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(54) Title: THE COMBINED USE OF TRIGLYCERIDES CONTAINING MEDIUM CHAIN FATTY ACIDS AND EXOGENOUS LIPOLYTIC ENZYMES AS FEED SUPPLEMENTS

(57) Abrégé/Abstract:
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(57) **Abstract:** The present invention relates to the use of triglycerides (TG) containing medium chain fatty acids (C4 to C12), combined with exogenous lipolytic enzymes (esterases or lipases) as a feed supplement for animals in order to prevent and/or alleviate the problems which are frequently met at this moment. This results in a marked improvement of the growth performances without the use of the classical, but contested, feed additives.

THE COMBINED USE OF TRIGLYCERIDES CONTAINING MEDIUM CHAIN FATTY ACIDS AND EXOGENOUS LIPOLYTIC ENZYMES AS FEED SUPPLEMENTS

Field of the Invention

The present invention relates to the use of triglycerides (TG) containing medium chain fatty acids (MCFA; C4 to C12), combined with exogenous lipolytic enzymes (esterases or lipases) as a feed supplement for animals, especially early weaned pigs in order to prevent and/or alleviate the problems which are frequently met at this moment. This results in a marked improvement of the growth performances without the use of the classical, but contested, feed additives.

Background of the Invention

Early weaning (3 to 4 weeks of age) of piglets has become a general practice in pig husbandry systems for increasing the productivity and maintaining the profitability. Early weaning, however, burdens the piglet with a lot of stresses, mainly of environmental, nutritional and immunological origin, combined with a more or less pronounced depression of feed intake and mobilization of body reserves. Maldigestion and malabsorption often aggravate the situation resulting in digestive upsets due to bacterial overgrowth and/or viral infections. These phenomena greatly interfere with the profitability of the enterprise. There is a vast body of literature covering these issues (e.g. VAN DER PEET, 1992; PARTRIDGE, 1993).

The currently used methods to handle those problems aim at the adaptation of the feed to the digestive capacity of the piglet, and/or by improving the acceptability of the feed by the use of specific ingredients (e.g. milk powder and derivates, such as whey and lactose, dried blood serum, flavors), all or not combined with an increase of the energy content of the feed. An increase of the energy content can be obtained among others by including easily digestible or metabolizable fats. The usefullness of medium chain triglycerides (MCTG) in this context is well documented both in neonatal (ODLE, 1999) and in weaned piglets (CERA et al., 1989). The reasons for the usefullness of MCTG is their specific digestive and metabolic fate, reviewed by BACH & BABAYAN. (1982).

Digestive upsets are prevented and/or treated by supplementing the feed with pharmaceutical antimicrobial substances (antibiotics, chemotherapeutics, called antibiotics further on). The combined effects of the above mentioned interventions mostly result in a pronounced improvement of the growth performances (called 'growth promotion' further on). This growth promotion is mainly due to, depending on the circumstances, an improved feed intake all or not combined with a better feed conversion (= kg feed / kg gain). However there is a growing concern about the use of antibiotics for growth promotion in animal production systems. Especially there is a well-considered fear for the risk of the emergence of cross-resistance to some last-resort antibiotics used in human medicine (CORPET,1996; WEGENER et al., 1998). Therefore most of those antibiotics (so called growth promotors) are already or will be banned in the near future in the EU which justify an urgent need for alternatives.

Because there is a general belief that the digestive pathology in early weaned pig is mainly caused by Gram – bacteria (especially *E. coli*) and that Gram + lactic acid bacteria (Bifidobacteria, Lactobacilli) have a protective and/or antagonistic effect against them, the currently proposed alternatives are selected for their anti-*E. coli* activity: eg. cupper and zinc compounds, selected organic acids (short chain fatty acids (SCFA, formic, acetic-, propionic acid), lactic , fumaric-, citric, malic, sorbic acid), probiotics (mainly lactic acid bacteria) and/or prebiotics (mainly bifidogenic oligosaccharides, so called NDO's). Cu- and/or Zn- compounds are effective but are not acceptable because their effect on the environment (pollution). Results obtained with pro- and/or prebiotics are unpredictable and generally spoken disappointing (CHESSON,1994).

Similar problems exist in other animal species and in animals of other age groups.

Only SCFA and the 'classical' organic acids are the most promising alternatives for the moment (ROTH et al., 1998). However rather high doses are needed, so that their usefullness is limited by the high cost, their corrosive nature and their averse taste which interferes greatly with the feed intake of the piglets

The antimicrobial effects of fatty acids (FA) in general and their salts (soaps) is already know for decades. A reevaluation of the antimicrobial effects of selected FA (and derivates) is given in the review of KABARA (1978). Special attention was thereby given to lauric acid (C12, a member of the MCFA-family) and derivatives.

Further literature data lead to conclude the relative important contribution of MCFA in the milk-lipid of certain animal species (e.g. rabbit, goat, horse), while in other species the concentrations were low or even nihil as in sow's milk (DIERICK, 1998, literature compilation, personal communication). In most mammals there is a more or less pronounced preduodenal (= not of pancreatic origin) lipolytic activity originating from lingual or gastric secretions. The activity of those lipases is independent of the presence of colipase and bile acids, is active and stable in a broad range of pH's and has a preference for MCFA in milk fat. The preduodenal lipase activity is high in preruminant calves and rabbits, moderate in piglets and absent in poultry (MOREAU et al., 1988). An excess MCFA can have important side-effects: indeed, there are data that they can be hypnotic in new born pigs (ODLE, 1999), and are a strong stimulus for CCK, an intestinal hormone with a pronounced satiating activity what could interfere with the feed intake (LEPINE et al., 1989). A lower feed intake could also be the result of the strong (goat-like) odour and averse taste of free MCFA, although data in this context are scarce and non-conclusive.

Summary of the Invention

The present invention aims at providing new feed supplements for animal feeds, particularly for early weaned pigs which can replace the commonly used (and contested) antibiotics and other growth enhancers.

The present invention is related to a feed supplement or feed composition whereby the feed supplement is a premix of feed additives (vitamins, minerals, antibiotics, among others) with a carrier for use as part (mostly 1 to 5%) of a complete food, and whereby the feed composition is the entire listing of the different feed ingredients used in a complete feed: an other term often used is "feed formula".

The present invention provides the use of at least one triglyceride (TG) containing medium chain fatty acids (MCFA: C4 to C12), combined with at least one exogenous lipolytic enzyme (esterase or lipase) as a feed supplement for animal feeds, especially for early weaned pigs in order to prevent and/or alleviate feeding problems which are frequently met at this moment. The addition of this combination of TG and exogenous lipolytic enzymes to feed surprisingly results in a physiological environment in the stomach which regulates and stabilizes the gastrointestinal microflora. This effect, combined with the fact that an easily digestible and metabolizable source of energy is provided, surprisingly results in a marked improvement of the growth which is comparable with the growth promotion obtained with the commonly used (and contested) antibiotics and other growth enhancers without negative side effects for the animal, the feed industry and the consumer.

Brief Description of the Drawings

Fig. 1 relates to the results obtained in example 1 and presents the *in vitro* released MCFA (expressed as g/100 g TG) for the different examined TG's (fig 1.a. coconut oil, fig. 1.b. MCTG1, fig 1.c. MCTG2, fig.1.d. butterfat) and selected enzymes. The enzymes, coded L1 to L6, were used in a dose of 10.000 ppm on the basis of TG. The release of FA was studied in buffered medium at pH 2, 3, 4 and 5 as being representative for the pH conditions prevailing *in vivo* in the stomach.

