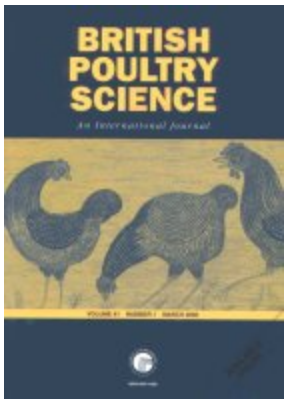

Lysolecithin, but not lecithin, improves nutrient digestibility and growth rates in young broilers

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Keywords:	Broilers, Fats and fatty acids, Lecithin, Lysolecithin, Performance, Digestibility

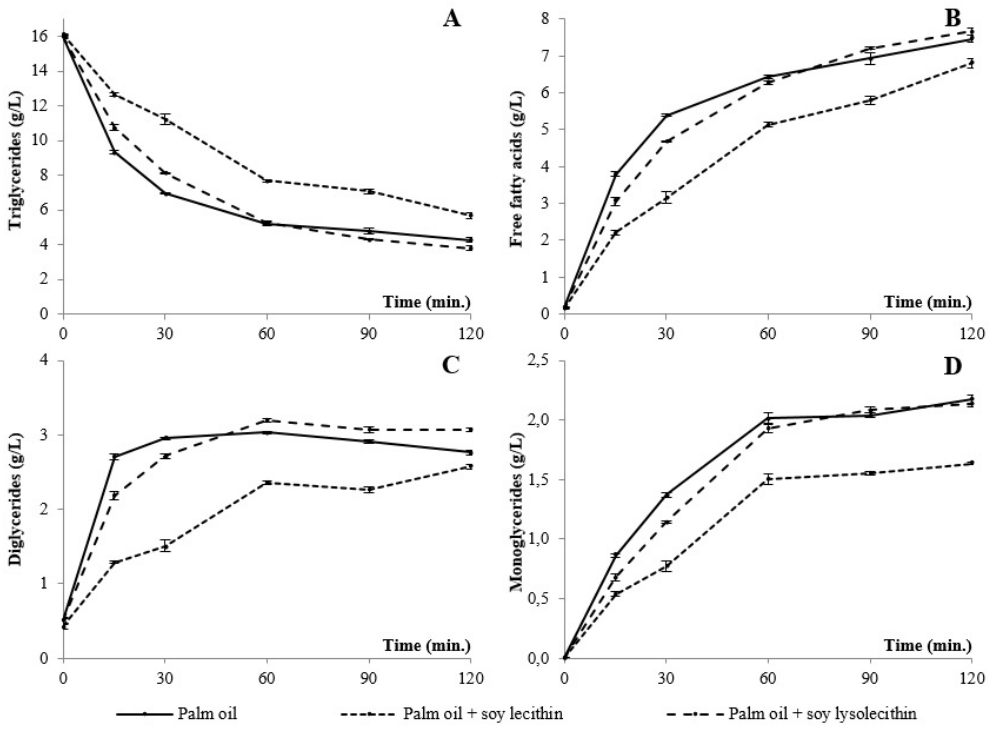

Figure 1. Hydrolysis of (A) triglycerides and accumulation of (B) free fatty acids, (C) diglycerides and (D) monoglycerides during the in vitro hydrolysis of palm oil (solid line), palm oil with soybean lecithin (dotted line) and palm oil with soybean lysolecithin (dashed line). The experimental treatments were carried out in triplicate. The mean concentrations of the lipids (mg/ml) are given over time (min), with error bars indicating the standard error values. (Experiment 1).

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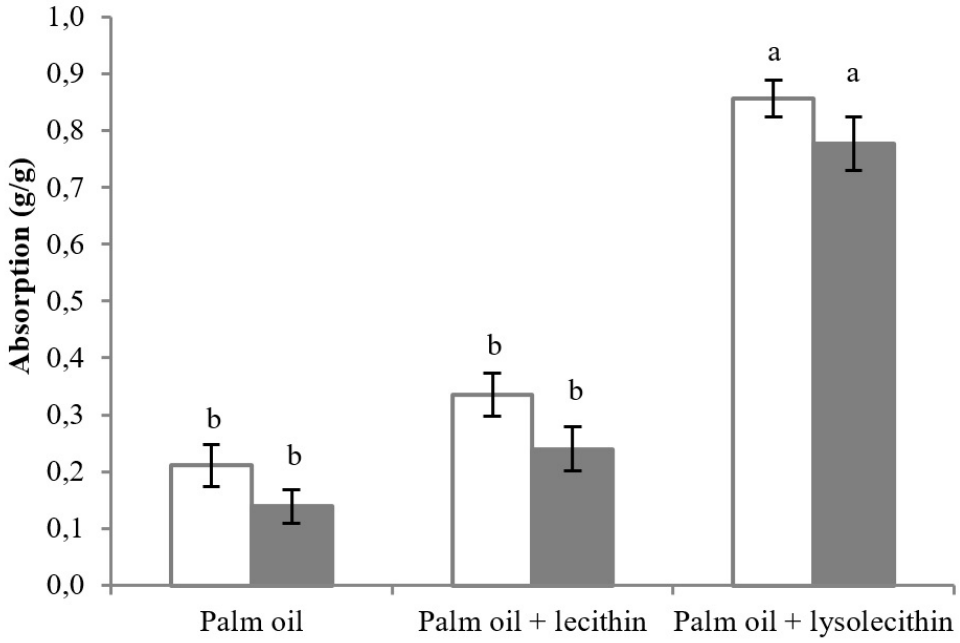

Figure 2. Absorption of monoglycerides (white bars) and free fatty acids (grey bars) generated during in vitro hydrolysis of palm oil, palm oil with soybean lecithin and palm oil with soybean lysolecithin by differentiated Caco-2 monolayers and expressed as percentage of applied monoglycerides end free fatty acids. Data are means of three or more observations per treatment, with error bars indicating the standard error values. (Experiment 1).

201x134mm (120 x 120 DPI)

Supplemental Information Table 1. Fatty acid composition (g/kg) in palm oil and the ether extract of the experimental starter diet (0-8 days of age) formulated with palm oil

Fatty acid[a]	Palm oil	Diet[b]
C14:0	10.1	4.7
C16:0	429.0	306.0
C18:0	45.7	37.2
C18:1 (ω-9)	401.6	339.3
C18:2 (ω-6)	102.1	289.4
C18:3 (ω-3)	3.8	11.3
Total unsaturated fatty acids	507.5	643.8
Total saturated fatty acids	492.4	356.2
Ratio unsaturated/saturated	1.03	1.81

[a] Fatty acids present at levels below 5 g/kg in all samples are not listed.

[b] Values represent the mean of the three experimental diets with standard error of the means of 0.1, 0.1, 0.4, 2.3, 1.6 and 0.2 g/kg for C14:0, C16:0, C18:0, C18:1, C18:2 and C18:3, respectively.

Supplemental Information Table 2. Phospho- and lysophospholipid contents[1] (g/kg) of soybean lecithin and lysolecithin

(Lyso)phospholipid	Soybean lecithin	Soybean lysolecithin
Phosphatidylcholine	125.3	90.4
Lysophosphatidylcholine	7.8	39.2
Phosphatidylinositol	88.0	81.9
Lysophosphatidylinositol	ND	13.0
Phosphatidylethanolamine	97.2	33.6
Lysophosphatidylethanolamine	3.3	40.4
Phosphatidic acid	72.2	13.8
Lysophosphatidic acid	3.0	26.2
Total phospholipids	382.7	219.7
Total lysophospholipids	14.1	118.8

[1] Contents of non-phospholipid compounds such as triglycerides, glycolipids, water and minerals are not presented.

Supplemental Information Table 3. Levels of moisture, impurities and unsaponifiables, and free fatty acids, U:S ratio, and predicted AME values for broilers <21 days in commercial oil samples used to make experimental diets (Experiment 3)

Oil Type	MIU, %	FFA, %	U:S ratio	AME, MJ/kg, <21 days	AME, MJ/kg, >21 days
Lecithin	3.70	24.8	3.7	32.30	34.31
Lecithin	3.60	24.6	4.1	32.72	34.56
Lecithin	3.50	18.7	3.7	32.89	34.73
Soybean	0.70	1.9	4.8	36.57	37.40
Soybean	--[1]	1.9	4.7	36.57	37.66

[1]Below limit of detection

Lysolecithin, but not lecithin, improves nutrient digestibility and growth rates in young broilers

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ABSTRACT

Young broilers have an underdeveloped ability for lipid digestion. The potential of lecithin and lysolecithin to improve lipid digestion and growth performance was investigated in 3 experiments: an *in vitro* model that mimics the intestinal conditions of the chick, a digestibility trial with chicks (5 to 7 days of age), and a performance trial until 21 days of age. In Experiment 1, palm oil (PO), palm oil with lecithin (PO+L), and palm oil with lysolecithin (PO+LY) were subjected to *in vitro* hydrolysis and applied to Caco-2 monolayers to assess lipid absorption. The *in vitro* hydrolysis rate of triglycerides was higher in PO+LY (k= 11.76 × 10-3 min-1) than in either PO (k= 9.73 × 10 3 min 1) or PO+L (k= 8.41 × 10 3 min 1), and the absorption of monoglycerides and free fatty acids was highest (P<0.01) for PO+LY. In Experiment 2, 90 broilers were assigned to three dietary treatments: a basal diet with 4% palm oil, and the basal diet supplemented with either 250 ppm lecithin or lysolecithin. ATTD of crude fat was higher in broilers supplemented with lysolecithin, but was lower in broilers supplemented with lecithin. DM digestibility and AMEn in birds supplemented with lysolecithin were significantly higher (3.03% and 0.47 MJ/kg, respectively). In Experiment 3, 480 broilers were randomly allocated to four dietary treatments: basal diet with soybean oil (2%), basal diet with lecithin (2%), soybean oil diet with 250 ppm lysolecithin, or lecithin oil diet with 250 ppm lysolecithin. Lecithin diets significantly reduced weight at day 10 and 21 compared with soybean oil. However, the addition of lysolecithin to lecithin-containing diets significantly improved bird performance. The results of these studies show that, in contrast to lecithin, lysolecithin is able to significantly improve the digestibility and energy values of feed in young broilers.

KEYWORDS: broiler; digestibility; fat; lecithin; lysolecithin; performance

INTRODUCTION

After hatch, lipid digestion in young birds is physiologically limited, and is a target for improvement and support. Roy et al. (2010) have ascribed the inefficient digestion and absorption of lipids by young chickens to a combination of a low duodenal secretion of lipase, a low level of lipase activity and a low rate of bile salt synthesis. Kroghdahl (1985) and Maiorka et al. (2004) showed, however, that lipase concentration and activity in young chickens (7 days of age) are modulated according to the inclusion level of fats in the diet. Moreover, in a study of Meng et al. (2004), lipase addition at 0.2 g/kg did not affect the apparent fat digestibility in broilers of one to 14 days of age. Therefore, the inability to utilize fats has been attributed to low bile salt concentrations in the intestines, rather than to deficiencies in lipase secretion or activity (Maiorka et al., 2004; Maisonnier et al., 2003). Although dietary supplementation of bile salt has been shown to improve lipid utilization in chickens (Krogdahl, 1985; Polin et al 1980), supplementation is generally not applied on a commercial scale due to economic drawbacks (Roy et al., 2010).

