Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

Published on 28 September 2026
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Cite this: *Food Funct.*, 2026, **17**, 9234

Matrix matters: the importance of the food matrix in bioaccessibility and basolateral recovery of microalgal-sourced DHA

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Omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA), are known for their positive health effects. To meet recommended intake levels, people commonly consume fish and microalgal oils as supplements, typically in the form of encapsulated triacylglycerols (TAGs). However, ingesting omega-3 LC-PUFAs dispersed in a food matrix may be beneficial, as this has been shown to improve digestion for other oil types. This study investigated the *in vitro* bioaccessibility and basolateral recovery of DHA in DHA-rich microalgae oil in various forms: bulk oil, encapsulated oil, powdered oil, and oil incorporated into a protein matrix. After adapting the INFOGEST protocol for lipid digestion, we assessed bioaccessibility after *in vitro* digestion using back titration and gas chromatography coupled to a flame ionization detector (GC-FID). We used a Caco-2 cell model, adapted to take into account the lipid adhesion to the plastic plate, to assess basolateral DHA recovery. Results showed that the digestion of encapsulated oil and bulk oil was similar (n = 3). Powdered oil showed higher bioaccessibility than bulk oil (47.0 ± 2.5% for powder and 9.5 ± 0.7% for bulk, n = 3). In the Caco-2 model, powdered oil showed higher basolateral recovery (4.8 ± 0.9%) than bulk oil (1.2 ± 0.2%), although the experimental model limits interpretation (n = 3). To evaluate matrix effects, we designed a vegan cheese-like matrix (VCM) using different proteins. DHA in the VCM was 38% more bioaccessible than in bulk oil (n = 3), and we observed no differences in basolateral recovery. Our findings highlight that food matrices play a critical role in DHA bioaccessibility and basolateral recovery, potentially indicating increased intestinal uptake. Rather than a one-size-fits-all approach, methodological choices, including digestion protocols, transport models, and quantification strategies, should be selected based on the specific research question.

Received 16th June 2026,
Accepted 26th September 2026

DOI: 10.1039/d6fo02980a

rsc.li/food-function

Introduction

Omega-3 long-chain polyunsaturated fatty acids (ω-3 LC-PUFAs) have been linked to numerous health benefits, including improved cardiovascular health and cognitive functioning in neuroinflammatory diseases, such as Alzheimer's Disease.[1] The essential ω-3 LC-PUFA alpha-linolenic acid (ALA) can be elongated and desaturated into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[2] These ω-3 LC-PUFAs can be incorporated into cellular structures and play other key roles in the body, such as regulating anti-inflammatory responses.[1,3] However, the human body converts ALA into EPA and DHA inefficiently because intake is unbalanced in favor of ω-6 fatty acids, which compete for the same conversion pathway. As such, EPA and DHA should primarily come from the diet.[2]

The European Food Safety Authority recommends an adequate daily intake of 250 mg EPA plus DHA for adults, with fatty fish and seafood as the primary dietary source.[4] Nevertheless, the modern Western diet is characterized by a low intake of ω-3 LC-PUFAs.[5] Vegetarian and vegan diets are especially low in, or completely devoid of, EPA and DHA.[6,7] To compensate for this low intake, diets can be complemented with enriched foods or ω-3 oil supplements, which typically contain fish oil. However, fish oil poses technological and sustainability challenges[8,9] and is unsuitable for vegetarians and vegans. Marine microalgae provide a sustainable, plant-based

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alternative: some species of microalgae are naturally rich in EPA and DHA[10,11] and can be cultivated without freshwater or arable land.[12,13]

In encapsulated oil supplements, ω-3 LC-PUFAs are typically present as triacylglycerols (TAGs) or ethyl esters (EEs), which often show relatively low bioavailability because of inefficient digestion and absorption in the gastrointestinal tract.[14,15] These long-chain fatty acids require emulsification and enzymatic hydrolysis before enterocytes can absorb them, so limited digestion can directly impair bioavailability. Studies comparing fish oil supplements to diets rich in fatty fish often report weaker health outcomes from supplements, suggesting that both the natural fish matrix and the chemical form of DHA, typically triglycerides (TAGs) in fish *versus* TAGs and ethyl esters (EEs) in supplements, play important roles in facilitating bioavailability.[16,17] Importantly, these effects should be distinguished, as the food matrix may influence DHA bioavailability by modulating lipid emulsification and digestion, whereas DHA's chemical form can independently affect its susceptibility to enzymatic hydrolysis and subsequent absorption.

A food matrix can influence lipid bioaccessibility and uptake, although its effects are complex and depend on multiple factors, including emulsion structure, matrix composition, lipid organization, and processing conditions.[18–20] Consequently, the effects of a food matrix on lipid bioaccessibility and bioavailability reported in the literature are inconsistent. Several studies show that a food matrix can enhance lipid digestion by improving emulsification and increasing access to digestive enzymes.[21,22] Specifically, research shows that ω-3 LC-PUFAs have higher bioavailability when incorporated into structured matrices such as nano-emulsions or gelled emulsions than in bulk oils.[7,23] However, not all studies are consistent; one study showed similar DHA bioavailability between algae oil capsules and a fortified snack.[24] Given that consumers can increase ω-3 LC-PUFAs intake by consuming fish rather than fish oil supplements, whereas vegetarians rely largely on supplements as their primary source of ω-3 LC-PUFAs, developing plant-based food products with enhanced ω-3 bioavailability may provide a valuable alternative.

