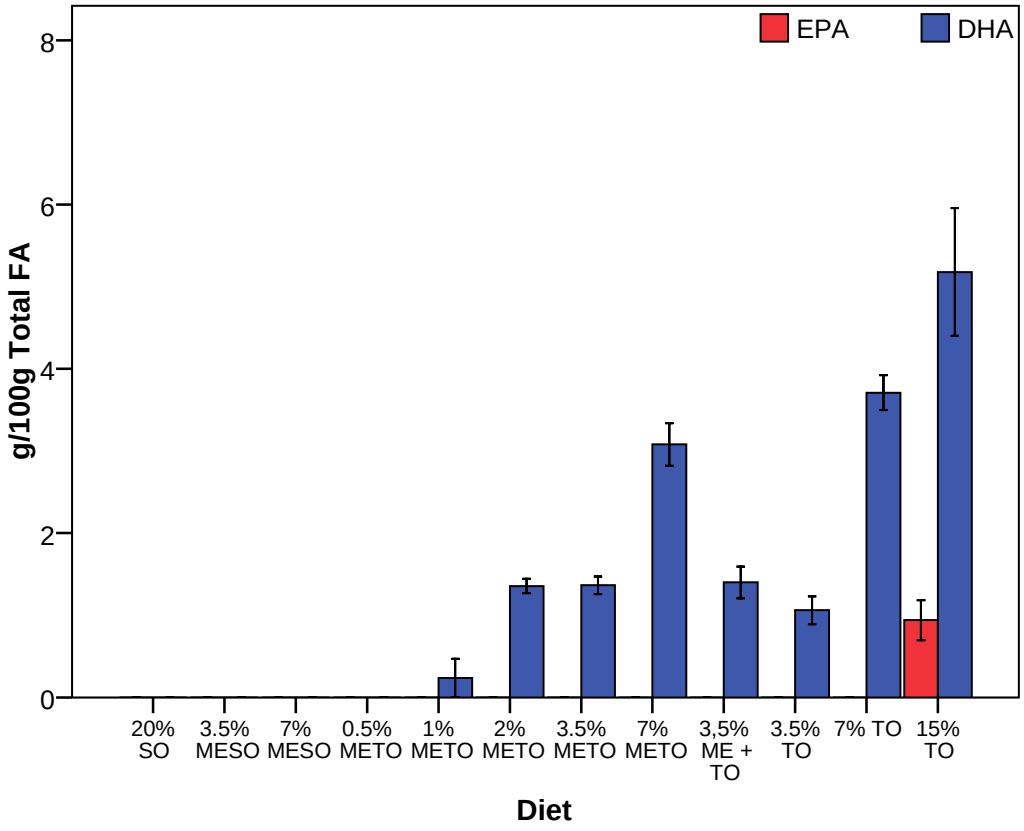
Adipose Tissue

Table 13: Fatty acid Profile from phospholipid of adipose tissue in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	45.7 ± 1.5	17.8 ± 1.0	36.4 ± 1.3	0.0 ± 0.0
3.5% MESO	41.0 ± 0.4	21.7 ± 0.2	37.2 ± 0.5	0.0 ± 0. 0
7% MESO	47.5 ± 1.3	19.9 ± 3.1	32.4 ± 4.5	0.0 ± 0.0
0.5% METO	42.9 ± 2.1	22.4 ± 1.5	33.8 ± 4.7	0.7 ± 3. 7
1% METO	36.4 ± 4.2	22.9 ± 2.5	39.4 ± 5.6	1.2 ± 3.9
2% METO	37.8 ± 0.5	26.6 ± 0.5	33.9 ± 0.1	1.5 ± 0.0
3.5% METO	35.1 ± 0.8	29.4 ± 0.9	33.8 ± 0.4	1.6 ± 0. 1
7% METO	36.8 ± 0.9	23.9 ± 1.7	32.9 ± 2.0	6.2 ± 3.1
3.5% ME+TO	31.4 ± 4.8	24.9 ± 3.2	38.2 ± 5.4	5.2 ± 3 .6
3.5% TO	34.9 ± 0.3	27.1 ± 0.5	36.8 ± 0.2	1.0 ± 0.1
7% TO	41.8 ± 2.9	19.5 ± 3.6	34.9 ± 0.7	3.7 ± 0.2
15% TO	42.1 ± 1.2	16.0 ± 2.2	35.6 ± 2.4	6.1 ± 0.8

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 44: DHA and EPA levels in Adipose phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