Fig 2. relates to the results obtained in example 2 and presents the total and selective bacterial counts (expressed as $\log_{10}$ Colony Forming Units, CFU per g fresh contents)in the stomach contents of cannulated pigs. Fig. 2.a, 2.b. and 2.c. give the results for the feeds with 5% coconut oil, MCTG1 and butterfat respectively. The first component of each figure presents the results obtained without lipolytic enzymes, the second and third block, the results with the addition of L2 and L5 (1000 ppm on feed basis) respectively. The first bar is the total count, the following bars are the number of lactobacilli, streptococci and *E. coli*. The results indicate that with each TG, the enzymes cause a reduction of the total count and the number of lactobacilli.

Fig. 3 relates to the results of the analysis of the fat fractions in the gastric contents (in g / 100 g contents) of the cannulated pigs used in example 2. The proportion of free

FA to total FA is given for the feeds with the different TG's used without enzyme (V1: coconut oil, V4: MCTG1; V7: butterfat) or with the supplementation of L2 or L5 (1000 ppm on feed basis). The free FA released without enzymes result from the activity of the endogenous preduodenal lipases. The results indicate that the lipolytic enzymes used grealty enhance the release of free FA from each TG tested.

Detailed description of the invention

The present invention relates to the use of at least one triglyceride (TG) containing medium chain fatty acids (MCFA), combined with at least one exogenous lipolytic enzyme (esterase or lipase) as a feed supplement for animal feeds in order to prevent and/or alleviate the problems which are frequently met at this moment.

The present invention thus also relates to a feed supplement composition which comprises at least one triglyceride (TG) containing medium chain fatty acids (MCFA) and at least one exogenous lipolytic enzyme (esterase or lipase).

Medium chain fatty acids according to the present invention include both even and odd fatty acids, such as fatty acids containing C4 (butyric acid, butanoic acid), C5 (valeric acid), C6 (caproic acid, hexanoic acid), C7 (heptanoic acid), C8 (caprylic acid, octanoic acid), C9 (pelargonic acid), C10 (capric acid, decanoic acid), C11 (undecanoic acid) or C12 (lauric acid, dodecanoic acid). The MCFA triglyceride component according to the present invention may be a naturally occuring triglycerides containing composition, such as butterfat and coconut oil. Alternatively, said triglyceride component may comprise one or more industrially prepared triglycerides or a mixture of naturally occuring and industrially prepared triglycerides. Said triglyceride may be prepared by interesterification of C4 to C12 chain fatty acids.

Examples of naturally ocurring substances which are rich in medium chain fatty acid containing triglycerides include but are not limited to coconut oil, palm kernel oil, babassu oil, cohune oil, tacum oil, cuphea oil derived from plant seeds, milk of mammalian species, such as milk from horse, rat, goat and rabbit, or butterfat.

Examples of commercial sources of chemically synthesized structured or tailor-made triglycerides containing medium chain fatty acid include but are not limited to those given in Table 10 or those exemplified in the Materials section of the Examples.

The lipolytic enzyme component according to the present invention may comprise a lipase or an esterase, a mixture of lipases or a mixture of esterases or a mixture of lipases and esterase. Said lipases or esterases may be naturally occuring or industrially prepared. Said lipolytic enzymes may be from microbial, mammalian or plant origin.

Examples of commercially available plant lipases include but are not limited to lipases from wheat, castor bean, rape, mustard and lupin.

Examples of commercially available microbial lipases include but are not limited to the lipases as given in Table 11 or those exemplified in the Materials section of the Examples.

Examples of commercially available esterases include but are not limited to pregastric esterase (PGE) from sublingual tissue of calf, kid and lamb, rennet paste from engorged abomasa of calf, kid and lamb, esterase from rabbit liver or porcine liver.

Preferably said triglyceride component according to the invention as defined above is added in a concentration of about 0.25% to about 10% to the feed.

Preferably said lipolytic enzyme component is added in a concentration of about 100 to about 10.000 ppm to the feed.

The use of a feed supplement composition according to the present invention is preferably as a feed supplement for animal feeds, particularly for early weaned pigs. The use of the feed supplement compositions according to the present invention do not, however, exclude the use of such compositions as a feed supplement for pigs of other age categories or as a feed supplement for other types of animals.

The present invention also relates to the use of a combination of at least one MCFA TG and at least one lypolytic enzyme according to the present invention for the preparation of a feed supplement preferably for early weaned piglets.

The present invention also relates to methods for the preparation of feed supplements according to the present invention comprising the step of mixing together of different MCFA TG and lipolytic enzyme components according to the invention.

The mechanism by which SCFA, MCFA and other organic acids excert antimicrobial activities is well documented in the literature. The current belief is that undissociated (RCOOH = non ionized) acids are lipid-permeable and in this way can pass across the microbial cell membrane and dissociate ($RCOOH \rightarrow RCOO^- + H^+$) in the more alkaline interior of the microorganism. This brings about an acidification of the intracellular pH below permissible levels for survival. In other words organic acids act as protonophores that increase the inward leak of H^+ so that efflux is not rapid enough to alkalinize the cytoplasm again. The physicochemical characteristics of the organic acids greatly influence their ability to act as protonophores: (molecular weight, pKa (dissociation constant), solubility). The physiological environment in which they are present (especially the pH in the different locations of the gastrointestinal tract) is also a very important factor. Further, the type of the microbial envelope (mainly peptidoglycan in Gram +, and lipopolysaccharide in Gram – bacteria) greatly influences the passage of the acids through the membrane.

First, In preliminary *in vitro* experiments, in which a broad range of organic acids (SCFA, MCFA and other commonly used organic acids in the feed and food industry) were tested for their antibacterial activity against the dominant bacteria of the small intestinal microflora, the present inventors unexpectedly found that the SCFA and the commonly used organic acids only were bacteriostatic at higher concentrations (0.02 to 0.04 M) for the Gram – flora (and to a lesser extent for Streptococci). However, with the MCFA an unexpectedly high bacteriostatic and bactericidal activity was found against both Gram + and Gram – bacteria. The antibacterial activity was pH dependent and highest at lower pH, thus when a relatively high proportion of the FA was in the undissociated form. In the same experiments a temptative minimal bacteriocidal concentration of 0.005 to 0.01 M was put forward .

Also unexpected was that by using a combination of MCFA, the antibacterial spectrum of the antibiotic growth promotors used in the intensive animal production could be mimicked totally.

The specific characteristics of MCTG as an easily available energy source are well documented. Their beneficial effect can be summarized as follow (BACH & BABAYAN, 1982):

-MCTG are digested, absorbed and transported rapidly in disorders were digestion and absorption are not optimal. Maldigestion and malabsorption are frequenty observed in newly weaned piglets, and are attributed to a sharp drop in the activity of most of the digestive enzymes. The deficiency of lipolytic enzymes shortly after weaning is very pronounced.

-MCTG are oxidized rapidly in the organism and are a source of abundant and rapidly available energy. However MCTG are ketogenic, what, when high doses are given, can have narcotic side effects. This side effect is certainly undesirable in piglets.

Also the depressive effect on the voluntary feed intake, by activation of CCK, is unwanted. Also unwanted (by the producer and/or the animal) is the strong unpleasant odour of the free MCFA which evaporate relatively easiliy.

In order to obtain the positive effects and to avoid the negative characteristics of MCFA, the inventors had the original idea to use a combination of a TG, containing sufficient MCFA, together with a lipolytic enzyme as a feed supplement, with the intention that sufficient MCFA should be released in the stomach to have a sterilizing effect, resulting in a lesser bacterial load in the small intestine and to prevent digestive upsets. This effect, combined with the extra easily available energy of the MCFA, and the supplementation of the natural lipase activity in the stomach and upper intestine by the exogenous lipolytic enzyme(s), resulted unexepectedly in a growth promotion making the use of antibiotics unnecessary. The expected gradual release and absorption of the free MCFA unexpectedly avoided the unwanted side effects.

In summary the invention describes the composition of a natural growth promoting feed supplement for the use in animals.

The following examples and drawings merely serve to illustrate the present invention and are not meant to be limiting in any manner.