One strategy for improvement of fat digestion is the dietary application of molecules with proven ability to improve lipid digestion, for example lecithin and its derivative lysolecithin. Huang et al. (2007) showed that in diets supplemented with 2% of soybean oil, replacement of 25% of the soybean oil with soybean lecithin (0.5% lecithin in the diet) improved crude fat digestion and performance in broilers. On the other hand, complete replacement of the soybean oil with lecithin (2% lecithin in the diet) resulted in adverse effects. Moreover, in a study of Blanch et al. (1996) the AME of a basal diet with tallow as the main fat source was not improved by the addition of 0.2% soybean lecithin.

Lysolecithins are produced by phospholipase to cleave one hydrophobic fatty acid from phospholipids (Joshi et al., 2006). This changes the stereochemical structure of the phospholipids in the lecithin into lysophospholipids. The resulting mixture, lysolecithin, has

an increased hydrophilic-lipophilic balance (Van Nieuwenhuyzen and Tomás, 2008) and lowere critical micelle concentration (0.02 to 0.2 mM/L). Both phospho- and lysophospholipids consist of a hydrophilic head group (phosphatidyl substituent) and a hydrophobic tail (fatty acid chains). But, due to the removal of one fatty acid, lysophospholipids are more hydrophilic and thus have better oil-in-water emulsifying properties than phospholipids (Joshi et al., 2006; Liu and Ma, 2011). In the animal, the pancreas secretes native phospholipase (EC 3.1.1.4) to convert the phospholipids secreted by the gall bladder into lysophospholipids (Karray et al., 2011). These lysophospholipids were shown to play an important role in mixed micelle formation (Lo and Tso, 2009). Therefore, it is hypothesized that lysolecithin possess a greater ability to improve the digestion of fats and oils than lecithin. Inclusion of 3.2% of rice bran lysolecithin in a broiler feed formulated with rice bran oil increased the crude fat digestibility (Raju et al., 2011). Additionally, Zhang et al. (2011) observed an increased fatty acid digestibility in broilers using 0.125% of soybean lysolecithin on soybean oil, tallow and poultry fat. These improvements in digestibility consistently lead to improved growth performance and efficiency in growing broilers (Wealleans et al., 2019), as well as in other species (Wang et al., 2019; Zhao et al., 2017; Papadopoulos et al., 2014).

The limited comparative studies available on lecithin and lysolecithin application in broiler diets have, however, all been performed with birds of 14 days of age or older. Therefore, the potential of both lecithin and lysolecithin from soybean to improve lipid digestion was investigated in 3 experiments: an *in vitro* model, a digestibility trial with young broilers (5 to 7 days of age), and a performance trial until 21 days of age.

METHODS AND MATERIALS

Experiment 1: In Vitro Fat Absorption

Crude palm oil (single batch) was sourced from a commercial feed mill in Belgium. The FA composition of the palm oil was analysed (ISO, 2002, 17764). Soybean lecithin and soybean lysolecithin were sourced from Kemin Europa NV (Herentals, Belgium). Lecithin and lysolecithin were analysed for their phospho- and lysophospholipid content by phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR, Spectral Service AG, Cologne, Germany).

The lipid hydrolysis model previously employed by Jansen et al. (2015) was slightly modified. Briefly, 1.00 g of lecithin or lysolecithin were first dispersed into 49.00 g of palm oil. Fasted state simulated intestinal fluid (FaSSIF) was prepared by adding 2.24 g of FaSSIF powder (Biorelevant.com Ltd, Croydon, United Kingdom) to 1 L of phosphate buffer (35 mM, pH 6.5) containing 106 mM NaCl. According to the manufacturer, the FaSSIF contained 3 mM bile salt (sodium taurocholate). Aliquots of 0.25 g of each of the respective fat treatments and 14.75 ml of FaSSIF were added into 50 ml centrifuge tubes. The content of each tube was mixed for 30 seconds with a high shear mixer (24000 rpm; IKA ultra-turrax T18, Staufen, Germany). Next, 24 mg of pancreatin (P7545, Sigma Aldrich) was added to each tube and they were incubated for two hours at 40 °C while shaking (250 rpm). The final contents in the digests were 106 mM NaCl, 1.6 g/L pancreatin, 1.6 g/L bile salts and 16.7 g/L palm oil. At 0, 15, 30, 60, 90 and 120 minutes of incubation, a 0.5 ml sample of each digest was taken and diluted in 9.5 ml tetrahydrofuran (HPLC grade, VWR International, Leuven, Belgium) to inactivate the enzymes and prepare the appropriate dilution for lipid analysis. Each digestion was performed in triplicate. Samples of the digests were submerged in liquid nitrogen and stored at -180 °C till absorption experiments started.

Human colonic adenocarcinoma cells (Caco-2) were obtained from the European Collection of Cell Cultures (Public Health England, Porton Down, Salisbury, UK). Caco-2 cell work stock was used between passages 54 and 60. Cells were cultured in Dulbecco's modified

eagle medium supplemented with 100 ml/L heat-inactivated fetal bovine serum (Hyclone, Thermo scientific, Leuven, Belgium), 10 ml/L non-essential amino acids, 100 U/ml of penicillin and 100 U/ml of streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and routinely passaged. Unless stated otherwise, the cell culture media and supplements were provided by Westburg (Leusden, The Netherlands).

Caco-2 cells were seeded on collagen-coated Transwell-COL inserts (1.12 cm², pore size 0.4 µm, Corning Costar Corporation, Cambridge, MA) in 24-well plates at a density of 2 x 10^5 cells per insert and incubated for 21 days to allow the cells to differentiate. During incubation the medium (apical and basal) was changed three times a week and the trans-epithelial electrical resistance was monitored (Millicell-ERS, Millipore, Overijse, Belgium). Next, the different digests obtained with the lipid hydrolysis model were diluted 25-fold in FaSSIF and applied at the apical side of the monolayer. Simultaneously, Hank's balanced salt solution was applied at the basal side of the monolayer. The digest concentration and the differentiation protocol were optimized during the development of the model. Similar to Vors et al. (2012), a 25-fold dilution of the digests was selected to avoid toxicity while still presenting sufficient monoglycerides (MG) and free fatty acids (FFA) to the monolayers. At the start and after 60 minutes of incubation, a sample of the apical fluid was taken and diluted twofold in tetrahydrofuran. Each absorption experiment performed in at least three replicates.

In each sample obtained during the *in vitro* lipid digestion, the degree of lipid hydrolysis was analyzed by HPLC. The lipids were separated into triglycerides (TG), diglycerides (DG), MG and FFA by a gel permeation column (PL 1110-6520, 5 µm 100A 300 x 7.5 mm, Agilent Technologies, Diegem, Belgium) and detected by an Evaporative Light Scattering Detector (ELSD 85, VWR International). tetrahydrofuran was used as the mobile phase at a flow rate of 0.5 ml/min. Likewise, samples obtained with the lipid absorption model were analysed for their MG and FFA content.

The hydrolysis of palm oil at each sample time was calculated and the apparent rate constant for TG hydrolysis and FFA, MG and DG release were determined as described by Jansen *et al*. (2015). The absorption of MG (g/g) in each well was calculated as follows:

$$\text{MG absorption} = \frac{MG_0 - MG_{60}}{MG_0}$$

where MG_0 and MG_{60} are the respective MG contents (g/L) before and after 60 minutes of incubation. Correspondingly, FFA absorption (g/g) was calculated from the respective FFA contents.

For statistical comparison of the in vitro hydrolysis, the apparent rate constants for TG hydrolysis and FFA, MG and DG release and the MG and FFA absorption were analysed as a one factorial arrangement. Analysis of variance (ANOVA) of the experimental treatments was done with STATGRAPHICS Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA), and means were separated by the least significant differences procedure. All statements of significance were based on a P-value equal to or less than 0.05.

Experiment 2: Digestibility Trial

A broiler digestibility trial was performed at the experimental research facility of the Laboratory of Livestock Physiology (Leuven, Belgium). The experiments were conducted in strict accordance with the European Communities Council Directive (2003/65/EC) and were approved by the Ethical Commission for Experimental Use of Animals of the KU Leuven (P213/2015). A total of 90 one-day-old male Ross 308 chickens were obtained from Belgabroed NV (Merksplas, Belgium) and assigned randomly at day zero, in groups of ten birds, to three dietary treatments: a basal diet without lecithin and lysolecithin, the basal diet supplemented with 250 ppm lecithin and the basal diet supplemented with 250 ppm

lysolecithin. Lecithin and lysolecithin were applied to diets at the same rate in order to allow direct comparison of the effects of phospholipids and lysophospholipids.

To prepare the experimental diet (Table 2), first all raw materials were milled together to obtain homogeneous batches. Next, the feed was divided into three batches and successively mixed in a small mixer with different premixes in order to produce the three experimental diets. Titanium dioxide (E171 titanium dioxide, IMCD Benelux N.V., Mechelen, Belgium) was added to all diets as an indigestible marker at an inclusion rate of 3 g/kg. All diets were fed as mash diets and also contained a commercial enzyme blend (KEMZYME Plus Concentrate Dry 50 ppm, Kemin Europa NV) and phytase (RONOZYME P-(CT) 100 ppm, DSM Nutritional products, Deinze, Belgium).

Birds were housed in nine digestibility cages (three replicates per treatment), consisting of a wire bottom with a plastic tray for excreta collection, two feed troughs and a drinking cup. The facility was kept under conventional EU conditions for lighting, heating and ventilation. The trial consisted of a pre-period of six days to minimize interference of egg yolk digestion (days 0 to 5) and a 48-hour collection period (days 5 to 7). Drinking water and feed were provided *ad libitum.* During the collection period, total excreta were collected, and pooled and homogeneous samples of the mixed wet excreta were freeze-dried and stored until analysis.

The titanium dioxide was determined using the method of Short et al. (1996) with modifications according to Myers et al. (2004). Samples of the feed and freeze-dried excreta were analyzed in the accredited laboratory of the Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium) for dry matter (DM), gross energy (GE), crude protein and crude fat according to EC (1971), ISO (1998, 9831), ISO (2005, 5983-2, N × 6.25) and ISO (1999, 6492), respectively. Additionally, the FA distribution of the ether extracts of the diet and excreta samples were determined (ISO, 2002, 17764).

The coefficient of total tract apparent digestibility (CTTAD) of DM, crude protein and crude fat were determined by the use of the concentrations of titanium dioxide in the excreta and in the feed and calculated as described by Jansen et al. (2015). For the titanium dioxide in the feed a single value, averaged over the diets, was used for all calculations. Average diet titanium recovery was within acceptable limits for all diets. The coefficients of total tract apparent digestibility of individual fatty acids (CTTADF) were calculated as follows:

$$CTTADF = \frac{\left[FA_{diet} - \left(FA_{excreta} \times \left(1 - CTTAD\ of\ \ crude\ fat\right)\right)\right]}{\left[FA_{diet}\right]}$$

where FA_{diet} and $FA_{excreta}$ are the respective FA contents (g/kg) analyzed in the ether extracts of the diet and excreta samples. The AME contents of the experimental diets were calculated from their respective titanium dioxide ratios and corresponding GE contents, as described by Jansen et al. (2015).

The values for CTTAD of DM and crude fat, CTTADF, N-retention and AMEn were analysed as a one factorial arrangement and subjected to ANOVA with STATGRAPHICS Centurion XVI software (Statpoint Technologies Inc.). Repeated measures techniques were not used. Means were separated by the least significant differences procedure. All statements of significance were based on a P-value equal to or less than 0.05.

Experiment 3: Performance Trial

All experimental procedures were conducted in strict accordance with the European Communities Council Directive (2003/65/EC) and were approved by the Ethics Committee of Nottingham Trent University.