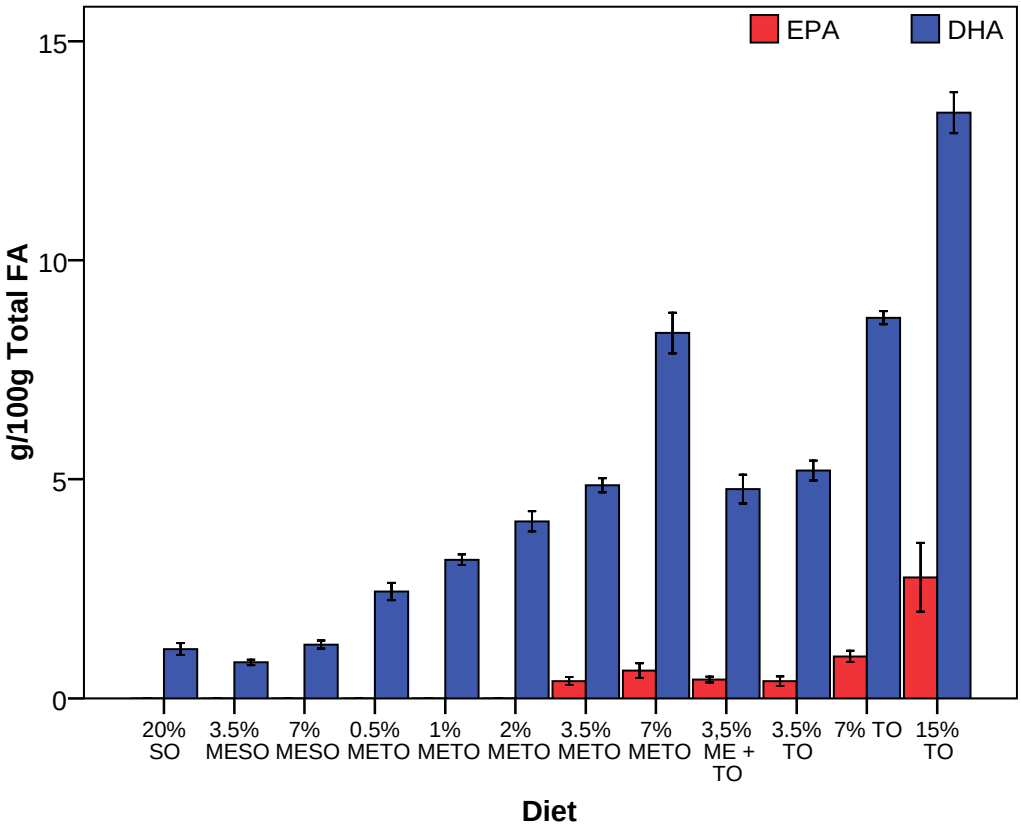
Small Intestine

Table 14: Fatty acid Profile from phospholipid of small intestine in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	57.4 ± 1.6	8.2 ± 0.2	32.4 ± 1.7	1.9 ± 0.1
3.5% MESO	53.3 ± 2.5	8.1 ± 1.8	37.2 ± 1.3	1.2 ± 0.1
7% MESO	54.8 ± 0.9	8.3 ± 0.4	34.8 ± 0.7	1.9 ± 0.0
0.5% METO	47.7 ± 2.7[b]	12.2 ± 1.6	33.8 ± 2.3	6.1 ± 3.1
1% METO	36.3 ± 0.8[b]	12.9 ± 0.3[a]	45.6 ± 0.7[b]	5.0 ± 0.2
2% METO	34.7 ± 0.7[b]	13.0 ± 0.9[a]	44.3 ± 0.6[b]	7.9 ± 0.3
3.5% METO	36.2 ± 1.0[b]	13.0 ± 0.4[a]	41.6 ± 3.8	9.0 ± 1.7[a]
7% METO	48.4 ± 2.2[a]	8.7 ± 0.4	31.6 ± 2.0	10.1 ± 0.3[a]
3.5% ME+TO	36.2 ± 1.1[b]	13.4 ± 0.4[b]	40.0 ± 2.0	10.2 ± 0.3[a]
3.5% TO	49.1 ± 0.6[a]	8.3 ± 0.3	32.8 ± 0.5	9.6 ± 0.2[a]
7% TO	48.8 ± 1.4[a]	8.9 ± 0.3	31.4 ± 1.2	10.7 ± 0.2[b]
15% TO	40.6 ± 1.6[a]	8.8 ± 0.2	32.7 ± 1.0	17.7 ± 0.9[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 45: DHA and EPA levels in Small Intestine phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