Examples

Materials

By way of example: the following fats (TG) were chosen to illustrate the present invention: butterfat, coconut oil, and two commercially available sources of MCTG: MCTG1 (Aldo MCT Kosher Food Grade) and MCTG2 (Stabilox-860), commercialized by LONZA Inc. (Fair Lawn, NJ 070410, USA) and LODERS-CROKLAAN BV (NL-1521 AZ Wormerveer) respectively. By way of example the following lipolytic enzymes were chosen to illustrate the present invention: L1: Lipozyme 10.000L, NOVO Nordisk A/S, 2880 Bagsvaerd, Denmark ; L2 : Lipase 10.000P, Biocatalysts Ltd., CF37 5UT

Pontypridd, Wales, UK ; L3 : TP 516P, Biocatalysts Ltd., CF37 5UT Pontypridd, Wales, UK ; L4 : LIPOMOD 224P, Biocatalysts Ltd., CF37 5UT Pontypridd, Wales, UK ; L5 : Lipase SAIKEN, NAGASE & Co, Chuo-ku, 103 Tokyo, Japan ; L6 : Lipase ITALASE C, SBI, Systems Bio-Industries, Inc., WI 53187-1609 Waukesha, USA. The codes L1 to L6 will be used further on. The selection of TG and lipolytic enzymes described in these examples does not exclude the potential usefullness of other TG and lipolytic enzymes and combinations of them for the purposes described in this invention.

Methods for extraction and analysis of different lipid compounds

A lipid extraction procedure using hexane/iso-propanol (3/2, v/v) avoiding any solvent evaporation step to prevent any loss of MCFA due to their great volatility was used.

Acid (H2SO4) catalyzed esterification of FA in the same extraction medium with formation of isopropyl esters (FAIPE) without loss of shorter esters or alteration of polyunsaturated higher FA was used. FAIPE appear in the upper hexane phase.

For the calculation of the concentration, quantitative capillary column (DB-225, 30m, ID 0,25 mm, Film 0,25 µm) GLC chromatography of individual FAIPE using 2 internal standards (C9 used for C4 to C12 acids and C17 used for C14 to C18:3 acids) was used. Coefficients of variation on the response factors amounted to 0,94 % for C9 and 2 ,51 % for C17.

Individual free FA was extracted from the lipid extract with a strong anion exchange resin Amberlyst 26 before esterification in the same medium and analysed by capillary GLC mean recovery of added free FA amounted to 101, 9 %.

Example 1

In vitro screening of MCFA containing TG's and lipolytic enzymes for lipolysis at different pH's (simulation of gastric conditions)

A selection of lipolytic enzymes to be tested, coded L1 to L6, was made which was based on their commercial availability and feasable price in commercial settings.

MCFA containing TG's were selected on the basis of their specific MCFA content in the fat as specified in table 1.

Table 1. MCFA concentration (g / 100 g FA) in the selected TG's

	C4	C6	C8	C10	C12
Butterfat	3.4	2.1	1.2	2.6	3.0
Coconut oil	0	0.7	8.5	6.2	48.8
MCTG 1	0	2.8	69.1	27.7	0.4
MCTG 2	0	0.2	57.5	42.3	0.0

The *in vitro* incubations were done in buffered circumstances at different pH's; a glycine buffer was used for incubations at pH 2 and pH 3; an acetate buffer was used for incubations at pH 4 and pH 5. Incubations were done for 180 min at 37°C in a shaking water bath. The parameters used for the incubations were chosen in order to simulate as closely as possible the *in vivo* conditions in gastric contents. The medium used for the incubations was made up of the following ingredients: 0.250 g of the selected TG + 2.250 g of a synthetic feed (based on starch, dextrose, casein and a vitamin-mineral premix) + 10 ml buffer solution + 0.5 ml pepsine solution (50 mg in 100 ml aqua dest) + 10000 mg/kg fat (= ppm) of the selected commercial lipolytic enzyme preparation. If necessary the fat was molten, otherwise there were no special preparations (dispersion or emulgation) of the fat.

The results of the incubations are given in figure 1a to 1d which presents the released MCFA in g/100 g TG for the different examined TG's. The hydrolytic activity was highest at pH 3 to 5 with each of the enzymes, which fits well with the pH normally occurring IN VIVO in the stomach of pigs. The amount of released free MCFA seems to be dependent on the amount present in the original source of TG. The amount of MCFA released was ± 3.5% with coconut oil, 10-15% with the two MCTG's and ±0.5% with butterfat.

Example 2

In vivo experiment with gastric cannulated pigs for the study of the release *in situ* of MCFA by endogenous and exogenous lipolytic enzymes

Three pigs (Belgian Landrace, stress negative, female) with an initial weight of ±8.5 kg were prepared with a gastric cannula using the technique of DECUYPERE et al. (1977). The cannulae were placed midway the curvatura major in the fundic region.

Three TG's (coconut oil, MCTG1 and butterfat, each) and 2 lipases (L2 and L5) were selected for the present experiment

Nine feeds were prepared using 95% of a commercial feed for piglets with 5% of the selected (eventually molten) TG's all or not supplemented with the selected lipases (see Table 2 for the codes used further on). The fats were simply poured on the meal and thoroughly mixed in a horizontal mixer. The concentration of the lipases was 1000 ppm of the commercial preparation in the feed

Table 2. Feeds used in experiment 2

Coconut oil:
- V1: 95% piglet feed + 5% coconut oil
- V2: idem + 1000 ppm L2
- V3: idem + 1000 ppm L5

MCTG1:
- V4: 95% piglet feed + 5% MCTG1
- V5: idem + 1000 ppm L2
- V6: idem + 1000 ppm L5

Butterfat:
- V7: 95% piglet feed + 5% butterfat
- V8: idem + 1000 ppm L2
- V9: idem + 1000 ppm L5

The composition of the piglet feed was based on maize, barley, dried acid whey, cassave, herringmeal, soybean oil, and was supplemented with a vitamin-mineral premix. The feed contained no growth promoting supplements. The proximate analysis of the feeds (V1, V4 and V7) in % of as given was: DM: 90.6, 90.7 and 90.8; total ash: 7.8, 7.9 and 8.5; crude protein: 15.1, 15.4 and 14.8; crude fat: 8.5, 8.3 and 8.3.

The feed was given dry, in three equal meals (9, 13 and 17h), at 85% of the ad libitum intake of pigs with a comparable weight.

The experiment had a 3 x 3 Latin square design.

The experiment had a successful course. There were no health problems nor feed refusals. Statistics were done using ANOVA (1997), differences were at $p< 0.01$ to $p<0.05$ (**) or $p<0.1$ (*)

Sampling of the gastric contents for the chemical analysis was done on 2 consecutive days, 2 times a day, 30 min after the 9 h and 13 h meal. The pH was measured directly, thereafter the samples were stored at –20°C till further analysis.

The sampling of the gastric contents for the bacteriological analysis was done during 1 day, 90 min after the 9 and 13h meal. The bacterial counts were done using the technique of VAN DER HEYDE et al. (1964). The media (all from OXOID, UK) used were RCM agar + hemin for the total count (48h, anaerobic), Rogosa agar for the Lactobacilli (48 h, anaerobic), Slanetz & Bartley agar for the fecal Streptococci (24 h, aerobic), and EMB agar for *E. coli* (24 h, aerobic). All incubations were at 37°C. Results are expressed as $\log_{10}$ CFU / g fresh contents (colony forming units)

The results of the experiment can be summarized as follows:

The pH of the stomach contents measured 30 and 90 min after feeding did not differ between the treatments (feeds) and ranged between 4.2 and 5.01. This is within the optimum range for the lipolytic activity of L2 and L5 as was found in the first experiment.

The results of the bacteriological counts are presented in table 3 and in fig 2.