Birds were sourced from PD Hook Cote hatchery, Oxford. Birds were feather sexed on day of hatch and collected by Nottingham Trent University (NTU) personnel to reduce travel stress. A total of 480-day-old male Ross 308 chicks were individually weighed before random

allocation to 80*80 cm mesh sided pens bedded on clean wood shavings. Unhealthy or unusually sized chicks were discarded from the trial upon arrival. Birds were individually weighed and only birds between 33 and 46 g were placed.

Birds were allocated to four dietary treatments in a 2 x 2 factorial design. Treatments were: basal diet with soybean oil (2%), basal diet with lecithin oil (2%), basal diet with soybean oil with 250 ppm lysolecithin, or basal diet with lecithin oil with 250 ppm lysolecithin. Compared to Experiment 2, where dietary fat type and level were used to create challenging diets that could elucidate clearly the mode of action of lysolecithin, in Experiment 3 the basal diets were formulated to reflect relevant commercial compositions and to meet all nutrient requirements of the birds (Table 5). The lecithin-based oil used in this study is commercially available as Leciol (Adams and Green, East Yorkshire, UK). The lysolecithin was supplemented as LYSOFORTE® EXTEND (Kemin Animal Nutrition and Health, Belgium). Each treatment consisted of 12 pens, with 10 birds per pen. Diets were manufactured by Target Feeds (Whitchurch, UK) and supplied bagged as crumb for both starter and grower. Feed and water were available *ad libitum*, with care taken to ensure the birds ate and drank as soon as possible after placement.

The lighting regimen was maintained in accordance with commercial practice with 15 minutes of dusk at the start and end of each dark period. Temperature was set at 31 °C on day 1 and reduced by approximately 1 °C per day until 21 °C was reached.

Bird observations were used to monitor the environment and if the birds appeared uncomfortable, the temperature and/or ventilation was altered accordingly. Birds were observed twice daily during the trial and any observations related to health recorded in a trial diary. Any dead birds were weighed, and reasons recorded if culled. Birds were weighed by pen on arrival, on day 10 and at the end of the trial on day 21. Initially, individual weighed bags of feed were prepared containing precisely weighed feed quantities for each phase. Each

pen of birds was fed from their designated bag for each phase. Extra feed was added to the bags as required and the quantity recorded. Total feed eaten was calculated as the difference between remaining feed in the bag and the amount weighed into the bag for each phase.

Energy conversion efficiency (ME MJ/kg gain) was calculated according to the following formula, as per Salah et al. (2004):

$$Calorie\ conversion = \frac{Total\ Feed\ Consumption\ \times Energy\ content\ of\ feed}{Sum\ weight\ of\ all\ birds\ at\ trial\ end}$$

Calorie conversion was calculated using the calculated ME contents on the feed, using both on the literature energy value of the two fat sources, as claimed by the manufacturers, and with the energy value as determined by the Wiseman equation (Wiseman et al., 1991).

Statistical analysis was carried out using SPSS v.24. After KS testing to confirm normality, data were analysed using one-way ANOVA to investigate the effect of dietary treatment on FCR, feed intake (per bird) and individual bodyweight gain for each weigh period of the study, and cumulatively. Where appropriate, Bonferroni post hoc testing was used to elucidate differences between diets/treatments.

RESULTS

Experiment 1: In Vitro Fat Absorption

In Experiment 1, the palm oil contained a high amount of saturated FA (492.4 g/kg), especially palmitic acid (429.0 g/kg), which was largely reflected in the FA in the ether extract of the diet (Supplemental Information Table 1). The total lysophospholipid fraction was confirmed to be much higher for soybean lysolecithin than for soybean lecithin (118.8 g/kg lysolecithin vs 14.1 lecithin; Supplemental Information Table 2). The majority of lysophospholipids in soybean lysolecithin were lysophosphatidylcholine (LPC) and lysophosphatidyl-ethanolamine.

The hydrolysis of TG and the accumulation of FFA, DG and MG during the *in vitro* hydrolysis of palm oil, palm oil with lecithin and palm oil with lysolecithin are shown in Figure 1. Over the whole incubation period of 120 min, the amounts of TG hydrolysed and FFA, DG and MG accumulated in palm oil with lecithin are markedly lower than those of palm oil and palm oil with lysolecithin. During the first 60 min of incubation, the amounts of TG hydrolysed and FFA and MG accumulated were slightly higher in palm oil without lysolecithin or lecithin than in palm oil with lysolecithin. After 120 min, however, the amounts of TG hydrolysed and FFA and DG accumulated was slightly higher in palm oil with lysolecithin than in palm oil without lysolecithin.

A comparison of the apparent first-order rate constants for TG hydrolysis and the accumulation of FFA, DG and MG for each treatment is presented in Table 1. Addition of lecithin or lysolecithin to the palm oil had significant ($P < 0.01$) impact on the rates of TG hydrolysis and FFA, DG and MG release. TG were hydrolysed faster when lysolecithin was added to the palm oil (k = 11.76 × 10-3 min-1) compared to palm oil without (lyso)lecithin (k = 9.73 × 10 3 min 1). In contrast, TG were hydrolysed slower when lecithin was added (k = 8.41 × 10 3 min 1). Similarly, the release of monoglycerides was the fastest in palm oil with lysolecithin (k = 3.07 × 10 3 min 1) and the slowest in palm oil with lecithin (k = 2.23 × 10 3 min 1). In contrast, DG release was the slowest in palm oil without lecithin or lysolecithin. There was no statistically significant difference observed in the rate of free fatty acid release between palm oil and palm oil with lecithin.

The absorption of MG and FFA generated during *in vitro* hydrolysis of palm oil, palm oil with lecithin and palm oil with lysolecithin is presented in Figure 2. The absorption of monoglycerides was significantly higher ($P < 0.01$) for palm oil with lysolecithin (85.6%) than for palm oil (21.1%) and palm oil with lecithin (35.5%). The overall absorption of FFA was slightly lower ($P > 0.1$) than that of MG. Nevertheless, the absorption of FFA was significantly

higher (P < 0.01) for palm oil with lysolecithin (77.8%) than for palm oil (13.9%) and palm oil with lecithin (24.0%).

Experiment 2: Digestibility Trial

For Experiment 2, during the collection period, the observed feed intake values were similar for all treatments with an average of 30.6 g per bird per day. Likewise, bodyweight gain values were similar for all treatments with an average of 26.35 g per bird per day. Apparent faecal digestibility values and AMEn are presented in Table 3.

There were significant differences (P<0.05) between the treatments on all parameters investigated. Compared to the basal diet, lysolecithin supplementation increased (P<0.01) the DM digestibility by 1.04%, CF digestibility by 2.25% and AMEn by 0.47 MJ/kg. On the other hand, supplementation of lecithin had no effect on dry matter digestibility or AMEn, but reduced (P<0.01) the crude fat digestibility by 3.2%. When compared to the basal diet, N-retention was not significantly affected by lecithin or lysolecithin supplementation. However, the N-retention of the basal diet supplemented with lysolecithin (27.75 g/kg DM) was higher (P<0.05) than that of the basal diet supplemented with lecithin (25.26 g/kg DM). The fatty acid distribution in the ether extracts of the faeces is presented in Table 4. There was no difference (P>0.05) between any of the dietary treatments in the fatty acid distribution in the faeces, nor in the ratio of unsaturated over saturated fatty acids.

Experiment 3: Performance Trial

Table 6 shows the performance of the birds across the whole experimental period of Experiment 3. During the starter phase, days 0-10, there were no significant differences in average daily gain, feed intake or FCR between the treatment groups. But, birds fed the diet with lecithin-based oil supplemented with lysolecithin were significantly heavier than those

fed the diet with lecithin-based oil alone. During the grower phase there was a significant difference in bird weight. Birds fed the diet with lecithin-based oil alone were significantly lighter in weight when compared to the birds fed the diet with lecithin-based oil supplemented with lysolecithin (p=0.047). Similarly, when the whole trial phase was evaluated there were significant differences in average daily gain. Birds fed diets with lecithin-based oil alone gained less on average daily (p=0.036) than the birds fed the diet with lecithin-based oil supplemented with lysolecithin (p=0.036).

The energetic values of each oil determined through analysis of chemical composition (Supplemental Information Table 3) demonstrated that, due to high levels of free fatty acids in the oils and an altered unsaturated: saturated ratio (Wiseman et al., 1991), the lecithin oil had a reduced predicted AME value for broilers <21 days compared to the soybean oil (36.57 MJ/kg soybean vs 32.64 MJ/kg lecithin). When calculating diet density based on the lipid analysis, therefore, rather than assumed energetic equivalence between the two oil sources, the effective energy conversion rate for soybean oil alone was 16.04 MJ/kg BWG, while the effective energy conversion rate for lecithin oil alone was worse, at 16.78 MJ/kg BWG. With the addition of lysolecithin, the energy conversion rate was improved across both fat sources (15.58 MJ/kg BWG soybean oil + lysolecithin; 16.23 MJ/kg BWG lecithin plus lysolecithin); the effective efficiency of use of the lecithin oil was brought closer to that of unsupplemented soya oil.

DISCUSSION

The structural differences in the composition of lecithin and lysolecithin leads to fundamentally diverse effects on both *in vitro* and *in vivo* lipid digestion. In Experiment 1, compared to palm oil without lecithin or lysolecithin, *in vitro* the apparent rate constants for triglyceride hydrolysis and monoglyceride release were significantly higher for palm oil supplemented with lysolecithin and significantly lower for palm oil supplemented with

soybean lecithin (Figures 1 and 2). Likewise, in Experiment 2, compared to the basal diet, the crude fat digestibility was significantly higher in broilers supplemented with lysolecithin, while it was significantly lower in broilers supplemented with lecithin. Though the limitations of this initial study require further data to confirm these findings, Zhang et al. (2011) postulated that while both lecithin and lysolecithin may act as an emulsifier within the first stages of lipid digestion, for lipid hydrolysis to take place, the pancreatic colipase-lipase complex first must adsorb onto the emulsion droplets (Reis et al., 2010). The adsorption and activity of lipase at the oil-water interface, however, is affected by various surface-active compounds such as phospholipids and lysophospholipids (Reis et al., 2010, 2009).

Possibly, the observed effect of lecithin on crude fat digestion in young broilers in Experiment 2 could be explained by a study of Chu et al. (2010) who showed that colipase and lipase adsorbed exclusively onto regions covered by phosphatidylcholine and bile salts and not to regions covered with phosphatidylcholine solely. This would suggest that, although phospholipids in lecithin may have aided the formation of smaller emulsion droplets, in the conditions of the young broiler insufficient bile salts are present – especially with diets containing high levels of palm oil where, due to the low amount of unsaturated fatty acids, digestibility is more challenging than that of other, more unsaturated, vegetable oils such as soybean oil (Tancharoenrat et al., 2013) – to facilitate the adsorption of the colipase-lipase complex to the surface of the droplet. The combination of the challenging basal fat type (palm oil) and level with the immature digestive system in young broilers led to a relatively low crude fat digestibility in the basal diet (69.52%) seen in Experiment 2; it is well known that relative digestibility coefficients of fat are limited when fats and oils are presented at a high level (Croom et al., 1999; Rampone, 1961), as the ability of the digestive system and liver for lipolysis and absorption become rate limiting. However, in contrast to the phospholipids contained in lecithin, it has been suggested that once in the small intestine, lysophospholipids

tend to dissolve into mixed micelles and in this way leave the interface (~~Malaki~~ Nik et al., 201~~2~~0). Moreover, through their participation in the formation of mixed micelles, lysophospholipids may play an additional role by displacing monoglycerides and FFA from the interface allowing the lipid hydrolysis process to continue (Lairon, 2009~~13~~). Recent studies have also demonstrated the effect of lysolecithin on the intestinal mucosa – Papadopoulos et al. (2018) reported significantly thinner mucosa in birds supplemented with lysolecithin than in unsupplemented birds, while Chen et al. (2019) reported elevated claudin-3 levels following lysolecithin supplementation, indicating better sealing of tight junctions (Milatz et al., 2010). Brautigan et al. (~~2016~~2017) also showed increased collagen deposition and villus height following lysolecithin supplementation. Together, these results suggest that as lysolecithin becomes incorporated into the epithelial cell walls it also improves mucosal absorptive capacity in a way lecithin cannot.