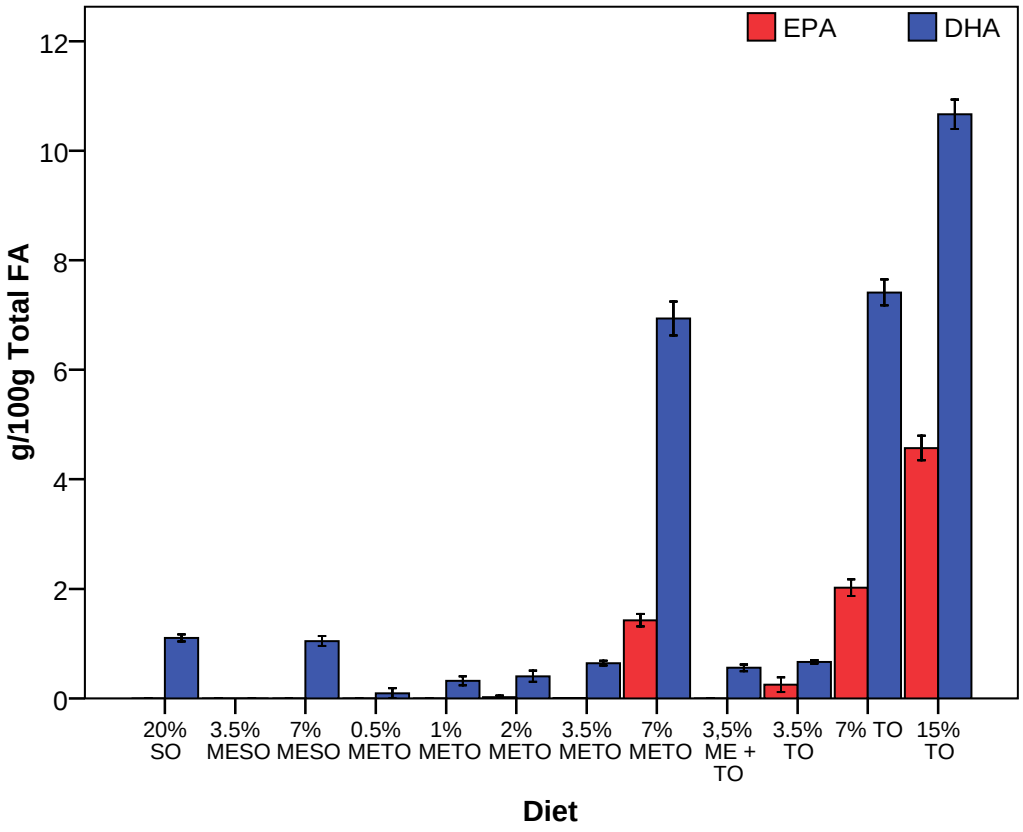
Proximal Colon

Table 15: Fatty acid Profile from phospholipid of proximal colon in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	50.0 ± 1.0	11.9 ± 0.1	36.8 ± 0.9	1.1 ± 0.0
3.5% MESO	50.5 ± 8.0	12.6 ± 4.3	36.5 ± 1.3	0.2 ± 4.5
7% MESO	47.5 ± 2.6	13.3 ± 1.3	37.3 ± 2.4	1.6 ± 3.5
0.5% METO	50.3 ± 0.6	14.8 ± 3.5	22.0 ± 1.1[b]	0.1 ± 0.1
1% METO	50.4 ± 0.4	13.3 ± 2.0	20.9 ± 0.1[b]	0.4 ± 0.1
2% METO	40.5 ± 5.6	13.7 ± 2.4	27.6 ± 3.8	0.7 ± 0.2
3.5% METO	42.3 ± 4.0	14.4 ± 2.6	24.9 ± 2.0[b]	4.5 ± 0.5
7% METO	48.2 ± 1.0	12.2 ± 0.3	30.5 ± 0.6	9.0 ± 0.3
3.5% ME+TO	35.1 ± 3.7	18.3 ± 2.6	29.1 ± 2.3	4.6 ± 0.5
3.5% TO	69.6 ± 5.1[b]	8.0 ± 6.7	18.1 ± 4.0[b]	4.1 ± 0.3
7% TO	48.2 ± 1.3	6.3 ± 0.7	34.6 ± 0.9	10.7 ± 0.1
15% TO	45.8 ± 0.5	12.3 ± 0.1	25.2 ± 0.3[b]	16.4 ± 0.3[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 46: DHA and EPA levels in Proximal Colonic phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