To accurately evaluate the effectiveness of different delivery structures, it is essential to choose analytical methods carefully to assess digestion and basolateral recovery. Particularly for complex formats such as encapsulated or powdered oils, standardized protocols are lacking or may be poorly suited. Existing analytical methods to assess the degree of digestion, such as back-titration, are simple and accessible but lack specificity for micellar lipid content, while more precise techniques, such as gas chromatography coupled to a flame ionization detector (GC-FID), require extensive optimization and validation.[25] Similarly, *in vitro* uptake models using Caco-2 cells are limited by issues such as lipid adhesion to plastic surfaces, which can significantly distort intestinal uptake (basolateral recovery) results. These methodological uncertainties highlight the need to comparatively validate available techniques and develop optimized workflows tailored to different oil delivery formats.

This study aims to investigate how different delivery forms of DHA from fish and algae, namely bulk oil, encapsulated oil, powdered oil, and oil incorporated into a structured protein matrix, affect their bioaccessibility and basolateral recovery in a standardized manner. To do so, we used various *in vitro* digestion methods based on the standardized INFOGEST protocol.[26] We compared a simple back-titration assay with a more advanced GC-FID-based micellar lipid quantification method to assess DHA bioaccessibility. We applied digesta to a Caco-2 Transwell® model to evaluate basolateral recovery and address practical challenges such as toxicity and lipid adhesion to plastic surfaces.[27] To further assess the effect of a structured matrix, we developed a vegan cheese-like protein matrix (VCM) and optimized the emulsifier composition to create a stable, digestible structure. By combining these models, we provide a framework for evaluating DHA bioaccessibility and basolateral recovery in various delivery formats.

Materials and methods

Materials

All materials were bought from Sigma-Aldrich Netherlands unless stated otherwise. Digestive enzymes and bile salts are: human salivary amylase (REF A1031), rabbit gastric extract (Lipolytech, France, REF RGE15), porcine pepsin (REF P7012), α-chymotrypsin from bovine pancreas (REF C4129), pancreatin from porcine pancreas (REF P7545), bovine bile (REF B3883), sodium taurocholate (REF T4009), and sodium glycodeoxycholate (REF G9910). All enzymatic activities were measured according to the INFOGEST 2.0 protocol.[26]

For GC-FID analysis, we used Triheptadecanoin (TAG C17:0/C17:0/C17:0, Larodan, Sweden, REF 33-1700) and Heneicosanoic Acid (C21:0, Larodan, REF 10-2100) as internal standards and PUFA2 (REF 47015-U), PUFA3 (REF 47085-U), FameMIX (REF CRM18920), and RM3 (REF O7256-1AMP) as external standards.

Encapsulated fish oil supplements used are MSC Visolie Omega 3 (Lucovitaal, Uden, Netherlands; capsule material: fish gelatin) and OMEGA-3 FISH OIL (Body & Fit, Amsterdam, Netherlands; capsule material: bovine gelatin). Encapsulated algae oil supplements used are Omega-3 Plantaardig (Flinndal, Haarlem, Netherlands; capsule material: modified corn starch) and DHA 550 ORIGINS® (Fermentalg, Libourne, France; capsule material: modified hydroxypropyl starch, E1440). All capsules were purchased at the local health food store, except for the DHA 550 ORIGINS®, which Fermentalg (Libourne, France) kindly provided (Table 1).

Fermentalg kindly provided the oil used for the bulk oil/powdered oil comparison: DHA 550-Y ORIGINS® (Fermentalg). The powdered oil: DHA ORIGINS® 170-P: Powder (Fermentalg). This powdered oil contains 307 mg g^{-1} oil and 188 mg g^{-1} DHA.

Ingredients used for preparation of the vegan cheese matrix were: Coconut oil (Biofood, Damhert Nutrition nv, Heusden-Zolder, Netherlands), algae oil (Alphamega3™ 400 MT algae oil), lupin protein concentrate (Inveja, REF FRALU-CON GF,

Table 1 Capsules used with their capsule material, oil source, and DHA content of the tested oil (mg g^{-1})

Commercial name	Company	Capsule material	Oil source	DHA concentration (mg g^{-1})
MSC visolie omega	Lucovitaal	Fish gelatin	Fish	166.8
OMEGA-3 FISH OIL	Body&fit	Bovine gelatin	Fish	99
Omega-3 plantaardig	Finndal	Corn starch	Micro algae	379.4
DHA 550 ORIGINS	Fermentalg	Hydroxypropyl starch	Micro algae	563.8

Twello, Netherlands), soy protein Isolate (FI&S, Maastricht, Netherlands), wheat protein isolate (Bob's Red Mill, Milwaukie, USA), trisodium citrate dihydrate (TSC, REF, S4641), calcium chloride dihydrate ($CaCl_2(H_2O)_2$, REF, C7902), NaCl (S9888), corn starch (Natuurlijk Natuurlijk, Ede, Netherlands), xanthan gum (Natuurlijk Natuurlijk), guar gum (Natuurlijk Natuurlijk), citric acid (REF 241), and potassium sorbate (Van Beekum Specerijen, Harderwijk, Netherlands).