Although in this proof of concept study lecithin had a negative effect on lipid hydrolysis, no adverse effect was observed on the absorption of generated monoglycerides and FFA by the differentiated Caco-2 monolayer (Figure 2). This also supports the argument that phospholipids likely reside at the interface of emulsion droplets and in this way do not participate in the absorption process at the enterocytes. In contrast, the absorption of monoglycerides and FFA was higher with digests from the palm oil supplemented with lysolecithin than with digests from palm oil and palm oil supplemented with lecithin. Similarly, in a study by Sugawara et al. (2001) the uptake of lipid-soluble carotenoids was greatly improved in Caco-2 cells exposed to micelles containing lysophosphatidylcholine in comparison to cells incubated with micelles containing phosphatidylcholine.

In addition to the improved CF digestibility, the DM digestibility and AMEn of basal diet supplemented with lysolecithin was also significantly higher than that of the basal diet and the basal diet supplemented with lecithin. This is in line with previous studies, which have

shown improvements in CF and N digestibility following lysolecithin supplementation, though the effect of fat source on response is still uncertain: Zaefarian et al. (2015) reported improved AMEn values in supplemented soy oil diets, but little effect of lysolecithin on diets formulated with tallow, while conversely, Jansen et al. (2015) reported improvement of the AMEn of diets formulated with pig lard but not those formulated with soybean oil. Across 33 studies, Wealleans et al. (2019) found little evidence that the magnitude of response to lysolecithin supplementation was consistently altered by dietary fat type. In the present study, the N-retention in the basal diet supplemented with lysolecithin was also significantly higher than that in the basal diet supplemented with lecithin. Furthermore, the AMEn improvement of 0.47 MJ/kg by lysolecithin supplementation can only be partially attributed to the 2.25% improvement in CF digestibility.

Honda et al. (2009) found that fats incorporated in the feed matrix could cover other nutrients, lowering their digestion. As a consequence of the improved CF digestibility, lysolecithins could in this way enhance the digestion of other nutrients. Another possible explanation for the large improvement in AMEn may be found at the enterocyte level. As supported by the improved absorption of MG and FFA in the present study, lysolecithin may enhance the uptake of multiple nutrients across the enterocyte membrane. Lysophospholipids are known to alter membrane structure by inducing local curvatures of the bilayer (Lundbaek, 2006; Wendel, 2000; Maingret et al., 2000). Moreover, they can affect proteins embedded in the membrane (Lundbaek, 2006; Maingret et al., 2000; Lundbaek and Andersen, 1994). In this way lysophospholipids could enhance the uptake of nutrients across the membrane of the microvilli in the intestinal epithelium; this is supported by the work of Brautigan et al. (~~2016~~2017), who reported increased villus height and collagen cross-linakges, driven by upregulation of collagen-related genes, in the intestinal epithelium of broilers supplemented with lysolecithin.

These differential effects on fat hydrolysis and overall digestibility between lecithin and lysolecithin are supported by the performance results of Experiment 3, although the basal diet formulations were substantially different – designed to be more commercially relevant – from those used in Experiment 2 Substituting soya oil for lecithin oil at the same concentration numerically reduced broiler growth and increased FCR until day 10 (1.45 soya oil vs 1.55 lecithin oil, P=0.161), while weight at day 10 was 3% lower in birds fed on the lecithin oil diet than diets formulated with soya oil. Though differences between unsupplemented soya and lecithin oil treatments were not statistically significant, the large difference in FCR during the starter phase is commercially important to overall production profitability. Similarly, Huang et al. (2007) reported that complete replacement of the soybean oil with lecithin (2% lecithin in the diet) resulted in adverse effects. The adverse effect of lecithin on bird performance was greatest in young birds, and after day 10 the gap in feed conversion ratio between soya oil and lecithin oil diets narrowed (6.9% increase in FCR lecithin vs soya to day 10, 3.1% increase in FCR lecithin vs soya day 10-21), with birds on all treatments performing similarly. Until 14 days of age, fat digestion is severely limited in chicks due to a lack of bile salt secretion (Krogdahl, 1985), after which the rates of synthesis increase fourfold. Therefore, it is likely that birds fed the lower available energy diets containing lecithin oil were able to achieve compensatory growth once bile salt synthesis reached sufficient levels and fat digestion improved (Krogdahl, 1985), thus closing the performance gap with those fed diets containing soya oil, despite energy conversion efficiency rates remaining lower throughout the trial. Future research on the comparative effects of lecithin and lysolecithin should continue to assess the effect on performance and digestibility until slaughter, when the bird is physiologically more mature. Meanwhile, the performance increases seen with supplemental lysolecithin are in line with previous research, which has demonstrated improved weight gain and feed conversion ratio (FCR) (Wealleans et al., 2019; Allahyari-Bake and Jahanian, 2017; Zaefarian

et al., 2015; Zampiga et al., ~~2013~~2016), while others have reported increases in apparent metabolizable energy (AME) (Majdolhosseini et al., 2019; Melegy et al., 2010; Jansen et al., 2015).

To conclude, this study presents evidence that lysolecithins – but likely not lecithin itself – are able to significantly improve the digestibility and energy values of feed, especially in young broilers. These improvements may be due to a combined effect of lysophospholipids on lipid hydrolysis and nutrient absorption, though further research is required to confirm the multifactorial mode of action

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609 **Table 1.** Effect of soybean lecithin and soybean lysolecithin on the apparent first-order rate
610 constant (k, × 10 3 min-1) of triglyceride hydrolysis and diglyceride, monoglyceride and free
611 fatty acid release during in vitro digestion of palm oil (Experiment 1)

	Triglyceride hydrolysis	Diglyceride release	Monoglyceride release	Free fatty acid release
Treatment[1]				
Palm oil	9.73[b]	1.15[c]	2.91[b]	10.85[b]
Palm oil + lecithin[2]	8.41[c]	1.58[b]	2.23[c]	9.49[b]
Palm oil + lysolecithin[3]	11.67[a]	1.70[a]	3.07[a]	12.49[a]
Pooled SEM	0.28	0.02	0.04	0.45
P-value	0.001	0.000	0.000	0.009

612 [a-c] Values within columns with different superscripts are significantly different ($P < 0.05$).

613 [1] Data are means of three observations per treatment.

614 [2,3] Lecithin and lysolecithin were applied at 1g, dispersed into 49 g of oil

615

Table 2. Ingredients and nutrient composition of the basal[1] experimental diet (Experiment 2)

Item (g/kg, unless noted)	Starter diet
Ingredient	
Corn	452.9
Wheat	100.0
Soybean meal (45.3% CP)	363.2
Palm oil	40.0
Monocalcium phosphate	13.6
Vitamin and mineral premix[2]	10.0
Methionine	3.0
Lysine HCl	2.3
Threonine	0.6
Limestone	6.8
NaCl	2.0
$NaHCO_3$	2.5
TiO_2	3.0
Calculated composition	
AMEn (MJl/kg)	11.41
Crude fiber	3.19
Lysine	1.15
Methionine + cysteine	0.86
Threonine	0.75
Calcium	0.85
Total phosphorus	0.69
Available phosphorus	0.40
Natrium	0.15
Analyzed composition[3]	
Dry matter	90.43
Crude protein	23.05
Crude fat	7.25
TiO_2	0.29
Gross energy (MJ/kg)	17.50

[1] For experimental treatments, lysolecithin was added on top of the specified basal diet at 250 ppm

[2] Supplied per kilogram of diet: manganese, 99 mg; zinc, 60 mg; iron, 49 mg; copper, 20 mg; iodine, 1.2 mg; selenium, 0.4 g; vitamin A, 13,500 IU (retinyl acetate); vitamin E, 55 IU (DL-α-tocopheryl acetate); cholecalciferol, 75 µg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_3, 30 mg; vitamin B_5, 15 mg; vitamin B_6, 4 mg; vitamin B_{12}, 2 mg; vitamin K, 2.5 mg; folic acid, 1 mg; biotin; 0.2 mg; choline, 600 mg; etoxyquine, 33 mg; butylated hydroxytoluene (BHT), 0.4 mg; propyl gallate, 0.12 mg; citric acid, 0.69 mg; sepiolite, 4 mg.

[3] Values represent the mean of the three experimental diets.

628 **Table 3.** Effect of soybean lecithin and soybean lysolecithin on the apparent faecal dry matter
629 and crude fat digestibility, nitrogen retention and AMEn of young broilers fed a palm oil rich
630 diet (Experiment 2)

	Digestibility (%)		N-retention	AMEn
	DM	Crude fat	(g/kg DM)	(MJ/kg DM)
Dietary treatment[1]				
Basal diet	69.85[b]	69.52[b]	27.03[ab]	13.03[b]
Basal diet + lecithin	69.47[b]	66.32[c]	25.26[b]	13.03[b]
Basal diet + lysolecithin	72.88[a]	71.77[a]	27.75[a]	13.50[a]
Pooled SEM	0.16	0.71	0.53	0.16
P-value	0.000	0.003	0.039	0.008

631 [a-c] Values within columns with different superscripts are significantly different ($P < 0.05$).

632 [1] Data are means of three observations per treatment.

633

Table 4. Fatty acid distribution (%) in the ether extracts of the faeces of young broilers fed a basal palm oil diet, the basal diet supplemented with lecithin and the basal diet supplemented with lysolecithin (Experiment 2)

Fatty acid[12]	Basal diet	Basal diet + lecithin	Basal Diet + lysolecithin	Pooled SEM	*P*-value
C16:0	40.59	40.47	41.23	0.69	NS
C18:0	6.29	6.89	6.91	0.15	NS
C18:1 (ω-9)	23.71	23.47	23.75	0.12	NS
C18:2 (ω-6)	26.01	26.46	25.55	0.75	NS
C18:3 (ω-3)	0.69	0.71	0.65	0.02	NS
Total unsaturated fatty acids	49.34	49.13	49.83	0.84	NS
Total saturated fatty acids	50.66	50.87	50.17	0.84	NS
Ratio unsaturated / saturated	1.03	1.04	1.01	0.03	NS

[1]Fatty acids that are not listed were present for less than 0.5% in all samples.

[2] Data are means of three observations per treatment.