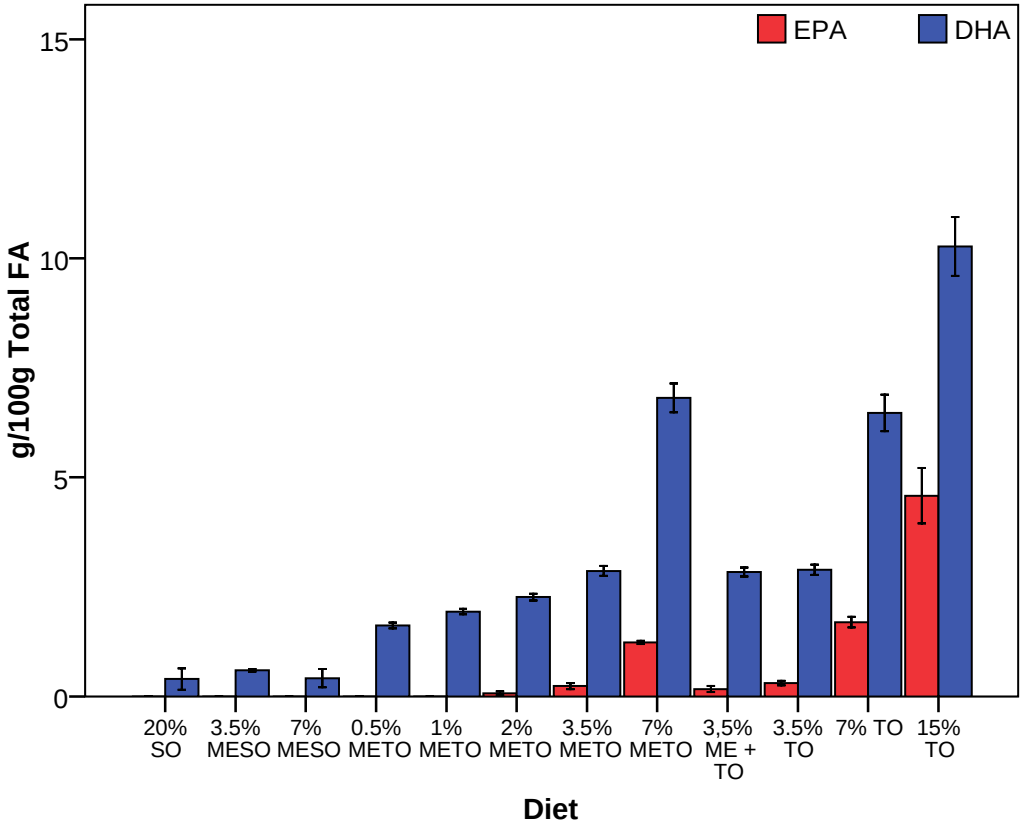
Distal Colon

Table 16: Fatty acid Profile from phospholipid of distal colon in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	52.0 ± 0.8	12.1 ± 0.3	35.3 ± 0.5	0.4 ± 0.2
3.5% MESO	46.1 ± 5.2	16.2 ± 1.5[a]	37.1 ± 0.7	0.5 ± 4.4
7% MESO	51.6 ± 0.7	12.1 ± 0.2	35.7 ± 0.5	0.4 ± 0.2
0.5% METO	44.5 ± 0.5	16.4 ± 0.4[b]	36.6 ± 0.3	2.4 ± 0.1
1% METO	45.6 ± 2.0	16.2 ± 0.9[b]	31.5 ± 2.4	6.4 ± 3.3
2% METO	44.0 ± 1.8	15.9 ± 0.7[a]	36.2 ± 1.4	3.7 ± 0.2
3.5% METO	42.4 ± 0.5	15.9 ± 0.4[a]	36.8 ± 0.5	4.7 ± 0.2
7% METO	48.4 ± 0.8	11.9 ± 0.3	31.0 ± 0.7	8.5 ± 0.3
3.5% ME+TO	52.2 ± 3.7	14.0 ± 1.1	27.2 ± 0.6[b]	6.4 ± 3.0
3.5% TO	56.4 ± 0.6	12.8 ± 0.3	27.1 ± 0.5[b]	3.5 ± 0.0
7% TO	47.1 ± 0.8	12.1 ± 0.2	32.0 ± 0.8	8.7 ± 0.3
15% TO	47.3 ± 1.4	12.0 ± 0.1	24.7 ± 1.7[b]	15.8 ± 0.7[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 47: DHA and EPA levels in Distal Colonic phospholipid



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

Long chain fatty acid profiles were also completed for the plasma (table 17 and figure 48) and faecal samples (table 18 and figure 49) collected from each group. The total omega-3 content in plasma followed a similar path to all other tissues measured, with a clear dose-response pattern emerging from the data and total omega-3 content increasing to a percentage of 18.6 ± 0.5 (SEM) in animals fed a 15% tuna oil diet. Levels of DHA alone corresponded to total omega-3 in terms of the dose- response pattern, though only animals fed diets containing 7% and 15% tuna oil had substantial traces of EPA in their plasma samples.

When analysing the level of omega-3 oils found in faeces, no clear dose-response pattern was observed and amounts of DHA and EPA were inconsistent when comparing between groups. Though the faecal samples from the 7% and 15% free tuna oil groups still recorded the highest level of DHA and total omega-3 PUFA, there was no clear trend observed with the remaining groups. Furthermore, the free and microencapsulated groups containing the equivalent dose of 7% oil gave differing levels of DHA present in samples.