Table 3. Bacteriological counts in the gastric contents of the piglets fed diets 1 to 9 ($\log_{10}$ CFU / g fresh contents: mean ± s.d) (n = 6).

	Total	Lacto.	Strepto.	*E. coli*
Coconut oil				
V1	6.4±0.8	6.0±0.8	4.3±1.0	2.3±1.2
V2	5.2±0.3**	5.0±0.3**	4.1±0.6	2.4±1.4
V3	5.3±0.6**	4.9±0.7**	2.7±1.6*	2.6±2.1
MCTG1				
V4	6.1±0.2	5.7±0.5	5.2±0.4	2.9±1.6
V5	4.2±0.5**	3.7±0.5**	0.0**	1.0±1.5**
V6	3.4±1.7**	2.7±1.4**	0.5±1.2**	0.5±1.2**
Butterfat				
V7	6.4±0.4	5.7±0.8	5.0±0.6	4.1±0.5
V8	5.6±0.9*	5.5±0.3	4.0±0.7*	3.4±0.1*
V9	5.7±0.5*	5.5±0.5	4.0±0.7*	4.5±1.4

*, ** : differences per TG within the column

The most important results are:

-with coconut oil, both L2 and L5 reduced 10 fold the total count and the number of lactobacilli

-with MCTG1, both enzymes had a very pronounced (mostly $p< 0.001$) effect and reduced the total count and the lactobacilli by a factor 100 to 1000; streptococci and *E. coli* were mostly reduced to non detectable levels

-with butterfat, there was a 10 fold reduction of the total count and the number of streptococci.

The results allow the conclusion that the combination of a MCFA containing TG and a lipolytic enzyme in the feed is able to suppress the total bacterial count and the dominant flora. This effect most likely is due to the release of free MCFA from the TG's used.

This statement was confirmed by the chemical analysis of the different fat fractions in the gastric contents collected during present experiment. The results of the analysis are given in fig. 3 in which the amount of total and free FA per 100 g fresh gastric contents are presented.

The results expressed as g free FA per 100 g total FA in the stomach contents, or in other words the degree (%) of hydrolysis of the TG, is given in table 4.

Table 4. Degree of hydrolysis (g free FA / 100 g total FA in fresh gastric contents) of the different TG's used in present experiment as influenced by L2 or L5

	control	+L2	+L5
Coconut oil	*V1* 16.5	*V2* 43.2	*V3* 44.8
MCTG1	*V4* 18.9	*V5* 58.5	*V6* 60.9
Butterfat	*V7* 16.8	*V8* 46.8	*V9* 45.8

The results for the individual FA (not given here) indicated that there was no preferential release of specific FA; in other words the release of individual FA is roughly proportional to their content in the TG used. Out of the results presented in fig.

3 and table 1 it can be concluded that the endogenous lipolytic activity in the stomach of the piglets hydrolyses ± 16-19 % of the TG. The addition of the exogenous lipolytic enzymes increases the hydrolysis about threefold.

It is striking and unexpected that the release of MCFA runs parallel with the degree of suppression of the bacterial load in the stomach: the most efficient suppression was observed with the combination MCTG1 + L5, which caused 60.9% hydrolysis of the TG in the stomach (corresponding with a concentration of ±1 % of free FA and 0.6% of MCFA), followed by coconut oil + L5 (0.8% FA acids and 0.3% MCFA) and butterfat + L5 (0.8% free FA and 0.06% MCFA).

Example 3.

Zootechnical experiment in commercial settings: Growth performance combined with *ex vivo* observations on the gastric contents

The aim of this experiment was to check if the above mentioned concept was applicable and suitable in commercial settings and to check, when a growth promotion was obtained, this was comparable with the growth promotion obtained in early weaned piglets with antibiotics or a combination of organic acids with proven effectiveness.

For this experiment 244 freshly weaned piglets (Seghers Hybrid F1, initial w eight ±6.5 kg) were divided according to litter, sex and weight in 4 groups: A: 68; B = 61; C = 60 and D = 55 piglets. The experiment was run in commercial settings in temperature controlled facilities.

The composition of the feeds used was based on barley, wheat, maize flakes, extruded maize, extruded soybeans, soy-flour, herring meal , 2.5% TG, and a commercial premix (mainly based on milk products, vitamins + minerals) for early weaned piglets (12.5%) . The treatments (A to D) differed in the used TG's and the used additives (see table 5). The feeds contained no growth promoting antibiotics. Feed A was a negative control, feed D a positive control containing a mix of commonly used organic acids The calculated proximate analysis of the feeds used was equalized. The formulated contents were (% fresh): DM: 90.0 à 88.8, crude protein: 18.7 à18.9, crude fat 6.9, total ash: 5.1-5.3 The energy content was (Nef97): 2463-

2475 kcal/kg, the ileal digestible amino acids were set at: Lys: 1.07%, Met + Cys: 0.65, Thre: 0.66, Try 0.19.

Table 5. Treatments used in the zootechnical experiment

Treatment	A	B	C	D
TG (2.5%)	soybean oil	MCTG2*	MCTG2	soybean oil
Lipase (L5)	-	-	1000ppm**	-
Supplement organic acids***	-	-	-	1.5%

* MCTG2 was selected upon commercial availability
**based on fresh feed
*** 0.25% citric acid + 0.75% fumaric acid , 0.5 % Na-formiate (as specified by the feed manufacturer)

The feed was prepared by a commercial feed company wich used a spray-equipment for fats and other liquid supplements. The feed was offered dry, ad libitum; water was continuously available via a nipple.

The experiment lasted 3 weeks. The piglets were weighed individually at the start of the experiment and weekly thereafter; feed intake was recorded daily per two pens (joint feed hopper for two pens with ±15 piglets each). Therefore statistics only could be done for the weights. The visual health condition of the pigs per pen was checked daily and coded on a scale from 0 (extremely bad) to 10 (excellent).

The zootechnical results on a weekly basis are presented in table 6

Table 6. Zootechnical performances of the piglets as influenced by the treatments (mean ± s.d.)

Treatment	week1	week2	week 3	week1 to 3	% of control
Feed intake (g/d)					
Feed A	156	365	472	331	100
Feed B	191	376	536	368	111
Feed C	180	391	533	361	110
Feed D	189	355	469	338	102
Daily growth (g/d)					
Feed A	127±57	127±57	300±133	185±81	100
Feed B	164±73**	160±70**	301±144	208±95*	112
Feed C	165±90**	161±88**	297±173	207±116*	111
Feed D	141±81**	123±73	280±111	181±71	98
Feed conversion (kg feed/kg growth)					
Feed A	1.23	2.88	1.57	1.79	100
Feed B	1.16	2.35	1.78	1.77	99
Feed C	1.09	2.43	1.79	1.74	97
Feed D	1.34	2.89	1.68	1.87	104

The visual health score (not given in detail) ranged between 4 and 9 on treatment A; for the other treatments the range was 8 à 9 without marked differences.

The daily growth did not differ between treatment A and D and between B and C. The most pronounced differences were obtained in the first two weeks after weaning during which the best growth performance (plus ±30% over the control) was obtained with treatment B and C. The better results obtained with the feeds B and C (MCTG2 without or with lipase) are due to an increase of the feed intake. The best feed conversion however was obtained with the feed containing MCTG2 + lipase. The improvement of the growth using a combination of a MCFA TG (MCTG2) and a lipase was in the same range as obtained with quinoxalines (additives with both a Gram + and Gram – spectrum) (Decuypere, meta analysis of literature data, unpublished results)

Two weeks after weaning 5 barrows of each experimental group were euthanized. Because the pigs were fed ad lib. there was no control of the feed intake. After dissection of the gastrointestinal tract, samples were taken from the stomach, and the upper (duodenum) small intestine. The samples were analysed chemically and bacteriologically in the same way as explained in the previous experiment. Only the total anaerobic count is reported here.