Table 5. Ingredients and nutrient composition of the basal experimental diets[1] (Experiment 3)

Item (g/kg, unless noted)	Soybean Oil	Lecithin Oil
Wheat	541.6	541.6
Pura[2]	100.0	100.0
Soybean Meal (46.6% CP)	304.0	304.0
Soybean Oil	20.0	--
Lecithin Oil	--	20.0
Limestone	5.6	5.6
Salt	1.6	1.6
Sodium Bicarbonate	2.8	2.8
DCP	12.4	12.4
Lysine HCl	2.7	2.7
DL Methionine	3.4	3.4
Threonine	1.0	1.0
Ronozyme P5000	0.15	0.15
Ronzyme WX	0.15	0.15
Vit/Min Premix[3]	4.63	4.63
Calculated composition, %		
ME, MJ/kg	12.609 MJ/kg	
Crude Protein	22.47	
Crude Fat	6.207	
Crude Fibre	3.38	
Dig Lys	1.247	
Dig Met	0.623	
Dig Met+Cys	0.938	
Ca	0.898	
Av P	0.449	
Na	0.150	

[1] Lysolecithins (as LYSOFORTE® EXTEND) were added on top of the basal diet formulations at 500 ppm

[2] Pura is a commercially available blend of whole rapeseed and pulses (field beans or peas) which has been ground, heat treated and pelleted. Typical analysis 21% oil, 21% protein.

[3]Supplied per kilogram of diet: manganese, 99 mg; zinc, 60 mg; iron, 49 mg; copper, 20 mg; iodine, 1.2 mg; selenium, 0.4 g; vitamin A, 13,500 IU (retinyl acetate); vitamin E, 55 IU (DL-α-tocopheryl acetate); cholecalciferol, 75 µg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_3, 30 mg; vitamin B_5, 15 mg; vitamin B_6, 4 mg; vitamin B_{12}, 2 mg; vitamin K, 2.5 mg; folic acid, 1 mg; biotin; 0.2 mg; choline, 600 mg; etoxyquine, 33 mg; butylated hydroxytoluene (BHT), 0.4 mg; propyl gallate, 0.12 mg; citric acid, 0.69 mg; sepiolite, 4 mg.

650 **Table 6.** Growth performance of young broilers from day 0-21 fed diets formulated with soya oil or lecithin oil, with or without supplemental
651 lysolecithin. (Experiment 3).

	Soya Oil	Soya Oil + Lysolecithin	Lecithin Oil	Lecithin Oil + Lysolecithin	Pooled SEM	P-value
Day 0-10[1]						
Weight at day 0, g	38.3	38.3	38.6	38.9	0.72	0.906
Average daily gain, g	21.2	21.5	20.8	22.0	0.41	0.198
Average daily feed intake, g	32.0	29.5	32.3	31.6	1.13	0.326
FCR	1.45	1.36	1.55	1.43	0.060	0.161
Day 10-21						
Weight at day 10, g	256.2^{ab}	258.9^{ab}	248.9^{b}	263.8^{a}	3.18	0.015
Average daily gain, g	61.3	61.4	59.4	63.4	0.11	0.105
Average daily feed intake, g	80.6	81.9	79.9	83.9	1.24	0.183
FCR	1.31	1.33	1.35	1.33	0.020	0.723
Day 0-21						
Weight at day 21, g	934.1^{ab}	941.6^{ab}	906.9^{b}	961.7^{a}	1.38	0.047
Average daily gain, g	42.5^{ab}	42.8^{ab}	41.3^{b}	43.9^{a}	0.50	0.036
Average daily feed intake, g	56.5	56.5	57.0	58.6	0.89	0.356
FCR	1.33	1.32	1.38	1.33	0.024	0.225
Energy conversion rate (MJ/kg BWG)	16.04	15.88	16.60	16.06	--	--

652 [1] Data are means of twelve observations per treatment.

Figure 1. Hydrolysis of (A) triglycerides and accumulation of (B) free fatty acids, (C) diglycerides and (D) monoglycerides during the in vitro hydrolysis of palm oil (solid line), palm oil with soybean lecithin (dotted line) and palm oil with soybean lysolecithin (dashed line). The experimental treatments were carried out in triplicate. The mean concentrations of the lipids (mg/ml) are given over time (min), with error bars indicating the standard error values. Statistical analysis of the treatments is performed based on apparent rate constants (see Table 1). For enzyme kinetic comparison, apparent rate constants are used. (Experiment 1).

Figure 2. Absorption of monoglycerides (white bars) and free fatty acids (grey bars) generated during *in vitro* hydrolysis of palm oil, palm oil with soybean lecithin and palm oil with soybean lysolecithin by differentiated Caco-2 monolayers and expressed as percentage of applied monoglycerides end free fatty acids. Data are means of three or more observations per treatment, with error bars indicating the standard error values. (Experiment 1).

Lysolecithin, but not lecithin, improves nutrient digestibility and growth rates in young broilers

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ABSTRACT

1. The potential of lecithin and lysolecithin to improve lipid digestion and growth performance was investigated in three experiments: 1. an *in vitro* model that mimics the intestinal conditions of the chick, 2. a digestibility trial with chicks (5-7 days of age), and 3. a performance trial until 21 days of age.
2. In experiment 1, palm oil (PO), palm oil with lecithin (PO+L), and palm oil with lysolecithin (PO+LY) were subjected to *in vitro* hydrolysis and applied to Caco-2 monolayers to assess lipid absorption.
3. The *in vitro* hydrolysis rate of triglycerides was higher in PO+LY ($k = 11.76 \times 10^3$/min) than in either PO ($k = 9.73 \times 10^3$/min) or PO+L ($k = 8.41 \times 10^3$/min), and the absorption of monoglycerides and free fatty acids was highest ($P<0.01$) for PO+LY. In experiment 2, 90 broilers were assigned to three dietary treatments: a basal diet with 4% palm oil, and the basal diet supplemented with either 250 ppm lecithin or lysolecithin.
4. ATTD of crude fat was higher in broilers supplemented with lysolecithin, but was lower in broilers supplemented with lecithin. DM digestibility and AMEn in birds supplemented with lysolecithin were significantly higher (3.03% and 0.47 MJ/kg, respectively).
5. In experiment 3, 480 broilers were randomly allocated to four dietary treatments: basal diet with soybean oil (2%), basal diet with lecithin (2%), soybean oil diet with 250 ppm lysolecithin, or lecithin oil diet with 250 ppm lysolecithin.
6. Lecithin diets significantly reduced weight at day 10 and 21 compared with soybean oil. However, the addition of lysolecithin to lecithin-containing diets significantly improved bird performance.
7. The results of these studies showed that, in contrast to lecithin, lysolecithin was able to significantly improve the digestibility and energy values of feed in young broilers.

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KEYWORDS: broiler; digestibility; fat; lecithin; lysolecithin; performance

INTRODUCTION

After hatch, lipid digestion in young birds is physiologically limited, and is a target for improvement and support. Roy *et al.* (2010) have ascribed the inefficient digestion and absorption of lipids by young chickens to a combination of a poor duodenal secretion of lipase, a low level of lipase activity and a reduced rate of bile salt synthesis. Kroghdahl (1985) and Maiorka *et al.* (2004) showed, however, that lipase concentration and activity in young chickens (at seven days of age) are modulated according to the inclusion level of fats in the diet. Moreover, in a study of Meng *et al.* (2004), lipase addition at 0.2 g/kg did not affect the apparent fat digestibility in broilers aged one to 14 days of age. Therefore, the inability to utilise fats has been attributed to low bile salt concentrations in the intestines, rather than to deficiencies in lipase secretion or activity (Maiorka *et al.*, 2004; Maisonnier *et al.*, 2003). Although dietary supplementation of bile salt has been shown to improve lipid utilisation in chickens (Krogdahl, 1985; Polin et al 1980), supplementation is generally not applied on a commercial scale due to economic considerations (Roy *et al.*, 2010).

One strategy for improving fat digestion is the dietary application of molecules with proven ability to improve lipid digestion, for example lecithin and its derivative, lysolecithin. Huang *et al.* (2007) showed that, in diets supplemented with 2% of soybean oil, replacement of 25% of the soybean oil with soybean lecithin (0.5% lecithin in the diet) improved crude fat digestion and performance in broilers. On the other hand, complete replacement of the soybean oil with lecithin (2% lecithin in the diet) resulted in adverse effects. Moreover, in a study of Blanch *et al.* (1996) the AME of the basal diet containing tallow as the main fat source was not improved by the addition of 0.2% soybean lecithin.

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Lysolecithins are produced by phospholipase which cleaves one hydrophobic fatty acid from phospholipids (Joshi *et al.*, 2006). This changes the stereochemical structure of phospholipids in lecithin into lysophospholipids. The resulting lysolecithin mixture has an increased hydrophilic-lipophilic balance (Van Nieuwenhuyzen and Tomás, 2008) and lower critical micelle concentration (0.02 to 0.2 mM/l). Both phospholipids and lysophospholipids consist of a hydrophilic head group (phosphatidyl substituent) and a hydrophobic tail (fatty acid chains). But, due to the removal of one fatty acid, lysophospholipids are more hydrophilic and thus have better oil-in-water emulsifying properties than phospholipids (Joshi *et al.*, 2006; Liu and Ma, 2011). In the animal, the pancreas secretes native phospholipase (EC 3.1.1.4) to convert the phospholipids secreted by the gall bladder into lysophospholipids (Karray *et al.*, 2011). These have been shown to play an important role in mixed micelle formation (Lo and Tso, 2009). Therefore, it can be hypothesised that lysolecithin possesses a greater ability to improve the digestion of fats and oils than lecithin. Inclusion of 3.2% of rice bran lysolecithin in a broiler feed formulated with rice bran oil increased the crude fat digestibility (Raju *et al.*, 2011). Additionally, Zhang *et al.* (2011) observed increased fatty acid digestibility in broilers using 0.125% of soybean lysolecithin in partial replacement for soybean oil, tallow and poultry fat. These improvements in digestibility consistently lead to improved growth performance and efficiency in growing broilers (Wealleans *et al.*, 2019), as well as in other species (Wang *et al.*, 2019; Zhao *et al.*, 2017; Papadopoulos *et al.*, 2014).

The limited comparative studies available on lecithin and lysolecithin application in broiler diets have all been performed with birds of 14 days of age or older. Therefore, the potential of both lecithin and lysolecithin from soybean to improve lipid digestion was investigated in three experiments: an *in vitro* model, a digestibility trial with young broilers (5-7 days of age), and a performance trial until 21 days of age.

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METHODS AND MATERIALS

Experiment 1: In vitro fat absorption

Crude palm oil (single batch) was sourced from a commercial feed mill in Belgium. The FA composition of the palm oil was analysed (ISO, 2002, 17764). Soybean lecithin and lysolecithin were sourced from Kemin Europa NV (Herentals, Belgium). Lecithin and lysolecithin were analysed for their phospho- and lysophospholipid content by phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR, Spectral Service AG, Cologne, Germany).

The lipid hydrolysis model, previously employed by Jansen *et al.* (2015), was slightly modified. Briefly, 1 g of lecithin or lysolecithin were first dispersed into 49 g of palm oil. Fasted state, simulated intestinal fluid (FaSSIF) was prepared by adding 2.24 g of FaSSIF powder (Biorelevant.com Ltd, Croydon, United Kingdom) into 1 L of phosphate buffer (35 mM, pH 6.5) containing 106 mM NaCl. According to the manufacturer, the FaSSIF contained 3 mM bile salt (sodium taurocholate). Aliquots of 0.25 g of each of the respective fat treatments and 14.75 ml of FaSSIF were added into 50 ml centrifuge tubes. The content of each tube was mixed for 30 seconds with a high shear mixer (24000 rpm; IKA ultra-turrax T18, Staufen, Germany). Next, 24 mg of pancreatin (P7545, Sigma Aldrich) was added to each tube and incubated for two hours at 40°C while shaking (250 rpm). The final contents in the digests were 106 mM NaCl, 1.6 g/l pancreatin, 1.6 g/l bile salts and 16.7 g/l palm oil. At 0, 15, 30, 60, 90 and 120 minutes of incubation, a 0.5 ml aliquot of each digest was taken and diluted in 9.5 ml tetrahydrofuran (HPLC grade, VWR International, Leuven, Belgium) to inactivate the enzymes and prepare the appropriate dilution for lipid analysis. Each digestion was performed in triplicate. Samples of the digests were submerged in liquid nitrogen and stored at -180°C until the absorption experiments started.