In all, no significant changes were observed with regard to the level of omega-3 PUFA measured in faeces with regard to either dose of tuna oil or the form of tuna oil used.

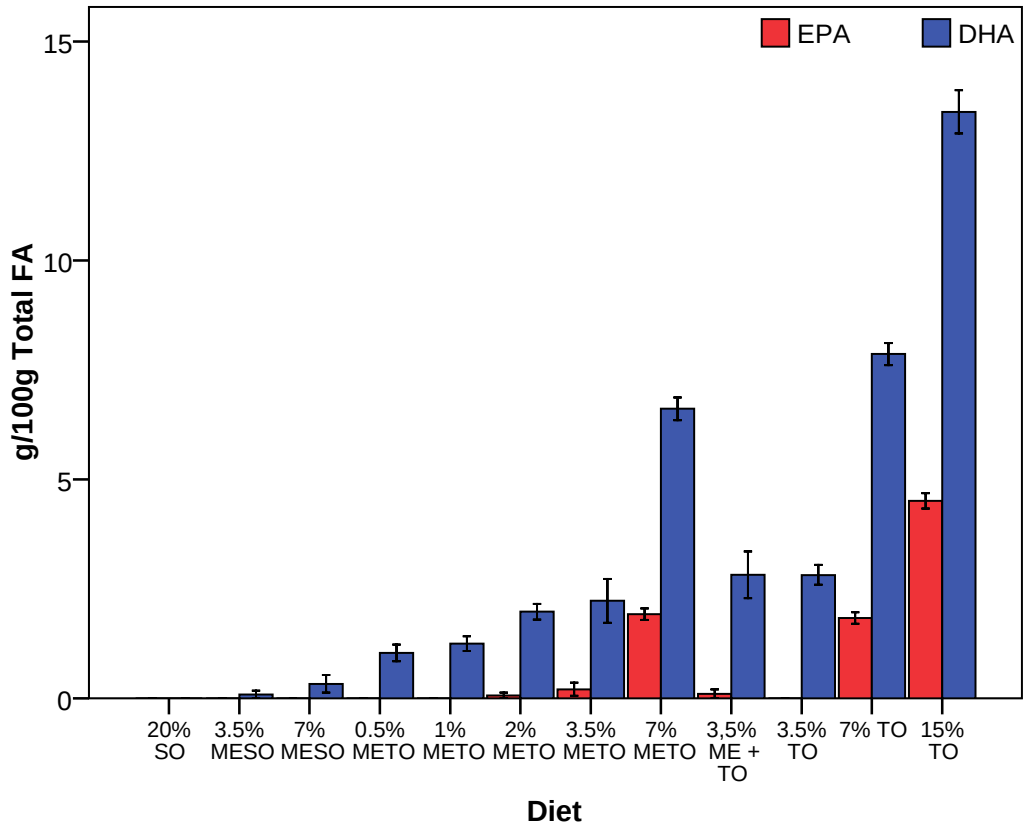
Plasma

Table 17: Fatty acid Profile from phospholipid of plasma in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	29.0 ± 1.1	13.9 ± 1.4	57.0 ± 1.1	0.0 ± 0.0
3.5% MESO	40.2 ± 2.8	12.1 ± 1.0	47.4 ± 2.7	0.1 ± 0.1
7% MESO	28.0 ± 1.9	14.5 ± 1.4	57.0 ± 2.3	0.3 ± 0.2
0.5% METO	20.8 ± 2.9	17.3 ± 2.0	60.0 ± 1.9	1.7 ± 0.3
1% METO	25.2 ± 4.2	15.5 ± 1.4	53.4 ± 6.8	5.7 ± 3.5
2% METO	20.9 ± 2.2	15.3 ± 1.3	60.2 ± 1.2	3.4 ± 0.3
3.5% METO	28.7 ± 4.1	15.9 ± 1.1	51.6 ± 3.3	3.7 ± 0.3
7% METO	29.5 ± 2.4	18.6 ± 1.5	43.1 ± 1.4[b]	8.6 ± 0.3[b]
3.5% ME+TO	40.3 ± 3.0	14.7 ± 1.0	40.9 ± 1.9[b]	3.9 ± 0.2
3.5% TO	41.4 ± 1.4	14.8 ± 0.9	40.1 ± 0.9[b]	3.5 ± 0.2
7% TO	34.6 ± 0.8	14.1 ± 1.1	41.1 ± 0.9[b]	10.0 ± 0.2[b]
15% TO	33.6 ± 0.6	16.7 ± 0.9	30.9 ± 1.3[b]	18.6 ± 0.5[b]