Methods

Vegan cheese-like matrix preparation. Vegan cheese-like matrices were formulated using three different protein sources: lupin protein concentrate (LPC), wheat protein isolate (WPI), and soy protein isolate (SPI), which were selected for preliminary screening because they are commonly used in plant-based cheese formulations and differ in their techno-functional properties, allowing identification of the formulation producing the smallest lipid droplets for subsequent experiments. Each protein source required slight modifications to the preparation method. A Vorwerk Thermomixer was used for preparation, maintained at 40 °C for SPI-VCM and WPI-VCM formulations and at room temperature for LPC-VCMs. A schematic overview of the process is shown in SI Fig. S1, and ingredient compositions (in g per 100 g product) are listed in SI Table S1.

In brief, the protein source, trisodium citrate dihydrate (TSC), and water were mixed at 3.5 rpm for 1 min. Subsequently, NaCl was added for the SPI-VCM, and citric acid for the LPC-VCM and WPI-VCM, and mixed at 3.5 rpm for 1 min. After mixing, corn starch, xanthan gum, guar gum, potassium sorbate, and, for the LPC-VCM and WPI-VCM, $CaCl_2$, were added and mixed at 3.5 rpm for 3 min. Then, coconut oil was added and mixed at 4.5 rpm for 3 min. Finally, 1 g per 100 g Alphamega3™ 400 MT algae oil, and, for the SPI-VCM, citric acid were added and mixed for 30 s. For digestion experiments followed by Caco-2 cell exposure, the amount of algae oil was increased to 6.25 g per 100 g to enable accurate quantification *via* GC-FID. The VCMs were transferred into silicone ice cube trays, covered in aluminium foil, and left to harden overnight in the refrigerator at 4 °C. Samples were removed from the refrigerator immediately prior to use.

Structural analysis of the vegan cheese matrix. To investigate the physical structure of the vegan cheese matrix after 1 day, confocal laser scanning microscopy (CLSM) was used. After the vegan cheese preparation, the cheeses were frozen at −20 °C for 24 h to stabilize the structure before slicing. Thin slices were made and subsequently stained with 0.5 mg mL^{-1} 1% Rhodamine B to stain proteins, and 0.5 mg mL^{-1} BODIPY to stain lipids in 70% ethanol. The samples were incubated overnight and scanned. The images were visually analyzed using ZEN (ZEISS, Oberkochen, Germany).

Static simulated digestion *in vitro*. For the digestion of capsules *vs.* bulk oil, and method comparison, we used our 'in-house' *in vitro* digestion protocol described in Tomassen *et al.* (2023).[28] For the digestion of capsules, we adapted the enzyme activities of the in-house protocol to match the INFOGEST 2.0 protocol:[26,29] 75 U mL^{-1} salivary amylase, 60 U mL^{-1} gastric lipase, 2000 U mL^{-1} pepsin, and pancreatin was adjusted to 2000 U mL^{-1} lipase. We added 5 mM sodium taurocholate and 5 mM sodium glycodeoxycholate, representing bile salts.

All *in vitro* digestions were performed in three independent experiments (n = 3 biological replicates, n = 4 for corn starch), with each digestion condition prepared independently.

Characterization of lipid digestion using back-titration. To quantify the degree of TAG hydrolysis of the encapsulated oil and bulk oil from the capsules after static *in vitro* digestion, the samples were back-titrated to pH 11.5 using the pH-stat technique.[30–32] The obtained NaOH volumes were used to calculate the percentage of released free fatty acids (FFAs) (equation below). The number of moles of NaOH required to neutralize the FFAs is divided by the number of moles of FFAs that could have been produced from the TAGs if they were all digested. This equation assumes that two FFAs are produced per TAG molecule.

$$\mathrm{FFA}\% = \frac{V\,\mathrm{NaOH} \times M\,\mathrm{NaOH} \times M_{\mathrm{w}}\,\mathrm{lipid}}{2 \times W\,\mathrm{lipid}}$$

where V NaOH is the volume (L) of titrant consumed to neutralize the FFAs produced, M NaOH is the molarity (M) of the NaOH solution used, M_w lipid is the average molecular mass (g mol^{-1}) of the TAGs (963.6 for fish in fish gelatin capsule, 870.4 for fish in bovine gelatin capsule, 952.2 for algae in corn starch capsule, 980.6 for algae in hydroxypropyl starch capsule, and 740.6 for the VCM and bulk oil), and W lipid is the total mass (g) of lipid present in the sample used for titration. To control for the other products in the digesta, the volume of NaOH from an empty digesta was subtracted from the oil samples, and the volume of NaOH needed to digest only the capsule material was subtracted from the encapsulated oil. All experiments were performed in triplicate (n = 3), except for corn starch (n = 4).

Simulated digestion *in vitro* using pH-stat. For all other *in vitro* digestion experiments, we used a method based on the INFOGEST 2.0 protocol,[26] carried out in a pH-stat (Metrohm,

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REF 888 Titrando) with titration vessels thermostated to 37 °C and flushed with N_2. All *in vitro* digestions were performed in three independent experiments on different days (n = 3 biological replicates), with each digestion condition prepared independently. For the VCM digestion, the VCM was made on three different days for n = 3.