The pH of the gastric contents was ±3.5, and ±5.7 in the duodenum; there were no differences between the treatments. The total anaerobic counts are reported in table 7.

Table 7. Total anaerobic count (log_{10} CFU / g fresh contents, ± s.d.) in the stomach and upper small intestine in piglets, 2 weeks after weaning as influenced by the different treatments. (n = 5)

Treatment	stomach	duodenum
A	7.0±0.2	6.4±0.5
B	7.0±0.2	6.1±0.8
C	5.9±0.5**	5.6±0.5**
D	6.9±0.2	5.9±0.4

The results indicate that the feed with the combination of MCFA TG (MCTG2) and lipase (L5) caused a significant ±10 fold suppression of the bacteriological load, both in the stomach and upper intestine. That the effect was somewhat lower than in the previous experiment with the gastric cannulated pigs could be due to the lower amount of MCTG used in present experiment (2.5% versus 5%) and/or the different feeding and sampling procedures. Nevertheless, the present experiment confirmed the results obtained in the cannulated pig reported in example 3. The same can be stated for the results of the analysis of the different fat fractions (g / 100 g fresh contents) and the degree of hydrolysis (g free FA / 100 g total FA) in the gastric contents which are given in table 8

Table 8. Concentrations of free FA and total FA in gastric contents (g / 100 g fresh contents, mean ± s.d.) and degree of hydrolysis (free FA / total FA in %) as influenced by the treatments in pigs two weaks after weaning (n = 5)

Treatment	A	B	C	D
Free FA	0.28±0.06	0.44±0.10	0.95±0.22	0.31±0.09
Total FA	1.05±0.08	1.25±0.22	1.35±0.28	1.07±0.17
% hydrolysis	26.7	35.2	70.4	28.9

Out of the results for the % hydrolysis it can be calculated that for feed B (MCTG2 and C (MCTG2 + L5) respectively, 0.3 and 0.4 % free MCFA are present in the stomach. In the experiment with the cannulated pigs the highest concentrations of free MCFA (and the strongest inhibition of the bacterial load, ±100 fold) were obtained with MCTG1 + L5 and coconut oil + L5, 0.60 and 0.30 % respectively.

The combined results of experiment 2 and 3 clearly indicate that there is a correlation between the amount of released free MCFA and the inhibitory effect on the gastric flora.

Experiment 4

In vitro evaluation of the optimal combination of different concentrations of MCTG with different doses of a selected lipolytic enzyme.

Because it is our opinion that growth promotion is related and proportional to the inhibition of the total bacterial load in the small intestine, the following *in vitro* experiment, in which an ± optimal combination of the content of a MCFA containing TG (MCTG1, MCTG2 and coconut oil) and a proven effective lipase (L5) was set up.

Four concentrations TG were used: 0, 2.5, 5 and 10%; for each concentration TG, the lipase (L5) was incorporated at 10.000, 1000 or 100 ppm. The medium contained also 2.5 g per incubation flask of the same synthetic feed (based on starch, dextrose, casein and a vitamin-mineral premix) as used in experiment 1 However in present experiment the TG was dispersed (using gum arabic and gum tragacanth) before adding to the medium. The incubations were done at pH 5 using an appropriate acetate buffer. Finally the medium (15 ml) was inoculated with 1 ml of a suspension of bacteria originating from the ileal contents of two canulated pigs fed a diet without growth promoting additives. Incubations were done for 180 min at 37°C in a shaking water bath. All incubations were done in duplo.

The methods for the analysis of fats and the bacterial counts were the same as used in previous experiments. Only the total anaerobic count is reported here. Because a relationship between the antibacterial acivity and the moleculair weight of the FA was expected, the results for the free fatty acids were also expressed on a molar basis. The results are given in table 9.

Table 9. Relationship between the *in vitro* release of free fatty acids (g% or moles in the medium) and the total anaerobic count ($\log_{10}$ CFU / ml medium) with different TG's (MCTG1, MCTG2 and coconut oil) and different doses (10.000, 1000 and 100 ppm) of a lipolytic enzyme (L5)

	free FA g %	free FA M	total count $\log_{10}$ CFU/ml
<u>MCTG1</u>			
Start	0	0	6.2
180 min, control	0	0	6.8
180 min, 10.000 ppm L5			
2.5% MCTG1	0.17	0.012	5.9
5% MCTG1	0.34	0.024	<1
10% MCTG1	0.63	0.044	<1
180 min, 1000 ppm L5			
2.5% MCTG1	0.11	0.008	6.1
5% MCTG1	0.20	0.014	4.8
10% MCTG1	0.39	0.027	3.8
180 min, 100 ppm L5			
2.5% MCTG1	0.09	0.006	6.5
5% MCTG1	0.13	0.009	6.5
10% MCTG1	0.22	0.015	6.2

MCTG2			
Start	0	0	6.3
180 min, control	0	0	7.0
180 min, 10.000 ppm L5			
2.5% MCTG2	0.17	0.012	5.5
5% MCTG2	0.30	0.021	3.4
10% MCTG2	0.58	0.040	1.8
180 min, 1000 ppm L5			
2.5% MCTG2	0.13	0.009	6.3
5% MCTG2	0.21	0.015	6.3
10% MCTG2	0.36	0.025	5.6
180 min, 100 ppm L5			
2.5% MCTG2	0.11	0.008	6.5
5% MCTG2	0.16	0.011	6.6
10% MCTG2	0.23	0.016	6.7
COCONUT OIL			
start	0	0	6.3
180 min, control	0	0	7.1
180 min, 10.000 ppm L5			
2.5% coc. oil	0.10	0.007	7.2
5% coc. oil	0.16	0.011	6.2
10% coc. oil	0.36	0.025	6.2
180 min, 1000 ppm L5			
2.5% coc. oil	0.07	0.005	6.4
5% coc. oil	0.13	0.009	6.5
10% coc. oil	0.22	0.015	6.4
180 min, 100 ppm L5			
2.5% coc. oil	0.05	0.003	6.9
5% coc. oil	0.08	0.006	7.0
10% coc. oil	0.13	0.009	7.0

The results can be summarized as follows:

-The amount of released FA is nearly proportional to the concentration of the TG, while a 10 fold increase of the dosis of the lipolytic enzyme used only doubled the concentration of the free FA. For each combination of a % TG and a given ppm lipolytic enzyme the release of FA follows the order: MCTG1 > MCTG2 > coconut oil.

-The higher the concentration of the free FA, the more pronounced the suppression of the number of bacteria. A minimal concentration of ±0.35 g % FA the medium looks necessary for a significant suppression of the flora; this corresponds with 0.025 M / liter. The order MCTG1 > MCTG2 > coconut oil corresponds with an increase of the moleculair weight of the quantitatively most important MCFA in the TG: MCTG1 = C8, MCTG2 = C10, coconut oil = C12.

-The used *in vitro* protocol offers an excellent tool for the screening of the numerous combinations of MCFA containing TG's and available lipolytic enzymes for their usefulness as feed supplements with a stabilizing or suppressive effect on the gastrointestinal microflora. This effect is generally accepted as the basis for obtaining a growth promotion.