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 48: DHA and EPA levels in Plasma



All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

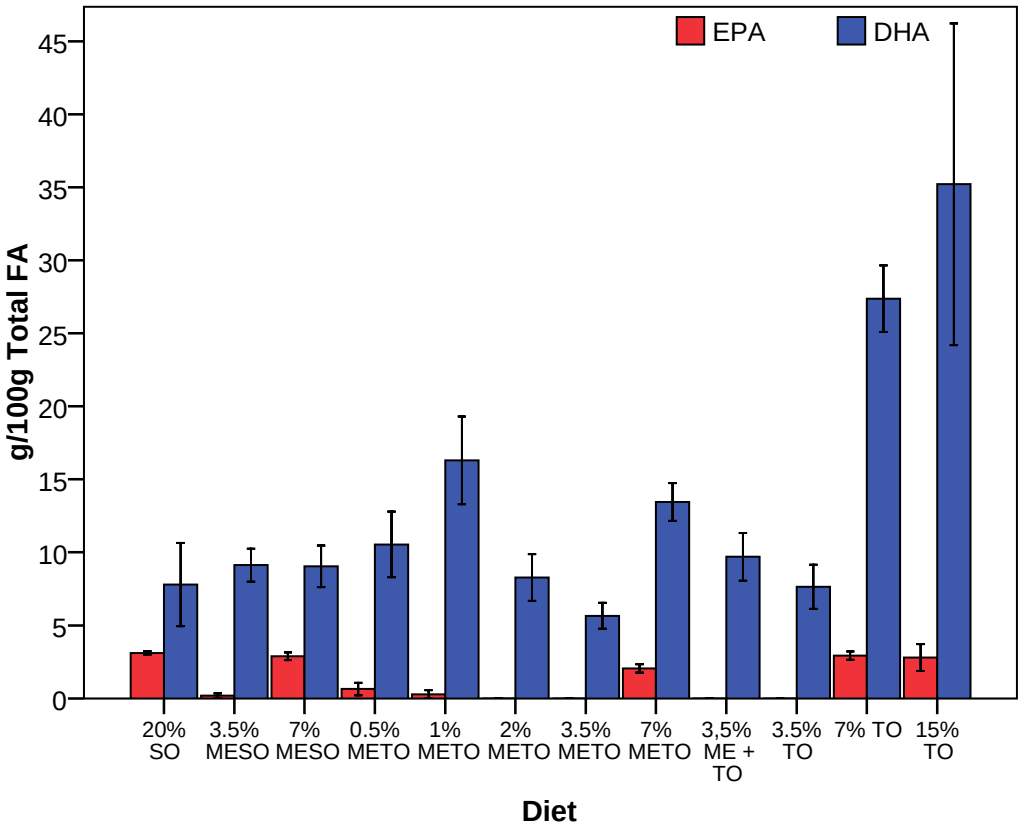
Faecal matter

Table 18: Fatty acid profile from faecal matter in rats fed different experimental diets.

	% FA			
Diet	SFA	MUFA	n6 PUFA	n3 PUFA
20% SO	64.3 ± 3.0	14.3 ± 0.6	17.8 ± 1.9	3.5 ± 1.1
3.5% MESO	71.1 ± 1.1	13.6 ± 0.6	12.4 ± 1.0	2.7 ± 0.7
7% MESO	67.0 ± 1.4	14.3 ± 0.6	15.3 ± 0.9	3.2 ± 0.4
0.5% METO	75.5 ± 2.2	12.1 ± 0.5	10.0 ± 1.2[a]	2.3 ± 1.0
1% METO	72.9 ± 1.4	12.4 ± 0.9	11.6 ± 1.0[a]	2.9 ± 0.4
2% METO	72.8 ± 2.8	11.4 ± 1.1	12.6 ±1.9	3.2 ± 0.3
3.5% METO	73.6 ± 1.5	12.9 ± 2.0	11.0 ± 0.6[a]	2.3 ± 0.5
7% METO	69.9 ± 6.3	20.8 ± 6.2	6.5 ± 0.7b	2.7 ± 0.4
3.5% ME+TO	71.3 ± 2.3	14.2 ± 1.4	11.6 ± 1.0[a]	2.7 ± 0.1
3.5% TO	74.0 ± 0.9	12.5 ± 1.1	10.6 ± 0.5[b]	2.7 ± 0.4
7% TO	76.0 ± 1.6	11.5 ± 1.5	8.2 ± 0.3[b]	4.0 ± 0.1
15% TO	78.3 ± 1.5[a]	9.1 ± 1.7	7.0 ± 0.6[b]	5.4 ± 1.4

All data expressed as means ± SEM for 60 rats (n=5).
Percentages calculated from full long chain fatty acid profile.
[a] p< 0.05, [b] p< 0.01 represents means significantly different from 20% SO control group by Tukey's ANOVA.