Porcine colipase was partly purified from pancreatin *via* the procedure described in Fernandez *et al.*[33] In brief, 10 g of porcine pancreatin was partly solubilized at 4 °C for 2 h in 100 mL of 20 mM Tris-HCl, 3.3 mM $CaCl_2$, 2 mM benzamidine, and 1 mM phenylmethanesulfonylfluoride (PMSF). The suspension was then centrifuged at 3000*g* at 4 °C for 30 min, and lipase activity was measured in the supernatant according to Grundy *et al.* (2021).[29] Subsequently, the supernatant was acidified to pH 2.0 and centrifuged at 3000*g* at 4 °C for 30 min, abolishing lipase activity. The resulting supernatant was neutralized to pH 5.0 with 1 M NaOH solution. Then, the supernatant was again centrifuged at 3000*g* at 4 °C for 30 min to remove precipitated proteins, and colipase was sampled in aliquots and stored at −20 °C.

Oral phase. The sample was mixed with water to a total weight of 5 g before the addition of simulated salivary fluid (SSF; 1 : 1, w/w). The initial sample weights were 4 g bulk algae oil (DHA 550 ORIGINS®), 2 g powdered algae oil reconstituted with 2 g water (1 : 1, w/w), 4 g VCM, or 1.14 g VCM-only oil, the latter corresponding to the amount of oil (0.25 g Alphamega3™ 400 MT algae oil + 0.89 g coconut oil) present in 4 g VCM. $CaCl_2$ was added, achieving a final concentration of 1.5 mM in SSF. When starch was present, 75 U mL^{-1} salivary amylase was added, and the mixture was incubated for 2 min.

Gastric phase. Simulated gastric fluid (SGF) was mixed with the bolus 1 : 1 (vol/vol) and $CaCl_2$ was added, achieving a final concentration of 0.15 mM. The pH was set to 3.0 using HCl (1 M). Subsequently, rabbit gastric extract and porcine pepsin were added to achieve activities of 60 U mL^{-1} gastric lipase and 2000 U mL^{-1} pepsin. The mixture was incubated for 2 h. A sample was taken at the end of the gastric phase and stored at −20 °C until further analysis.

Intestinal phase. In the last phase, simulated intestinal fluid (SIF) was added to the gastric phase, aiming at a final ratio of 1 : 1 (vol/vol). $CaCl_2$ was added to get a final concentration of 0.6 mM. For bile salts, we added 5 mM sodium taurocholate and 5 mM sodium glycodeoxycholate, or 10 mM of bovine bile, the pH was set to 7.0 using NaOH (1 M). Meanwhile, pancreatin was dissolved in SIF, sonicated for 1 min, and centrifuged at 3000*g* for 5 min at 4 °C. After adjusting the pH, co-lipase and the supernatant from the pre-treated pancreatin were added to achieve a lipase activity of 2000 U mL^{-1}. This mixture was incubated for 2 h. The end volume of the digesta was measured and supplemented to 50 mL with buffer (composed of SSF, SGF, and SIF). Samples were either flushed with nitrogen and stored at −20 °C until further analysis or centrifugated at 4000*g* for 30 min at room temperature to obtain the aqueous phase (bioaccessible layer) for GC-FID analysis.

During static digestion, the volume of NaOH required to maintain the pH at 7 was recorded over time. This was used to monitor lipid digestion, applying the same formula as in the back-titration method. For the VCM, a control sample without oil was included to account for pH changes arising from non-lipid components of the matrix.

For the micellar layer, we calculated the theoretical highest possible FA and DHA content using the formula below.

$$\text{Theoretical highest possible FA or DHA content} = \frac{\text{gram FA or DHA per gram product} \times \text{grams of starting material}}{\text{end volume digestion in mL}}$$

where grams of starting material are: 4 g bulk algae oil (DHA 550 ORIGINS®), 2 g powdered algae oil, 4 g VCM, or 1.14 g VCM-only oil (bulk oil), end volume of digestion is 50 mL.

Assessment of omega-3 basolateral recovery by a Caco-2 Transwell® system. To assess DHA basolateral recovery, we used an *in vitro* model representing intestinal cells: the Caco-2 Transwell® system. The DHA recovered in the basolateral compartment after incubation is referred to as basolateral recovery, which may reflect intestinal transport and uptake. Caco-2 cells (ATCC-HTB-37) were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g L^{-1}) and 25 mM HEPES (Gibco, REF 42430-025), supplemented with 10% fetal bovine serum (HyClone) at 37 °C with 5% CO_2 and used between passages 30 and 40. After reaching 95% confluency, the cells were cultured on 0.4 μm Transparent Transwell® membranes (Greiner Bio-one, REF 662640) for three weeks to facilitate differentiation into small-intestinal-like cells. During the final week, cells were apically serum-starved to minimize lipid interference.[28] Confirmation of cellular differentiation was conducted through measurement of *trans* epithelial electrical resistance (TEER) using the EVOM™ manual, proceeding only when TEER exceeded a predetermined threshold of 235 Ω cm^{-2} for 24-well inserts. Subsequently, 50× diluted digested samples were applied to the apical side of the cells and measured with TEER measurements at time points 0, 1, 3, 6, and 24 h to confirm membrane integrity. TEER measurements were normalized against media controls set to 100%. After 24 h, the basolateral media of technical triplicates were pooled and stored at −20 °C until further analysis. Experiments were performed with three biological replicates (n = 3 independent cell passages, or n = 4 for the bulk *vs.* VCM experiment), each with three technical replicates (n = 3 wells per passage).