Table 10. Examples of commercial sources of chemically synthesized structured lipids (1)

Product	Composition	Company
Aldo MCT	C8, C10	Lonza Inc., Fair Lawn, USA
Stabilox-860	C8, C10	Loders-Croklaan BV, Wormerveer, NL
Caprenin	C6:0, C8:0, C22:0	Proctor & Gamble, Cincinatti, OH:
Salatrim	C3:0, C4:0, C18:0	Nabisco Foods Group, East Hanover, NJ
Captex	C8:0, C10:0, C18:2	Abitec, Columbus, OH
Captex 300	C8, C10	Capital City Products, Columbus, OH
Captex 810B	C8, C18	Capital City Products, Columbus, OH
Tripelargonate	C9	Capital City Products, Columbus, OH
Mixed odd chain	C7, C9	Abbott Laboratories, North Chicago, IL
Neobee	C8:0, C10:0, LCFA	Stepan Co, Maywood, NJ
Neobee M5	C8:0, C10:0	Stepan Co, Maywood, NJ
Neobee 1095	C10:0	Stepan Co, Maywood, NJ
Coconado	C8:0	Kao Co, Wakayama, Japan
Coconard-RK	C8, C10, C12	Kao Co, Wakayama, Japan
MCTG	C4,C5,C6,C7,C8,C10	Karlshamns Lipid Specialties, Columbus, OH
MCTG	C8, C10	Mead Johnson & Co, Evansville, IN

(1) source : tested products + literature compilation

Table 11. Examples of experimental or commercially available microbial lipases (1)

Origin	Organism	Company
Yeast	Candida sp.	
	Candida rugosa*	Amano, Biocatalysts, Boehringer Mannheim Fluka, Genzyme, Sigma, Meito Sankyo
	Candida antartica A/B	Boehringer Mannheim, Novo Nordisk
	Candida lipolytica	
	Candida paralipolytica	
	Saccharomyces lipolytica	
Fungal		
	Thermomyces lanuginosus**	Novo Nordisk, Boehringer Mannheim, Amano
	Rhizomucor Miehei	Novo Nordisk, Biocatalysts, Amano
	Rhizopus sp.	Nagase, Tokyo, Japan
	Rhizopus delemar	
	Rhizopus oryzae	
	Rhizopus niveus	Alltech,
	Rhizopus arrhizus	Sigma
	Rhizopus javanicus	Amano
	Aspergillus sp.	
	Aspergillus niger	Finnfeeds International, Amano
	Aspergillus usamii	
	Aspergillus oryzae	Novo Nordisk
	Mucor sp.	
	Mucor javanicus	
	Mucor lipolyticus	
	Penicillium sp.	
	Penicillium roquefortii	Amano
	Penicillium cyclopium	Amano
	Penicillium simplissimum	
	Penicillium camembertii	
	Geotrichum candidum	Amano
	Neurospora crassa	
	Ustilago maydis	
	Fusarium solani	
Bacterial	Burkholderia cepacia***	Amano, Fluka, Boehringer Mannheim
	Pseudomonas sp.	
	Pseudomonas alcaligenes	Genencor
	Pseudomonas mendocina	Genencor
	Pseudomonas fluorescens	
	Pseudomonas aeroginosa	Amano
	Pseudomonas spp.	Finnfeeds International; Karlan, CA, USA
	Chromobacterium viscosum****	Asahi, Tokyo, Japan; Biocatalysts; Karlan, CA,USA; Toyo Jozo Shizuoka, Japan
	Staphylococcus sp.	
	Staphylococcus aureus	
	Staphylococcus carnosus	
	Staphylococcus hyicus	
	Achromobacter lipolyticum	
	Acinetobacter	
	Propionibacterium acnes	
	Bacillus sp.	

* formerly named Candida cylindracea
** formerly named Humicola lanuginosus
*** formerly named Pseudomas cepacia
**** C. viscosum is identical to Burkholderia glumae

(1) source : tested products + literature compilation

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Claims

1. A feed composition containing at least one triglyceride containing C4 to C12 medium chain fatty acids and at least one lipolytic enzyme, for use as a medicament

2. A feed composition containing at least one triglyceride containing C4 to C12 medium chain fatty acids and at least one lipolytic enzyme, for use as an antimicrobial agent.

3. A feed composition containing at least one triglyceride containing C4 to C12 medium chain fatty acids and at least one lipolytic enzyme, for preventing digestive upsets.

4. The feed composition according to any of claims 1 to 3, wherein said triglyceride is a naturally occurring triglyceride.

5. The feed composition according to any of claims 1 to 3, wherein said triglyceride is an industrially prepared triglyceride composition.

6. The feed composition according to any of claims 1 to 3, wherein said triglyceride is a mixture of naturally occurring triglycerides and industrially prepared triglycerides.

7. The feed composition according to any of claims 5 or 6, wherein said triglyceride is prepared by interesterification of C4 to C12 chain fatty acids.

8. The feed composition according to any of claims 1 to 7, wherein said lipolytic enzyme is a lipase.

9. The feed composition according to any of claims 1 to 7, wherein said lipolytic enzyme is an esterase.

10. The feed composition according to any of claims 1 to 7, wherein said lipolytic enzyme is a mixture of lipase and esterase.

11. The feed composition according to any of claims 1 to 10, wherein said triglyceride is present in a naturally occurring or industrially prepared medium chain fatty acids containing triglyceride composition and said lipolytic enzyme is present in a commercially available lipolytic enzyme composition.

12. The feed composition according to any of claims 1 to 11, wherein said triglyceride component is added in a concentration ranging from 0.25% to 10% to the feed and said lipolytic enzyme component is added in a concentration ranging from 100 to 10.000 ppm, to the feed.

13. Use of a feed composition according to any of claims 1, 4 to 12, for the preparation of a medicament for prophylactic or therapeutic treatment of growth impairment.

14. Use of the feed composition of any one of claims 1 to 12 to formulate a feed, comprising 1 to 5% of the feed composition, so that the feed comprises at least one triglyceride containing medium chain fatty acids and at least one exogenous lipolytic enzyme.

15. Use of the feed composition of any one of claims 1 to 12 to prepare a feed suitable for production or companion animals.

16. Use according to claim 15, wherein the animals are early weaned piglets.

Fig. 1. In Vitro release of MCFA (g / 100g TG) at different pH's from the different triglycerides tested