Figure 49: DHA and EPA levels in Faeces

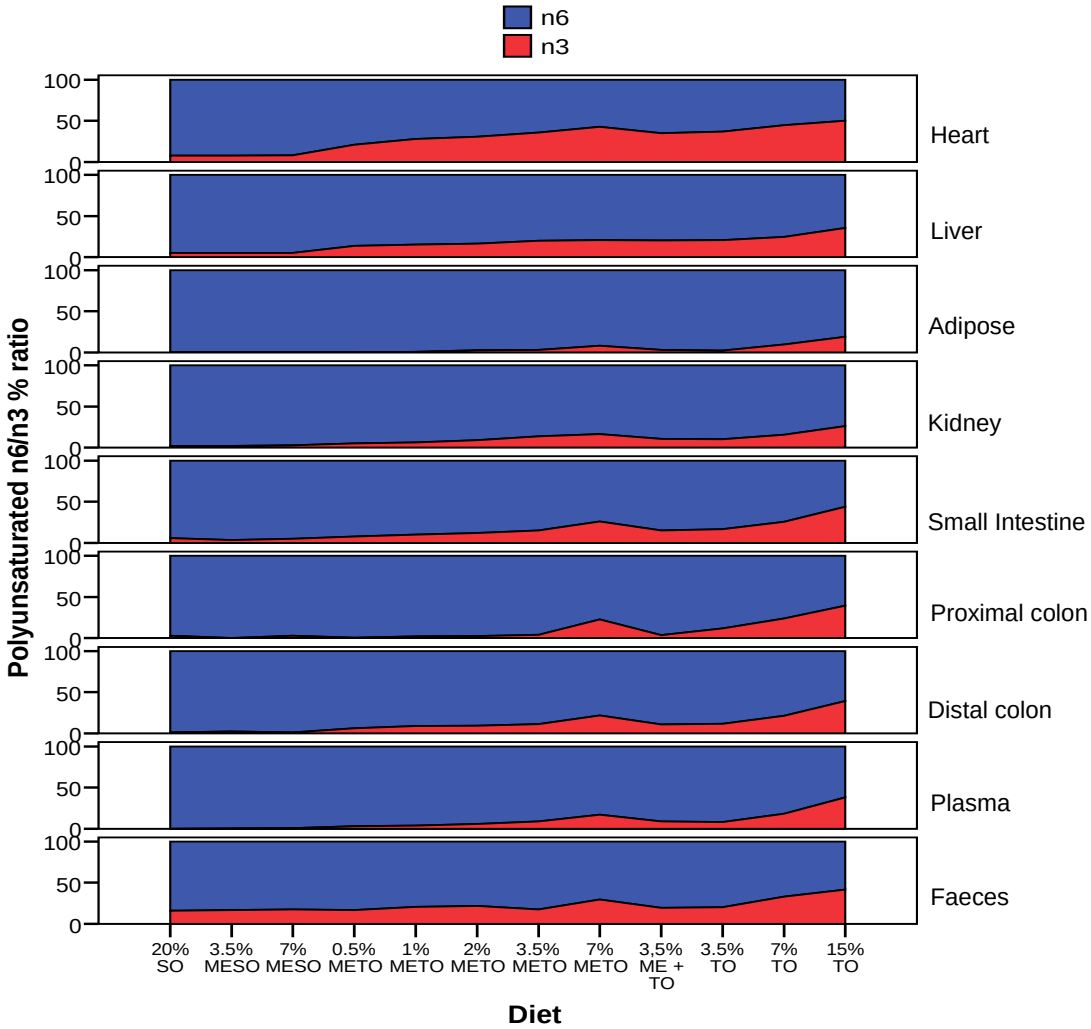


All data expressed as means ± SEM for 60 rats (n=5).
EPA and DHA levels calculated from full long chain fatty acid profile.

5.1.4.6. Omega-6: omega-3 ratio

Figure 50 summarises the omega-6:omega-3 PUFA ratio in each dietary groups for all tissue types and samples collected. A dose-response relationship can be seen for each tissue with regards to both the lower dose ME diets and the higher dose free oil diets. The phospholipid membrane of the heart most readily incorporated omega-3 PUFA in exchange for omega-6 PUFAs, leading to a considerable decrease in the omega-6:omega-3 ratio with a lower dose tuna oil diet, while also effectively resulting in an omega-6:omega-3 of 1:1 ratio in animals fed 15% tuna oil. Adipose tissue was the least affected tissue, while the omega-6: omega-3 ratio in colonic tissue was most affected in animals fed a diet of 7% tuna oil and above.