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Human colonic adenocarcinoma cells (Caco-2) were obtained from the European Collection of Cell Cultures (Public Health England, Porton Down, Salisbury, UK). Caco-2 cell work stock was used between passages 54 and 60. Cells were cultured in Dulbecco's modified eagle medium supplemented with 100 ml/l heat-inactivated foetal bovine serum (Hyclone, Thermo scientific, Leuven, Belgium), 10 ml/l non-essential amino acids, 100 U/ml of penicillin and 100 U/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and routinely passaged. Unless stated otherwise, the cell culture media and supplements were provided by Westburg (Leusden, The Netherlands).

Caco-2 cells were seeded on collagen-coated Transwell-COL inserts (1.12 cm^2, pore size 0.4 µm, Corning Costar Corporation, Cambridge, MA) in 24-well plates at a density of 2 x 10^5 cells per insert and incubated for 21 days to allow the cells to differentiate. During incubation the medium (apical and basal) was changed three times a week and the trans-epithelial electrical resistance was monitored (Millicell-ERS, Millipore, Overijse, Belgium). Next, the different digests obtained with the lipid hydrolysis model were diluted 25-fold in FaSSIF and applied at the apical side of the monolayer. Simultaneously, Hank's balanced salt solution was applied at the basal side of the monolayer. The digest concentration and the differentiation protocol were optimised during the development of the model. Similar to Vors *et al.* (2012), a 25-fold dilution of each digest was selected to avoid toxicity while still presenting sufficient monoglycerides (MG) and free fatty acids (FFA) to the monolayers. At the start and after 60 minutes of incubation, a sample of the apical fluid was taken and diluted twofold in tetrahydrofuran. Each absorption experiment performed in at least three replicates.

In each sample obtained during the *in vitro* lipid digestion, the degree of lipid hydrolysis was analysed by HPLC. The lipids were separated into triglycerides (TG), diglycerides (DG), MG and FFA by a gel permeation column (PL 1110-6520, 5 µm 100A 300 x 7.5 mm, Agilent Technologies, Diegem, Belgium) and detected by an Evaporative Light Scattering Detector

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(ELSD 85, VWR International). Tetrahydrofuran was used as the mobile phase at a flow rate of 0.5 ml/min. Likewise, samples obtained with the lipid absorption model were analysed for their MG and FFA content.

The hydrolysis of palm oil at each sample time was calculated and the apparent rate constant for TG hydrolysis and FFA, MG and DG release were determined as described by Jansen *et al.* (2015). The absorption of MG (g/g) in each well was calculated as follows:

$$\text{MG absorption} = \frac{MG_0 - MG_{60}}{MG_0}$$

where MG_0 and MG_{60} are the respective MG contents (g/l) before and after 60 minutes of incubation. Correspondingly, FFA absorption (g/g) was calculated from the respective FFA contents.

For statistical comparison of the *in vitro* hydrolysis, the apparent rate constants for TG hydrolysis and FFA, MG and DG release and the MG and FFA absorption were analysed as a factorial arrangement. Analysis of variance (ANOVA) of the experimental treatments was done with STATGRAPHICS Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA), and means were separated by the least significant difference procedure. All statements of significance were based on a P-value equal to or less than 0.05.

Experiment 2: Digestibility

A broiler digestibility trial was performed at the experimental research facility of the Laboratory of Livestock Physiology (Leuven, Belgium). The experiments were conducted in strict accordance with the European Communities Council Directive (2003/65/EC) and were approved by the Ethical Commission for Experimental Use of Animals of the KU Leuven (P213/2015). A total of 90, one-day-old male Ross 308 chickens were obtained from Belgabroed NV (Merksplas, Belgium) and assigned randomly at day zero, in groups of ten

birds, to three dietary treatments; a basal diet without lecithin and lysolecithin, the basal diet supplemented with 250 ppm lecithin and the basal diet supplemented with 250 ppm lysolecithin. Lecithin and lysolecithin were applied to diets at the same rate in order to allow direct comparison of the effects of phospholipids and lysophospholipids.

To prepare the experimental diet (Table 1), all raw materials were milled together to obtain homogeneous batches. Next, the feed was divided into three batches and successively mixed in a small mixer with different premixes in order to produce the experimental diets. Titanium dioxide (E171, IMCD Benelux N.V., Mechelen, Belgium) was added to all diets as an indigestible marker at an inclusion rate of 3 g/kg. All diets were fed in mash form and contained a commercial enzyme blend (KEMZYME Plus Concentrate Dry 50 ppm, Kemin Europa NV) and phytase (RONOZYME P-(CT) 100 ppm, DSM Nutritional products, Deinze, Belgium).

Table 1 here

Birds were housed in nine digestibility cages (three replicates per treatment), constructed with a wire bottom and a plastic tray for excreta collection, two feed troughs and a drinking cup. The facility was maintained under conventional EU conditions for lighting, heating and ventilation. The trial consisted of a pre-period of six days to minimise interference of egg yolk digestion (days 0 to 5) and a 48-hour collection period (days 5 to 7). Drinking water and feed were provided *ad libitum*. During the collection period, total excreta were collected, and pooled and homogeneous samples of the mixed wet excreta were freeze-dried and stored until analysis. The titanium dioxide content was determined using the method of Short *et al.* (1996) with modifications according to Myers *et al.* (2004). Samples of the feed and freeze-dried excreta were analysed in the accredited laboratory of the Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium) for dry matter (DM), gross energy (GE), crude protein

and crude fat according to EC (1971), ISO (1998, 9831), ISO (2005, 5983-2, N × 6.25) and ISO (1999, 6492), respectively. Additionally, the FA distribution of the ether extract of the diets and excreta samples were determined (ISO, 2002, 17764).

The coefficient of total tract apparent digestibility (CTTAD) of DM, crude protein and crude fat were determined by the use of the concentrations of titanium dioxide in the excreta and in the feed, and calculated as described by Jansen *et al.* (2015). For the titanium dioxide in the feed a single value, averaged over the diets, was used for all calculations. Average titanium recovery was within acceptable limits for all diets. The coefficients of total tract apparent digestibility of individual fatty acids (CTTADF) were calculated as follows:

$$CTTADF = \frac{\left[FA_{diet} - \left(FA_{excreta} \times \left(1 - CTTAD\ of\ \ crude\ fat\right)\right)\right]}{\left[FA_{diet}\right]}$$

where FA_{diet} and $FA_{excreta}$ are the respective FA contents (g/kg) analysed in the ether extract of the diets and excreta samples. The AME contents of the experimental diets were calculated from their respective titanium dioxide ratios and corresponding GE contents, as described by Jansen *et al.* (2015).

The values for CTTAD of DM and crude fat, CTTADF, N-retention and AMEn were analysed as a one factorial arrangement and subjected to ANOVA with STATGRAPHICS Centurion XVI software (Statpoint Technologies Inc.). Repeated measures techniques were not used. Means were separated by the least significant difference procedure. All statements of significance were based on a P-value equal to or less than 0.05.

Experiment 3: Performance

All experimental procedures were conducted in strict accordance with the European Communities Council Directive (2003/65/EC) and were approved by the Ethics Committee of Nottingham Trent University.

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Birds were sourced from PD Hook Cote hatchery, Oxford, UK. Birds were feather sexed on day of hatch and collected by Nottingham Trent University (NTU) personnel to reduce travel stress. A total of 480-day-old male Ross 308 chicks were individually weighed before random allocation to 80 x 80 cm mesh sided pens bedded on clean wood shavings. Unhealthy or unusually sized chicks were discarded from the trial upon arrival. Birds were individually weighed and only birds between 33 and 46 g were placed in trial cages.

Birds were allocated to four dietary treatments in a 2 x 2 factorial design. Treatments were; basal diet with soybean oil (2%), basal diet with lecithin oil (2%), basal diet with soybean oil with 250 ppm lysolecithin or basal diet with lecithin oil with 250 ppm lysolecithin. Compared to experiment 2, where dietary fat type and level were used to create challenging diets that could elucidate clearly the mode of action of lysolecithin, in experiment 3 the basal diets were formulated to reflect relevant commercial compositions and to meet all nutrient requirements of the birds (Table 2). The lecithin-based oil used in this study was commercially available as Leciol (Adams and Green, East Yorkshire, UK). The lysolecithin was supplemented as Lysoforte® Extend (Kemin Animal Nutrition and Health, Belgium). Each treatment consisted of 12 pens, with 10 birds per pen. Diets were manufactured by Target Feeds (Whitchurch, UK) and supplied bagged as mash for both starter and grower. Feed and water were available *ad libitum*, with care taken to ensure the birds ate and drank as soon as possible after placement. The lighting regimen was maintained in accordance with commercial practice with 15 minutes of dusk at the start and end of each dark period. Temperature was set at 31°C on day 1 and reduced by approximately 1°C per day until 21°C was reached.

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Bird observations were used to monitor the environment and if the birds appeared uncomfortable, the temperature and/or ventilation was altered accordingly. Birds were observed twice daily during the trial and any observations related to health recorded in a trial diary. Any dead birds were weighed, and reasons recorded if culled. Birds were weighed by pen on arrival, on day 10 and at the end of the trial (day 21). Initially, individual weighed bags of feed were prepared containing weighed feed quantities for each phase. Each pen of birds was fed from their designated bag for each phase. Extra feed was added to the bags as required and the quantity recorded. Total feed eaten was calculated as the difference between remaining feed in the bag and the amount weighed into the bag for each phase.

Energy conversion efficiency (ME MJ/kg gain) was calculated according to the following formula, as per Salah *et al.* (2004):

$$Calorie\ conversion = \frac{Total\ Feed\ Consumption\ \times Energy\ content\ of\ feed}{Sum\ weight\ of\ all\ birds\ at\ trial\ end}$$

Calorie conversion was calculated using the calculated ME contents on the feed, using both on the literature energy value of the two fat sources, as claimed by the manufacturers, and with the energy value as determined by Wiseman *et al.* (1991).

Statistical analysis was carried out using SPSS v.24. After testing to confirm normality, data were analysed using one-way ANOVA to investigate the effect of dietary treatment on FCR, feed intake (per bird) and individual bodyweight gain for each weigh period of the study, and cumulatively. Where appropriate, Bonferroni *post hoc* testing was used to elucidate differences between diets/treatments.

RESULTS

Experiment 1: In vitro fat absorption

In experiment 1, palm oil contained a high amount of saturated FA (492.4 g/kg), especially palmitic acid (429.0 g/kg), which was largely reflected in the FA in the ether extract of the diet

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(Supplemental Table 1). The total lysophospholipid fraction was confirmed to be much higher for soybean lysolecithin than for soybean lecithin (118.8 g/kg lysolecithin *vs.* 14.1 lecithin; Supplemental Table 2). The majority of lysophospholipids in soybean lysolecithin were lysophosphatidylcholine (LPC) and lysophosphatidyl-ethanolamine.