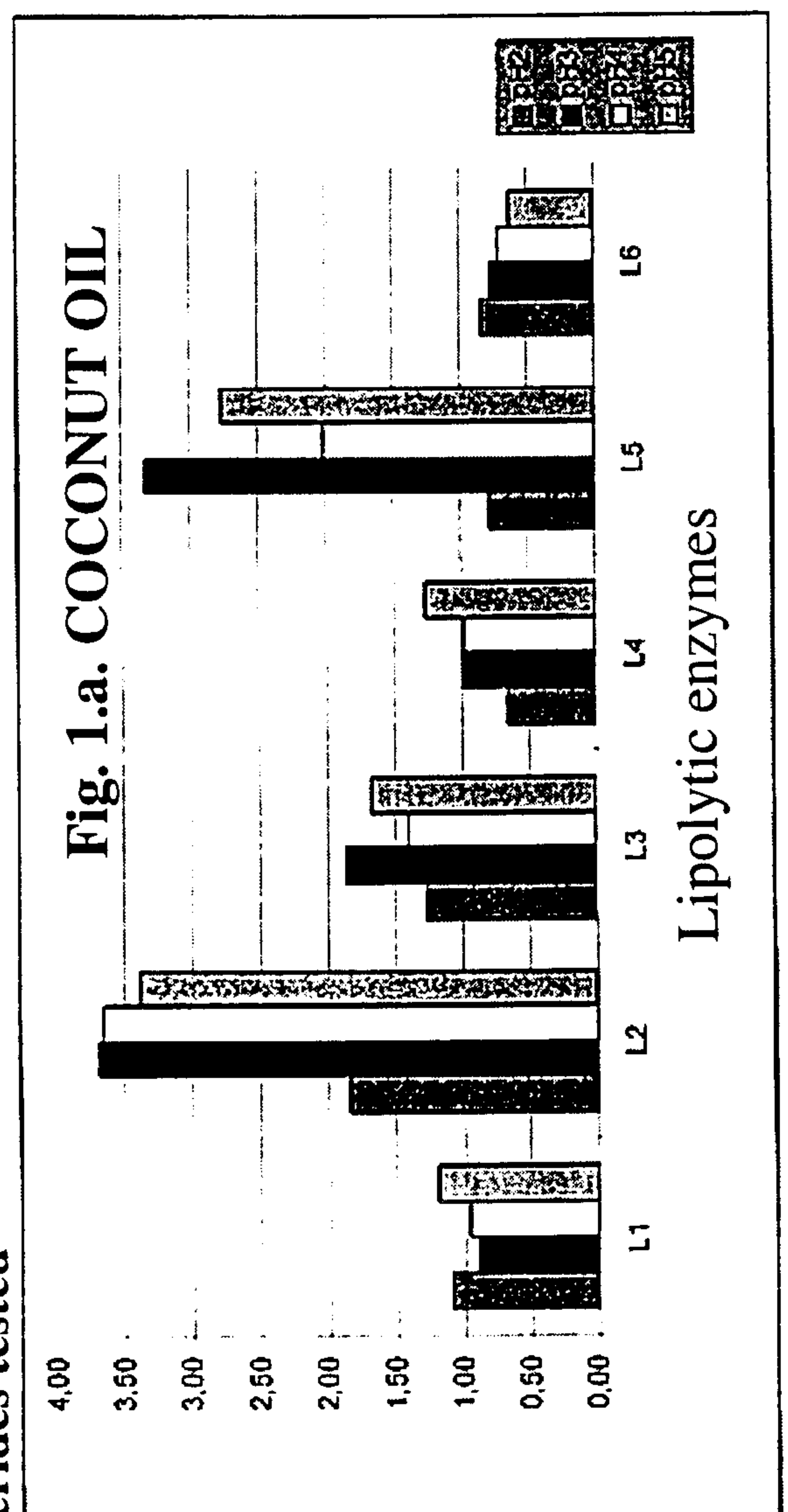


Fig. 1.a. COCONUT OIL

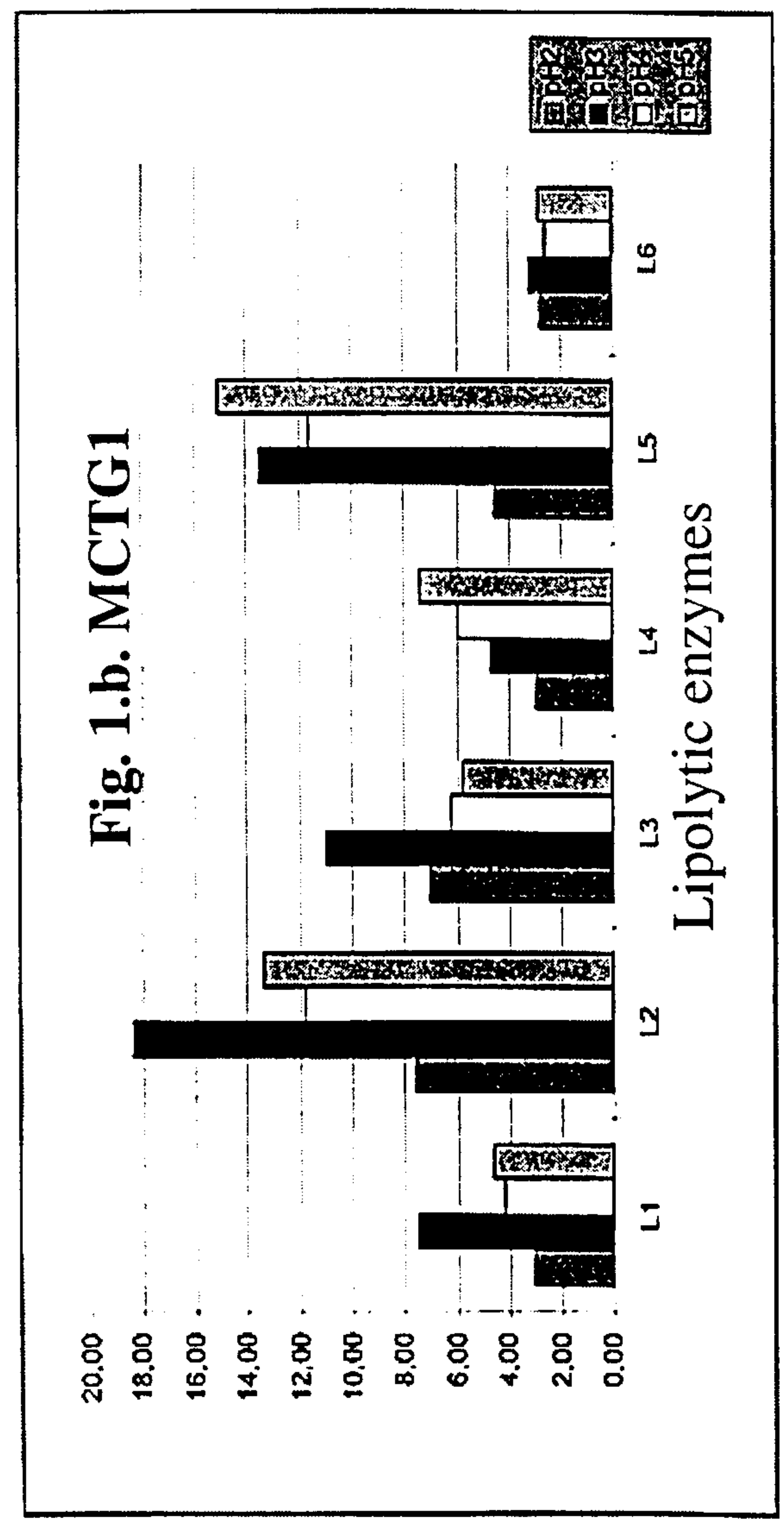
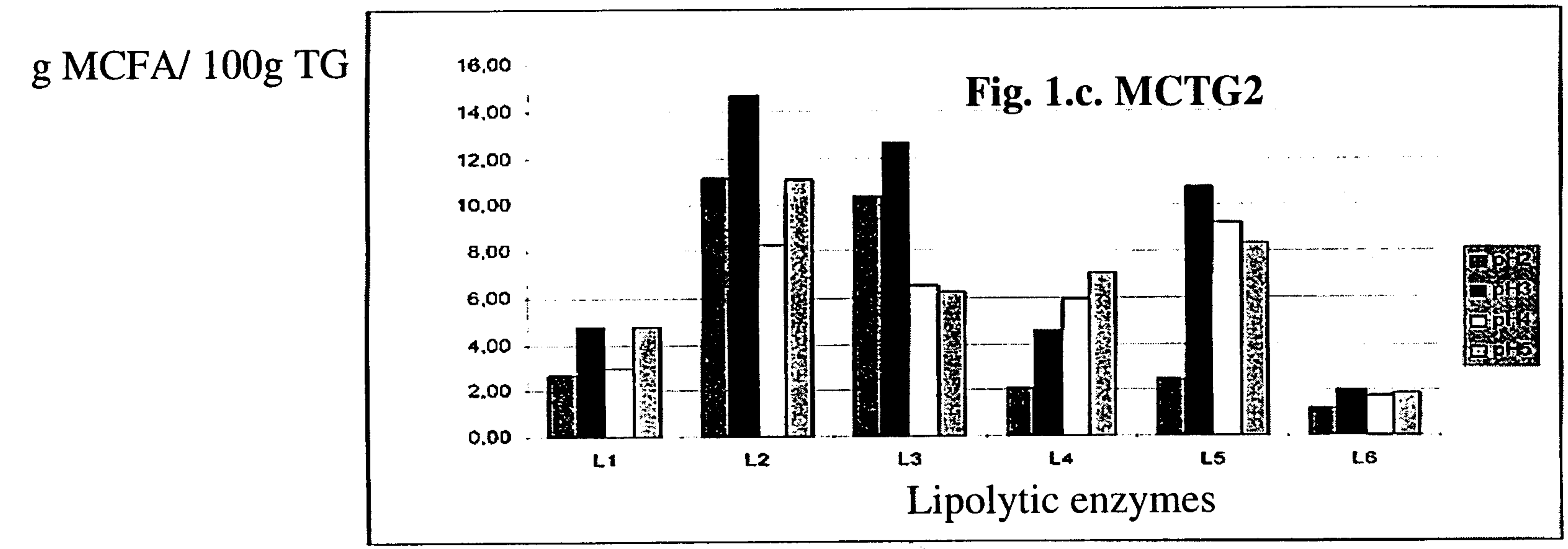