Figure 50: Polyunsaturated n6/n3 ratio across all diets in all samples measured



Data expressed as a percentage of n3 (red) and n6 (blue) PUFA for each dietary group (n=5). Data separated into 9 rows summarising ratio results for each tissue type and sample measured.

5.1.5. Discussion

This chapter described the effects of the novel microencapsulated product when tested in the rat-AOM model. The safety of this product was established as no negative effects, such as weight or hair loss, were observed in animals fed a diet of either the microencapsulated sunflower or tuna oil.

The microencapsulated oil did not have any substantial effects on the short chain fatty acid profile when consumed in the diet at a level of 3.5% or lower. A diet containing 7% microencapsulated oil however, did have an affect on the SCFA profile of both the caecum and faeces. Butyrate levels in particular increased significantly in these animals. It was hypothesised that the outer capsule of this ME product, which was designed from a specific protein and carbohydrate complex, was the dietary component that was most likely to be contributing to this butyrate increase.

Carbohydrates such as resistant starch are known to influence fermentation variables in the colon and increase butyrate levels [195] and butyrate promotes colonocyte health and has pro-apoptotic properties [77, 195, 196]. This result is therefore an important detail to consider when testing the chemoprevention properties of the encapsulated product. If modulation of apoptosis or other acute endpoints is observed, the effect of butyrate on this response will have to be considered as well as the products direct delivery capabilities.

Increases in levels of branched-chain fatty acids (BCFAs) such as isobutyric and isovaleric acids are indicative of an increase in protein fermentation [197]. As no changes were observed in the trace amounts of these particular BCFAs, it can be implied that the microencapsulated product did not significantly alter the rates of protein fermentation in the caecum or colon.

The primary reason for testing this microencapsulated product was to determine if the *in vitro* results showing direct delivery of oil to the colon could be replicated in an *in vivo* model. Following the analysis of the long chain fatty

acid profiles it was apparent that microencapsulated tuna oil did not increase the omega-3 PUFA content in the phospholipid of any of the key tissues tested.

It was initially hypothesised that a potential increase in omega-3 would preferentially occur in the distal colon as a result of tuna oil being delivered directly to the colonic epithelium via the ME product. This hypothesis was not supported as the LCFA profile of the colonic phospholipid were comparable in groups fed either free or microencapsulated tuna oil. Yet the concept of direct delivery to the colon using the ME product can not yet be dismissed.

It was decided that the LCFA profiles would be measured in order to demonstrate any differential effects of delivery or uptake of omega-3 caused by the ME product. However, analysing these profiles after 4 weeks of feeding may have masked the potential of the microencapsulated product to deliver oil directly to the colon. It is possible that in the 4 week feeding time frame, lipid levels may have been equilibrated throughout the body tissues of the animals. This may have effectively masked any difference in delivery between the two different forms of dietary oil.

The only way to avoid this issue is to perform a shorter feeding experiment or carry out a timed experiment that measures the release of oil *in vivo* throughout the digestive tract. Nevertheless, even if these experiments support the suggestion of direct oil delivery to colon one can question the effectiveness of this product if within a short period of time lipid levels simply equilibrate throughout the body.

Regardless of this products ability to alter the phospholipid membrane it is still possible that microencapsulated tuna oil still may have protective properties against the initiation of carcinogenesis. It is possible that such effects occur through mechanisms other than an influence on the membrane phospholipid. Therefore, the effect of encapsulating fish oil on the acute host responses to a genotoxin will still be explored in chapter 6.

Regardless of the effect of microencapsulation of lipid profiles, a clear dose-response pattern was observed in all tissues measured. It was clearly evident that an increase in tuna oil in the diet corresponded to an increase of total omega-3 levels in the phospholipid membrane of tissues and also in the individual DHA and EPA fatty acids. This relationship was observed in all samples with the exception of the faecal samples.

It is possible that this relationship is not as evident in faecal matter, however, given the strong relationship shown in all other samples, it is also likely that the process of faecal collection may have altered results. Though fresh faecal samples were collected and frozen as quickly as possible, there is a chance that lipids in these samples in particular may have been subjected to the process of oxidation. In addition, it is also possible that the method of extracting and analysing the lipid content of faecal matter may be more difficult when compared to tissues. As a result this protocol may have to be refined accordingly.

Other general observations made with regard to the tissue type and the readiness in which they incorporated omega-3 PUFAs showed that the heart was the organ that incorporated the highest levels of both DHA and EPA. This was followed by the liver and digestive organs including the small intestine, proximal and distal colon. While relatively lower amounts of omega-3 PUFAs were in incorporated into kidney tissue, this organ was of particular interest in that the EPA levels were almost equivalent to the tissues DHA levels. This suggests that the conversion rate from EPA to DHA is not as efficient in the kidney when compared to other organs.