The hydrolysis of TG and the accumulation of FFA, DG and MG during the *in vitro* hydrolysis of palm oil, palm oil with lecithin or with lysolecithin are shown in Figure 1. Over the whole incubation period of 120 min, the amounts of TG hydrolysed and FFA, DG and MG accumulated in palm oil with lecithin are markedly lower than those of palm oil and palm oil with lysolecithin. During the first 60 min of incubation, the amounts of TG hydrolysed and FFA and MG accumulated were slightly higher in palm oil without lysolecithin or lecithin, than in palm oil with lysolecithin. After 120 min, however, the amounts of TG hydrolysed and FFA and DG accumulated was slightly higher in palm oil with lysolecithin than in palm oil without lysolecithin.

Fig 1 here

A comparison of the apparent first-order rate constants for TG hydrolysis and the accumulation of FFA, DG and MG for each treatment is presented in Table 3 Addition of lecithin or lysolecithin to the palm oil had significant ($P<0.01$) impact on the rates of TG hydrolysis and FFA, DG and MG release. TG were hydrolysed faster when lysolecithin was added to the palm oil ($k = 11.76 \times 10^3$/min) compared to palm oil without (lyso)lecithin ($k = 9.73 \times 10^3$/min). In contrast, TG were hydrolysed slower when lecithin was added ($k = 8.41 \times 10^3$/min). Similarly, the release of MG was the fastest in palm oil with lysolecithin ($k = 3.07 \times 10^3$/min) and the slowest in palm oil with lecithin ($k = 2.23 \times 10^3$/min). In contrast, DG release was the slowest

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in palm oil without lecithin or lysolecithin. There was no statistically significant difference observed in the rate of free fatty acid release between palm oil and palm oil with lecithin.

Table 3 here

The absorption of MG and FFA generated during *in vitro* hydrolysis of palm oil, palm oil with lecithin or with lysolecithin is presented in Figure 2. The absorption of MG was significantly higher ($P<0.01$) for palm oil with lysolecithin (85.6%) than for palm oil (21.1%) or palm oil with lecithin (35.5%). The overall absorption of FFA was slightly lower ($P>0.1$) than that of MG. Nevertheless, the absorption of FFA was significantly higher ($P<0.01$) for palm oil with lysolecithin (77.8%) than for palm oil alone (13.9%) or with lecithin (24.0%).

Fig 2 here

Experiment 2: Digestibility

In experiment 2, during the collection period the observed feed intake values were similar for all treatments with an average of 30.6 g per bird per day. Likewise, bodyweight gain values were similar for all treatments with an average of 26.35 g per bird per day. Apparent faecal digestibility values and AMEn are presented in Table 4.

Table 4 here

There were significant differences ($P<0.05$) between the treatments for all parameters investigated. Compared to the basal diet, lysolecithin supplementation increased ($P<0.01$) the DM digestibility by 1.04%, CF digestibility by 2.25% and AMEn by 0.47 MJ/kg. On the other

hand, supplementation of lecithin had no effect on dry matter digestibility or AMEn, but reduced (P<0.01) crude fat digestibility by 3.2%. When compared to the basal diet, N-retention was not significantly affected by lecithin or lysolecithin supplementation. However, N-retention of the basal diet supplemented with lysolecithin (27.75 g/kg DM) was higher (P<0.05) than that of the basal diet supplemented with lecithin (25.26 g/kg DM). Fatty acid distribution in the ether extracts of the faeces is presented in Table 5. There was no difference (P>0.05) between any of the dietary treatments in the fatty acid distribution in the faeces, nor in the ratio of unsaturated over saturated fatty acids.

Table 5 here

Experiment 3: Performance Trial

Table 6 shows the performance of the birds across the whole experimental period of experiment 3. During the starter phase, days 0-10, there were no significant differences in average daily gain, feed intake or FCR between the treatment groups. However, birds fed the diet containing lecithin-based oil supplemented with lysolecithin were significantly heavier than those fed the diet with lecithin-based oil alone. During the grower phase there was a significant difference in bird weight. Birds fed the diet with lecithin-based oil alone were significantly lighter in weight when compared to the birds fed the diet with lecithin-based oil supplemented with lysolecithin (P=0.047). Similarly, when the whole trial phase was evaluated there were significant differences in average daily gain. Birds fed diets with lecithin-based oil alone gained less daily (P=0.036) than the birds fed the diet with lecithin-based oil supplemented with lysolecithin (P=0.036).

Table 6 here

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The energetic values of each oil determined through analysis of chemical composition (Supplemental Information Table 3) demonstrated that, due to high levels of FFA in the oils and an altered unsaturated: saturated ratio (Wiseman *et al.*, 1991), the lecithin oil had a reduced predicted AME value for broilers <21 days compared to the soybean oil (36.57 MJ/kg soybean *vs*. 32.64 MJ/kg lecithin). When calculating diet density based on lipid analysis, rather than assuming energetic equivalence between the two oil sources, the effective energy conversion rate for soybean oil alone was 16.04 MJ/kg BWG, while lecithin oil alone was worse, at 16.78 MJ/kg BWG. With the addition of lysolecithin, the energy conversion rate was improved across both fat sources (15.58 MJ/kg BWG soybean oil + lysolecithin; 16.23 MJ/kg BWG lecithin plus lysolecithin); the effective efficiency of use of the lecithin oil was brought closer to that of unsupplemented soya oil.

DISCUSSION

The structural differences in the composition of lecithin and lysolecithin leads to fundamentally diverse effects on both *in vitro* and *in vivo* lipid digestion. In experiment 1, compared to palm oil without lecithin or lysolecithin, the apparent rate constants *in vitro* for triglyceride hydrolysis and monoglyceride release were significantly higher for palm oil supplemented with lysolecithin and significantly lower for palm oil supplemented with soybean lecithin (Figures 1 and 2). Likewise, in experiment 2, compared to the basal diet, crude fat digestibility was significantly higher in broilers supplemented with lysolecithin, but was significantly lower in broilers supplemented with lecithin. Though the limitations of this initial study require further data to confirm these findings, Zhang *et al.* (2011) postulated that, while both lecithin and lysolecithin may act as an emulsifier within the first stages of lipid digestion, for hydrolysis to take place, the pancreatic colipase-lipase complex first must be adsorbed onto the emulsion

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droplets (Reis *et al.*, 2010). The adsorption and activity of lipase at the oil-water interface, however, is affected by various surface-active compounds such as phospholipids and lysophospholipids (Reis *et al.*, 2010; 2009).

Possibly the observed effect of lecithin on crude fat digestion in young broilers in experiment 2 could be explained by a study of Chu *et al.* (2010), who showed that colipase and lipase adsorbed exclusively onto regions covered by phosphatidylcholine and bile salts and not to regions covered with phosphatidylcholine solely. This suggested that, although phospholipids in lecithin may have aided in the formation of smaller emulsion droplets, the young broiler produces insufficient bile salts. This is especially the case in diets containing high levels of palm oil where, due to the low amount of unsaturated fatty acids, digestibility is more challenging than with other, more unsaturated, vegetable oils, such as soybean oil (Tancharoenrat *et al.*, 2013) to facilitate the adsorption of the colipase-lipase complex to the surface of the droplet. The combination of the challenging basal fat type (palm oil) and level with the immature digestive system in young broilers led to a relatively low crude fat digestibility in the basal diet (69.52%) seen in experiment 2. It is well known that relative digestibility coefficients of fat are limited when fats and oils are present at a high levels (Croom *et al.*, 1999; Rampone, 1961), as the ability of the digestive system and liver for lipolysis and absorption become rate limiting. However, in contrast to the phospholipids contained in lecithin, it has been suggested that, once in the small intestine, lysophospholipids tend to dissolve into mixed micelles and, in this way, leave the interface (Nik *et al.*, 2010). Moreover, through their participation in the formation of mixed micelles, lysophospholipids may play an additional role by displacing MG and FFA from the interface, allowing lipid hydrolysis to continue (Lairon, 2009). Recent studies have demonstrated the effect of lysolecithin on the intestinal mucosa. Papadopoulos *et al.* (2018) reported significantly thinner mucosa in birds supplemented with lysolecithin than in unsupplemented birds, while Chen *et al.* (2019)

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reported elevated claudin-3 levels following lysolecithin supplementation, indicating better sealing of tight junctions (Milatz *et al.*, 2010). Brautigan *et al.* (2017) showed increased collagen deposition and villus height following lysolecithin supplementation. Together, these results suggested that, as lysolecithin becomes incorporated into the epithelial cell walls, it improves mucosal absorptive capacity in a way lecithin cannot.

Although in this 'proof of concept' study lecithin had a negative effect on lipid hydrolysis, no adverse effect was observed on the absorption of generated MG and FFA by the differentiated Caco-2 monolayer (Figure 2). This supported the argument that phospholipids likely reside at the interface of emulsion droplets and do not participate in the absorption process in enterocytes. In contrast, the absorption of MG and FFA was higher with digests from the palm oil supplemented with lysolecithin than with digests from palm oil and palm oil supplemented with lecithin. Similarly, in a study by Sugawara *et al.* (2001) the uptake of lipid-soluble carotenoids was greatly improved in Caco-2 cells exposed to micelles containing lysophosphatidylcholine in comparison to cells incubated with micelles containing phosphatidylcholine.

In addition to the improved CF digestibility and DM digestibility, AMEn of the basal diet supplemented with lysolecithin was significantly higher than that of the basal diet or basal diet supplemented with lecithin. This is in line with previous studies, which have shown improvements in CF and N digestibility following lysolecithin supplementation, although the effect of fat source on response is still uncertain: Zaefarian *et al.* (2015) reported improved AMEn values in supplemented soy oil diets, but little effect of lysolecithin on diets formulated with tallow. Conversely, Jansen *et al.* (2015) reported improvements in AMEn of diets formulated with pig lard but not those formulated with soybean oil. Across 33 studies, Wealleans *et al.* (2019) found little evidence that the magnitude of response to lysolecithin supplementation was consistently altered by dietary fat type. In the present study, N-retention

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for the basal diet supplemented with lysolecithin was significantly higher than in the basal diet supplemented with lecithin. Furthermore, the AMEn improvement of 0.47 MJ/kg by lysolecithin supplementation was only partially attributed to the 2.25% improvement in CF digestibility.

Honda *et al.* (2009) found that fats incorporated in the feed matrix could encapsulate other nutrients, lowering their digestion. As a consequence of improved CF digestibility, lysolecithins could, in this way, enhance the digestion of other nutrients. Another possible explanation for the large improvement in AMEn may be found at the enterocyte level. As supported by the improved absorption of MG and FFA in the present study, lysolecithin may enhance the uptake of multiple nutrients across the enterocyte membrane. Lysophospholipids are known to alter membrane structure by inducing local curvatures in the bilayer (Lundbaek, 2006; Wendel, 2000; Maingret *et al.*, 2000). Moreover, they can affect proteins embedded in the membrane (Lundbaek, 2006; Maingret *et al.*, 2000; Lundbaek and Andersen, 1994). In this way lysophospholipids could enhance the uptake of nutrients across the membrane of the microvilli in the intestinal epithelium, which is supported by the work of Brautigan *et al.* (2017), who reported increased villus height and collagen cross-linkages, driven by upregulation of collagen-related genes in the intestinal epithelium of broilers supplemented with lysolecithin.