Fig. 1.b. MCTG1

Fig. 1. (followed) In Vitro release of MCFA (g / 100g TG) at different pH's from the different triglycerides tested



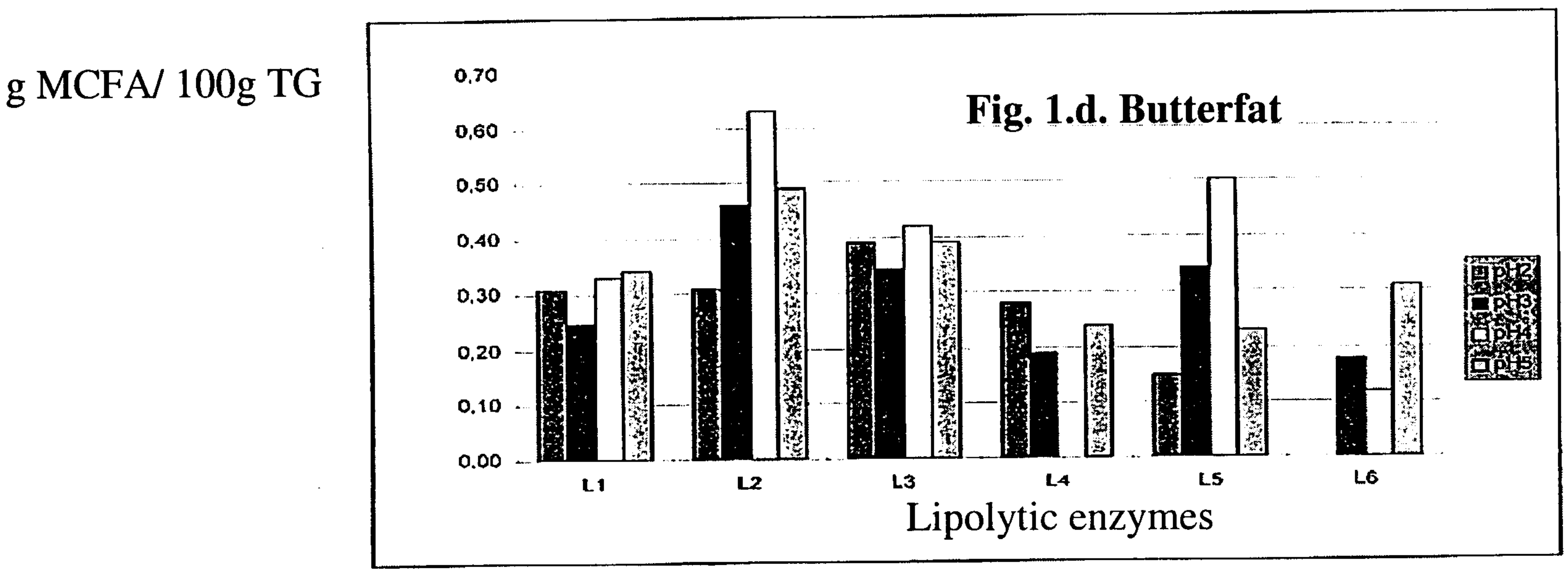


Fig. 2. In Vivo bacterial counts in the stomach contents of cannulated piglets fed three different TG and two lipases

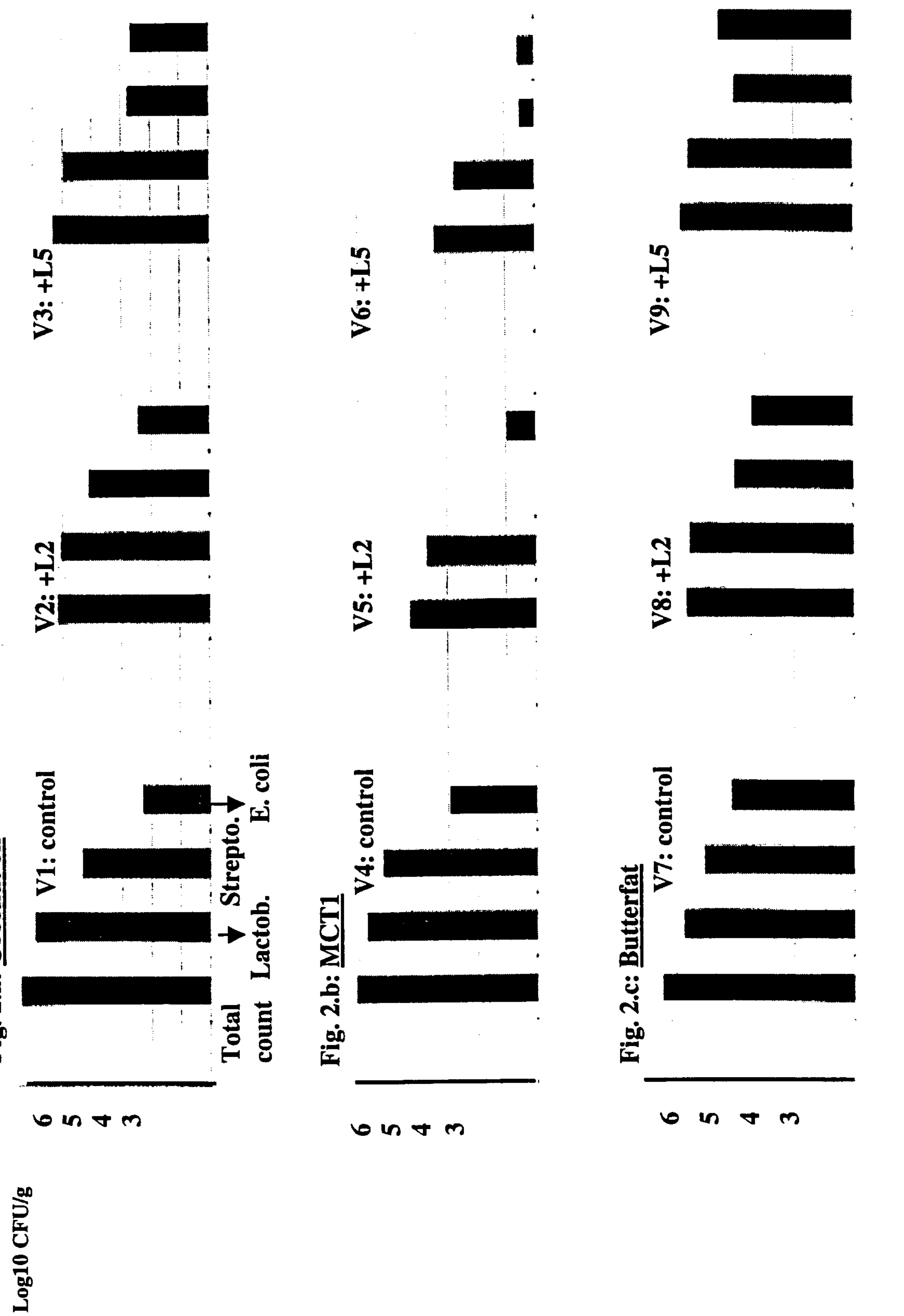


Fig. 3. In Vivo concentration of total, TG-bound and free fatty acids (FFA) in stomach contents of cannulated pigs fed three TG and two lipases