The maximum FA or DHA possible in the Caco-2 media was calculated with the following formula:

$$\text{Theoretical highest possible FA or DHA content Caco-2 media } (\mu g) = \frac{C_{\text{FA/DHA,product}}(\text{mg g}^{-1}) \times m_{\text{product}}(\text{g})}{V_{\text{digest}}(\text{mL}) \times \text{DF}} \times V_{\text{apical}}(\text{mL}) \times 1000$$

where $C_{\text{FA/DHA,product}}$ is the concentration of FA or DHA in the oil or powder used (mg g^{-1}), m_{product} is the mass of product added to the digestion (g), V_{digest} is the final volume of the digesta (50 mL), DF is the dilution factor applied to the digesta before addition to the Caco-2 cells (50×), and V_{apical} is the volume of diluted digesta added to the apical compartment of

the Caco-2 Transwell (0.150 mL). The factor 1000 converts mg to μg.

For the powdered oil, the DHA concentration per gram of powder was calculated by multiplying the DHA concentration measured in the extracted oil by the fat content of the powder (30.7%).

To assess how much and where fatty acids (FAs), specifically DHA, adhered to the plastic surfaces of the Caco-2 Transwell® system, experiments were conducted with and without seeded Caco-2 cells. A total of 150 μL of a 50× diluted digest in cell culture medium was either collected directly for GC-FID analysis (to determine the initial FA content; ng initial) or applied to the apical compartment of the insert. This validation experiment was performed in technical triplicate ($n = 3$).

The percentage of FA or DHA that adhered to plastic surfaces or was in the cells and the distribution per compartment, were calculated using the following equations:

$$\%\ \text{of FA or DHA that sticks to plastic or present in cells} = 100\% - \left(\frac{100}{\text{ng initial}} \times (\text{ng basolateral} + \text{apical compartment})\right)$$

$$\%\ \text{of FA or DHA per compartment} = \frac{100}{\text{ng initial}} \times \text{ng FA or DHA in compartment/extract}$$

ApoB ELISA. To measure apolipoprotein B (ApoB) levels as a marker for chylomicron production, we used the APOB ELISA kit according to the manufacturer's protocol (antibodies-online.com, REF ABIN612664). ApoB levels were measured in three biological replicates, each analyzed in technical duplicate.

Fatty acid methyl ester preparation. To quantify the FAs present in our oils, digesta, and cell culture media, we used GC-FID. All derivatizations were performed in Kimax tubes pre-washed twice with chloroform/methanol (2 : 1 vol/vol).

Alkali derivatization for pure oil samples. Two mg of internal standard TAG standard C17:0/C17:0/C17:0 diluted in chloroform/methanol (2 : 1 vol/vol) was added to a Kimax tube, and the solvent was evaporated under N_2 flow. After evaporation, 10 mg of oil was added, followed by 2 mL hexane. The samples were vortexed until fully dissolved, and 0.5 mL 0.5 N NaOH solution in methanol was added to each sample. After vortexing, the samples were left to react for 15 min and centrifuged for 1 min at 3000*g*. The upper hexane phase with the FAMEs was diluted 5× in hexane before GC-FID measurements.

Acid derivatization for digesta and basolateral media. After centrifugation of sampled digesta, four layers were obtained (from top to bottom): an undigested oil layer, a cream phase, an aqueous/micellar (bioaccessible) phase – containing the released fatty acids – and a pellet with indigestible compounds. Approximately 100 mg of the aqueous/micellar phase was weighed in Kimax tubes and stored at −20 °C until further analysis. For the analysis of the basolateral media, 500 mg was freeze-dried overnight to remove all the water before derivatization. For the plastic extraction experiment, all cell culture media were removed. The outside of each Transwell® insert was rinsed by pipetting ACN around the exterior, and the insert was transferred to a clean 24-well plate. ACN was then added to the apical compartment of the Transwell® in the new plate, while acetonitrile was also added directly to the basolateral compartment in the original plate. Both were incubated for 1 h on an orbital shaker at 300*g*. After incubation, the ACN from both compartments was collected into a Kimax tube and evaporated under N_2 flow.

The FFA standard C21:0 (0.25 mg mL^{-1} for digesta and 0.0025 mg mL^{-1} for cell media) was dissolved in toluene and 400 μL of this mixture was added to the samples. Then, 600 μL of freshly prepared 8% HCl in methanol was added together with 3 mL methanol. The tubes were sealed hermetically, vortexed for 10 s, and heated in a heating block at 90 °C for 1 h. After the tubes were cooled down to room temperature, 1 mL of MilliQ water and 2 mL of hexane were added and the tubes were vortexed. The two phases were left to equilibrate for about 10 min. For the digesta, the hexane layer was directly collected in GC vials for GC-FID analysis. For basolateral cell culture media, the upper hexane phase was collected and concentrated 20 times under N_2 flow before GC-FID analysis.