These differential effects on fat hydrolysis and overall digestibility between lecithin and lysolecithin were supported by the performance results of experiment 3, although the basal diet formulations were substantially different, and designed to be more commercially relevant, from those used in experiment 2 Substituting soya oil for lecithin oil at the same concentration numerically reduced broiler growth and increased FCR until day 10 of age (1.45 soya oil *vs.* 1.55 lecithin oil, P=0.161), while weight at day 10 was 3% lower in birds fed the lecithin oil diet compared to those formulated with soya oil. Although differences between

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unsupplemented soya and lecithin oil treatments were not statistically significant, the large difference in FCR during the starter phase was commercially important to overall production profitability. Similarly, Huang *et al.* (2007) reported that complete replacement of the soybean oil with lecithin (2% lecithin in the diet) resulted in adverse effects. This was greatest in young birds, and, after day 10, the gap in feed conversion ratio between soya oil and lecithin oil diets narrowed (6.9% increase in FCR lecithin *vs*. soya to day 10, 3.1% increase in FCR lecithin *vs*. soya day 10-21), with birds on all treatments performing similarly. Until 14 days of age, fat digestion was severely limited in chicks due to a lack of bile salt secretion (Krogdahl, 1985), after which the rates of synthesis increased fourfold. Therefore, it was likely that birds fed the lower available energy diets containing lecithin oil were able to achieve compensatory growth once bile salt synthesis reached sufficient levels and fat digestion improved (Krogdahl, 1985), thus closing the performance gap with those fed diets containing soya oil, despite energy conversion efficiency rates remaining lower throughout the trial.

Future research on the comparative effects of lecithin and lysolecithin should continue to assess the effect on performance and digestibility until slaughter, when the bird is physiologically more mature. Meanwhile, the performance increases seen with supplemental lysolecithin were in line with previous research, which demonstrated improved weight gain and FCR (Wealleans *et al.*, 2019; Allahyari-Bake and Jahanian, 2017; Zaefarian *et al.*, 2015; Zampiga *et al.*, 2016), while others have reported increases in AME (Majdolhosseini *et al.*, 2019; Melegy *et al.*, 2010; Jansen *et al.*, 2015).

To conclude, this study presented evidence that lysolecithins, but likely not lecithin itself, are able to significantly improve the digestibility and energy values of feed, especially in young broilers. These improvements may be due to a combined effect of lysophospholipids on lipid hydrolysis and nutrient absorption, although further research is required to confirm any multifactorial mode of action

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Table 3. Effect of soybean lecithin and soybean lysolecithin on the apparent first-order rate constant (k × 10^3/min) of triglyceride hydrolysis and diglyceride, monoglyceride and free fatty acid release during *in vitro* digestion of palm oil (Experiment 1)

	Triglyceride hydrolysis	Diglyceride release	Monoglyceride release	Free fatty acid release
Treatment[1]				
Palm oil	9.73[b]	1.15[c]	2.91[b]	10.85[b]
Palm oil + lecithin[2]	8.41[c]	1.58[b]	2.23[c]	9.49[b]
Palm oil + lysolecithin[3]	11.67[a]	1.70[a]	3.07[a]	12.49[a]
Pooled SEM	0.28	0.02	0.04	0.45
P-value	0.001	0.000	0.000	0.009

[a-c] Values within columns with different superscripts are significantly different (P<0.05).

[1] Data are means of three observations per treatment.

[2,3] Lecithin and lysolecithin were applied at 1g, dispersed into 49 g of oil

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Table 1. Ingredients and nutrient composition of the basal[1] experimental diet (Experiment 2)

Item (g/kg, unless noted)	Starter diet
Ingredient	
Corn	452.9
Wheat	100.0
Soybean meal (45.3% CP)	363.2
Palm oil	40.0
Monocalcium phosphate	13.6
Vitamin and mineral premix[2]	10.0
Methionine	3.0
Lysine HCl	2.3
Threonine	0.6
Limestone	6.8
NaCl	2.0
$NaHCO_3$	2.5
TiO_2	3.0
Calculated composition	
AMEn (MJl/kg)	11.41
Crude fibre	3.19
Lysine	1.15
Methionine + cysteine	0.86
Threonine	0.75
Calcium	0.85
Total phosphorus	0.69
Available phosphorus	0.40
Sodium	0.15
Analysed composition[3]	
Dry matter	90.43
Crude protein	23.05
Crude fat	7.25
TiO_2	0.29
Gross energy (MJ/kg)	17.50

[1] For experimental treatments, lysolecithin was added on top of the specified basal diet at 250 ppm

[2] Supplied per kilogram of diet: manganese, 99 mg; zinc, 60 mg; iron, 49 mg; copper, 20 mg; iodine, 1.2 mg; selenium, 0.4 g; vitamin A, 13,500 IU (retinyl acetate); vitamin E, 55 IU (DL-α-tocopheryl acetate); cholecalciferol, 75 µg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_3, 30 mg; vitamin B_5, 15 mg; vitamin B_6, 4 mg; vitamin B_{12}, 2 mg; vitamin K, 2.5 mg; folic acid, 1 mg; biotin; 0.2 mg; choline, 600 mg; etoxyquine, 33 mg; butylated hydroxytoluene (BHT), 0.4 mg; propyl gallate, 0.12 mg; citric acid, 0.69 mg; sepiolite, 4 mg.

[3] Values represent the mean of the three experimental diets.

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Table 4. Effect of soybean lecithin and soybean lysolecithin on the apparent faecal dry matter and crude fat digestibility, nitrogen retention and AMEn of young broilers fed a palm oil rich diet (Experiment 2)

	Digestibility (%)		N-retention	AMEn
	DM	Crude fat	(g/kg DM)	(MJ/kg DM)
Dietary treatment[1]				
Basal diet	69.85[b]	69.52[b]	27.03[ab]	13.03[b]
Basal diet + lecithin	69.47[b]	66.32[c]	25.26[b]	13.03[b]
Basal diet + lysolecithin	72.88[a]	71.77[a]	27.75[a]	13.50[a]
Pooled SEM	0.16	0.71	0.53	0.16
P-value	0.000	0.003	0.039	0.008

[a-c] Values within columns with different superscripts are significantly different (P<0.05).

[1] Data are means of three observations per treatment.

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Table 5. Fatty acid distribution (%) in the ether extracts of the faeces of young broilers fed a basal palm oil diet, the basal diet supplemented with lecithin and the basal diet supplemented with lysolecithin (Experiment 2)

Fatty acid[12]	Basal diet	Basal diet + lecithin	Basal Diet + lysolecithin	Pooled SEM	*P*-value
C16:0	40.59	40.47	41.23	0.69	NS
C18:0	6.29	6.89	6.91	0.15	NS
C18:1 (ω-9)	23.71	23.47	23.75	0.12	NS
C18:2 (ω-6)	26.01	26.46	25.55	0.75	NS
C18:3 (ω-3)	0.69	0.71	0.65	0.02	NS
Total unsaturated fatty acids	49.34	49.13	49.83	0.84	NS
Total saturated fatty acids	50.66	50.87	50.17	0.84	NS
Ratio unsaturated / saturated	1.03	1.04	1.01	0.03	NS

[1]Fatty acids that are not listed were present for less than 0.5% in all samples.

[2] Data are means of three observations per treatment.

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Table 2. Ingredients and nutrient composition of the basal experimental diets[1] (Experiment 3)

Item (g/kg, unless noted)	Soybean Oil	Lecithin Oil
Wheat	541.6	541.6
Pura[2]	100.0	100.0
Soybean Meal (46.6% CP)	304.0	304.0
Soybean Oil	20.0	--
Lecithin Oil	--	20.0
Limestone	5.6	5.6
Salt	1.6	1.6
Sodium Bicarbonate	2.8	2.8
DCP	12.4	12.4
Lysine HCl	2.7	2.7
DL Methionine	3.4	3.4
Threonine	1.0	1.0
Ronozyme P5000	0.15	0.15
Ronzyme WX	0.15	0.15
Vit/Min Premix[3]	4.63	4.63
Calculated composition, %		
ME, MJ/kg	12.609 MJ/kg	
Crude Protein	22.47	
Crude Fat	6.207	
Crude Fibre	3.38	
Dig Lys	1.247	
Dig Met	0.623	
Dig Met+Cys	0.938	
Ca	0.898	
Av P	0.449	
Na	0.150	

[1] Lysolecithins (as LYSOFORTE® EXTEND) were added on top of the basal diet formulations at 500 ppm

[2] Pura is a commercially available blend of whole rapeseed and pulses (field beans or peas) which has been ground, heat treated and pelleted. Typical analysis 21% oil, 21% protein.

[3]Supplied per kilogram of diet: manganese, 99 mg; zinc, 60 mg; iron, 49 mg; copper, 20 mg; iodine, 1.2 mg; selenium, 0.4 g; vitamin A, 13,500 IU (retinyl acetate); vitamin E, 55 IU (DL-α-tocopheryl acetate); cholecalciferol, 75 µg; vitamin B_1, 2 mg; vitamin B_2, 5 mg; vitamin B_3, 30 mg; vitamin B_5, 15 mg; vitamin B_6, 4 mg; vitamin B_{12}, 2 mg; vitamin K, 2.5 mg; folic acid, 1 mg; biotin; 0.2 mg; choline, 600 mg; etoxyquine, 33 mg; butylated hydroxytoluene (BHT), 0.4 mg; propyl gallate, 0.12 mg; citric acid, 0.69 mg; sepiolite, 4 mg.

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Table 6. Growth performance of young broilers from day 0-21 fed diets formulated with soya oil or lecithin oil, with or without supplemental lysolecithin. (Experiment 3).

	Soya Oil	Soya Oil + Lysolecithin	Lecithin Oil	Lecithin Oil + Lysolecithin	Pooled SEM	P-value
Day 0-10[1]						
Weight at day 0, g	38.3	38.3	38.6	38.9	0.72	0.906
Average daily gain, g	21.2	21.5	20.8	22.0	0.41	0.198
Average daily feed intake, g	32.0	29.5	32.3	31.6	1.13	0.326
FCR	1.45	1.36	1.55	1.43	0.060	0.161
Day 10-21						
Weight at day 10, g	256.2^{ab}	258.9^{ab}	248.9^{b}	263.8^{a}	3.18	0.015
Average daily gain, g	61.3	61.4	59.4	63.4	0.11	0.105
Average daily feed intake, g	80.6	81.9	79.9	83.9	1.24	0.183
FCR	1.31	1.33	1.35	1.33	0.020	0.723
Day 0-21						
Weight at day 21, g	934.1^{ab}	941.6^{ab}	906.9^{b}	961.7^{a}	1.38	0.047
Average daily gain, g	42.5^{ab}	42.8^{ab}	41.3^{b}	43.9^{a}	0.50	0.036
Average daily feed intake, g	56.5	56.5	57.0	58.6	0.89	0.356
FCR	1.33	1.32	1.38	1.33	0.024	0.225
Energy conversion rate (MJ/kg BWG)	16.04	15.88	16.60	16.06	--	--

[1] Data are means of twelve observations per treatment.

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Figure 1. Hydrolysis of (A) triglycerides and accumulation of (B) free fatty acids, (C) diglycerides and (D) monoglycerides during the in vitro hydrolysis of palm oil (solid line), palm oil with soybean lecithin (dotted line) and palm oil with soybean lysolecithin (dashed line). The experimental treatments were carried out in triplicate. The mean concentrations of the lipids (mg/ml) are given over time (min), with error bars indicating the standard error values. Statistical analysis of the treatments is performed based on apparent rate constants (see Table 1). For enzyme kinetic comparison, apparent rate constants are used. (Experiment 1).

Figure 2. Absorption of monoglycerides (white bars) and free fatty acids (grey bars) generated during *in vitro* hydrolysis of palm oil, palm oil with soybean lecithin and palm oil with soybean lysolecithin by differentiated Caco-2 monolayers and expressed as percentage of applied monoglycerides end free fatty acids. Data are means of three or more observations per treatment, with error bars indicating the standard error values. (Experiment 1).

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