GC-FID acquisition method. GC-FID analysis of the samples was carried out using a Thermo Scientific TRACE 1300 Series Gas Chromatograph (Waltham, US) equipped with a split/splitless injector and a flame ionization detector. The carrier gas used was hydrogen. The separation was conducted on a Famewax column (30 m × 0.25 mm ID × 0.25 μm dF). The oven program was: 200 °C isothermal for 1 min, 30 °C min^{-1} to 250 °C, isothermal for 4 min. The hydrogen carrier flow was 35 mL min^{-1}, and the injector operated in CT split mode at 240 °C. The detector temperature used was 240 °C.

Individual FAs were identified by comparison with external standards consisting of a wide variety of polyunsaturated FAs with known elution order. The data were analyzed using Xcalibur FreeStyle.

Results are expressed as concentration in mg g^{-1} or ng mL^{-1} using the following formula:

$$\text{Concentration (mg g}^{-1}) = ((\text{AUC}_{\text{FA}} \times m_{\text{IS}})/\text{AUC}_{\text{IS}})/m_{\text{oil}}$$

where AUC_{FA} and AUC_{IS} are the peak area for the FA of interest and the internal standard, respectively; m_{IS} is the mass of internal standard in mg; m_{oil} is the mass of oil in g.

Statistical analysis

The results are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism 10. Data distribution was assessed using the Shapiro–Wilk normality test prior to applying parametric statistical tests. For comparisons between two independent groups, two-tailed unpaired Student's *t*-tests were used when the data met the assumptions for parametric analysis. For experiments involving two experimental factors, ordinary two-way ANOVA followed by Šidák's multiple-comparisons test was performed. The significance threshold was set at $p \leq 0.05$. The number of biological replicates (n) is indicated

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in the respective figure legends. Biological replicates were considered the experimental unit; technical replicates, where applicable, were averaged before statistical analysis.

Results

Encapsulation does not affect lipid bioaccessibility of most capsule types, except hydroxypropyl starch

Using static digestion, we observed that capsule composition determined degradation patterns and oil release. Fish-based and bovine-based gelatine capsules swelled and deformed upon contact with the physiological buffer. Oil release occurred from halfway to the end of the gastric phase, respectively, before the capsule was fully degraded. Corn starch and hydroxypropyl starch-based capsules swelled, turned opaque, and released oil more gradually through partial detachment of capsule halves. We observed variability in oil-release timing, with starch-based capsules showing greater heterogeneity. Observations during the intestinal phase were limited because digestive transparency decreased.

To assess whether the encapsulation material influenced overall ω-3 digestion, we used *in vitro* digestion to determine oil bioaccessibility, after first chemically characterizing the oils. The DHA content differed considerably between the supplements, ranging from 99 mg g^{-1} in the bovine gelatin capsule to 563 mg g^{-1} in the hydroxypropyl starch capsule, with intermediate values of 166 mg g^{-1} and 380 mg g^{-1} in the fish gelatin and corn starch capsules, respectively (SI Table S2). Bioaccessibility was expressed as FFA release (%) and determined by back-titration of the digesta to pH 11.5 (Fig. 1). Encapsulation of DHA-rich oil in hydroxypropyl starch capsules significantly increased FFA % (p = 0.0225) compared with bulk oil. However, the capsules showed a large standard deviation in FFA release %. This supports the previous observation of the heterogeneity of starch-based capsules. Furthermore, lipid bioaccessibility did not differ significantly between the encapsulated oil and its bulk oil counterparts for fish gelatin, bovine gelatin, and corn starch.

Powdered algae oil shows higher bioaccessibility of DHA and total FA compared to bulk oil during *in vitro* digestion

We evaluated the digestion efficiency of ω-3 LC-PUFA-rich algae oil for total FA and DHA by comparing two forms: bulk oil and powder, both containing the same algae oil in different quantities. Because of matrix complexity, we could not perform back titration. Therefore, we analyzed the micellar layer after digestion, which predominantly contains FFAs and MAGs, which are the bioaccessible forms of digested TAGs, making it the most physiologically relevant fraction to assess bioaccessibility.

First, we calculated the maximum FA and DHA content per gram of bioaccessible layer using the FA and DHA concentrations of the samples (Fig. 2a). The bulk oil showed higher FA content (75.0 mg FA and 46.3 mg DHA per g) than the powder (12.3 mg FA and 7.0 mg DHA per g). This would indicate that the bulk oil form of DHA has a much higher initial concentration available before digestion. In the second analysis, we measured the actual bioaccessible DHA concentration after digestion (Fig. 2b and SI Table S3). The bulk oil outperformed the powder, although not significantly for DHA, yielding 10.3 ± 2.0 mg FA per g and 4.4 ± 0.3 mg DHA per g, while the powder yielded 5.5 ± 0.2 mg FA per g and 3.3 ± 0.2 mg DHA per g. These absolute values reflect the total amount of lipid available for biological exposure. To correct for the difference in initial DHA concentration before digestion, we calcu-

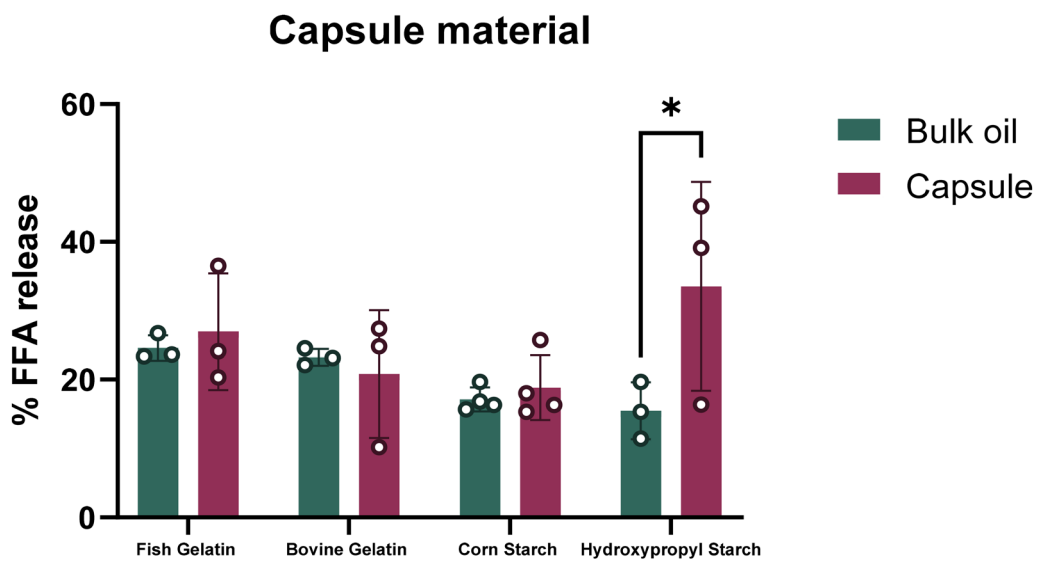

Fig. 1 Free fatty acid release percentage (% FFA release) of bulk oils and the same encapsulated oils of different encapsulation materials. From left to right: fish oil in fish gelatin capsule (n = 3), fish oil in bovine gelatin (n = 3), algae oil in corn starch (n = 4), and algae oil in hydroxypropyl starch (n = 3). Error bars represent SD. Data were analyzed using an ordinary two-way ANOVA followed by Sidak's multiple comparisons test. *p < 0.05.

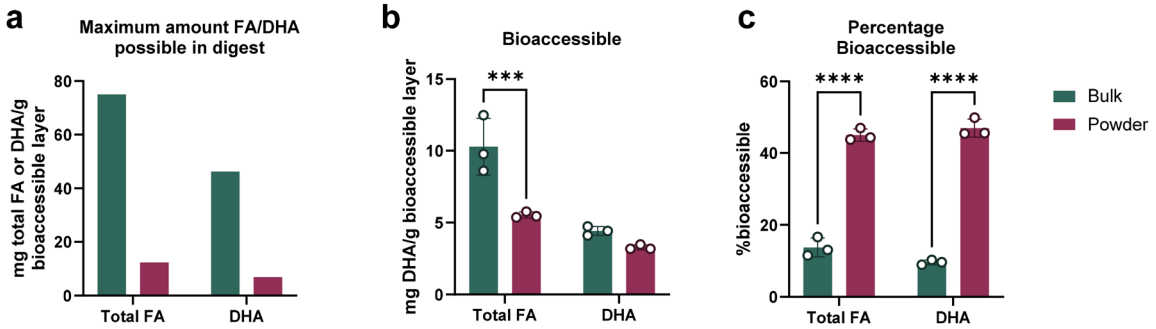

Fig. 2 (a) Theoretical calculation of the maximum amount (mg) of total fatty acid (FA) and docosahexaenoic acid (DHA) per g micellar phase of the digesta after centrifugation (bioaccessible layer), illustrating differences in the initial total FA and DHA content between bulk and powder. (b) Bioaccessible mg total FA and DHA per g bioaccessible layer, reflecting the absolute amount of lipid available for biological exposure (n = 3). (c) Percentage bioaccessible total FA and DHA, normalized to the initial FA and DHA content to determine the relative digestion efficiency of each formulation independent of differences in initial lipid content (n = 3). Error bars represent SD. Data were analyzed using an ordinary two-way ANOVA followed by Sidak's multiple comparisons test. ***: $p < 0.001$, ****: $p < 0.0001$.

lated the percentage of bioaccessible FAs while correcting for content (Fig. 2c and SI Table S3). The powder form showed a significantly higher percentage of bioaccessibility than the bulk oil for both total FA (45.5 ± 1.7% for powder and 13.7 ± 2.6% for bulk) and DHA (47.0 ± 2.5% for powder and 9.5 ± 0.7% for bulk). This normalization accounts for differences in the initial DHA content, allowing direct comparison of digestion efficiency between formulations. These results suggest that the powdered oil matrix facilitates greater total FA and DHA release during digestion than the bulk oil form.

Optimization of the Caco-2 Transwell® model for intestinal uptake assessment after digestion for DHA measurements

To evaluate basolateral recovery and predict intestinal transport and uptake of DHA-rich oils, we used a Caco-2 Transwell® model and quantified the FAs that passed to the basolateral compartment by GC-FID. Before this, we validated the GC-FID method to ensure the cell culture medium did not interfere with measurements. Specifically, we spiked DMEM with pure FFA DHA and analyzed the spiked DMEM directly or freeze-dried it. Thereafter, DHA recovery was quantified. We observed that a 500 μL DMEM volume did not affect FA quantification, whereas 2 mL DMEM led to overestimation, which freeze-drying resolved (SI Fig. S2a). We further validated the impact of freeze-drying in an experimental setting using digesta, confirming no significant effect on DHA quantification in the fully diluted digesta, apical, or basolateral compartments (SI Fig. S2b). Consequently, we freeze-dried the digesta to eliminate potential media interference.

To optimize digesta dilution for apical application in the Caco-2 model, we tested a range of dilutions (10×, 20×, 30×, 40×, 50×, 75×, 100×, 150×, 200×, and 400×) and monitored TEER at 0, 1, 3, 6, and 24 h. A 10× dilution significantly reduced TEER (30.8 ± 2.6% relative to the media control), indicating cytotoxicity (SI Fig. S3a, b, d and e), whereas cytotoxicity was not observed at other dilutions. DHA quantification in the basolateral compartment revealed a linear response between 50× and 150× dilutions (SI Fig. S3c). To maximize DHA concentration while maintaining linearity, we selected a 50× dilution for subsequent experiments.

Given the potential cytotoxicity associated with bile salts in the INFOGEST protocol, we compared the INFOGEST protocol with standard bovine bile, the INFOGEST protocol with individual bile salts (5 mM sodium glycodeoxycholate and 5 mM taurocholic acid sodium), and an in-house digestion protocol with individual bile salts (±4 mM sodium glycodeoxycholate and ±4 mM taurocholic acid sodium) in terms of total FA and DHA bioaccessibility, Caco-2 cell viability (TEER), chylomicron formation, and basolateral recovery. While the standard bovine bile tended to increase DHA bioaccessibility, total FA levels remained comparable across conditions (SI Fig. S4a and b). TEER measurements confirmed no cytotoxicity in Caco-2 cells across all digestion methods (SI Fig. S4e and f). Chylomicron formation increased with individual bile salts and the in-house protocol compared with standard bile salts (SI Fig. S4c). In the basolateral compartment, both versions of the INFOGEST protocol yielded higher DHA levels than the in-house digestion method (SI Fig. S4d). Therefore, we used the adapted INFOGEST protocol, which uses individual bile salts rather than standard bovine bile, for further experiments.

Lipid adherence to inserts prevents complete mass balance

Because lipids tend to stick to plastic, we tested whether we could recover our initial FA concentrations, specifically DHA. To investigate this, we used inserts with and without Caco-2 cells, added digesta to the apical side of the Transwell®, and measured FA content in all media compartments of the insert. Indeed, a large amount of FA stuck to the insert (Fig. 3a).

To reduce this, we added BSA to the basolateral compartment beforehand to see whether it would 'coat' the wells and prevent sticking. After 24 h, we collected media from the apical and basolateral compartments separately, extracted plastic-bound lipids from each compartment, and measured total FA and DHA content. DHA and total FAs were below the detection limit in Caco-2 cells (data not shown). Because DMEM contains background FAs that contribute to the measured FA and

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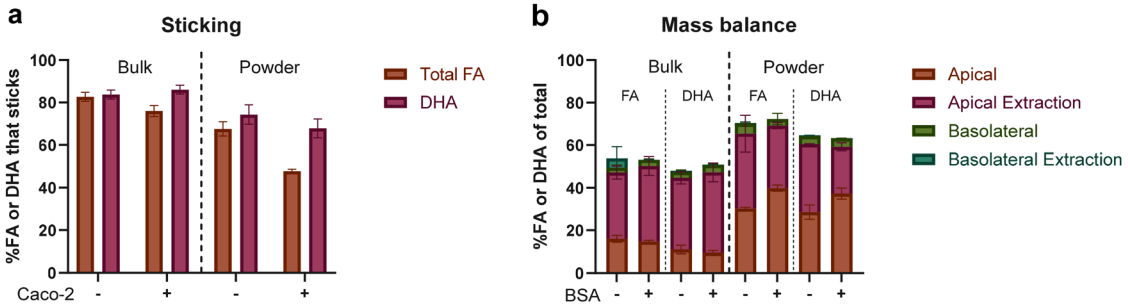

Fig. 3 (a) Percentage of total fatty acids (FA) or DHA adhering to the plastic surface of inserts, either with differentiated Caco-2 cells (+) or empty inserts (–), for bulk oil and powdered oil digests. (b) Mass balance showing the percentage recovery of total FA or DHA in each compartment of inserts containing differentiated Caco-2 cells. Fatty acids were quantified from the cell culture medium and from extracts of the insert plastic for both compartments, for both bulk and powdered oil digests, with or without basolateral addition of bovine serum albumin (BSA) (n = 3).

DHA concentrations, we subtracted the values measured in the used DMEM before calculating the percentages of total FA and DHA recovered in each compartment and after extraction. Both total FA and DHA tend to remain in the apical compartment, and adding basolateral BSA provided no benefit (Fig. 3b). We could not achieve a 100% mass balance. Therefore, although samples can be compared directly within the same set of experiments, we cannot draw conclusions about intestinal DHA uptake in *in vitro* models.

Basolateral recovery of DHA-rich algae oil in bulk and powder form

To assess basolateral recovery from bulk and powdered DHA-rich oils, we applied digested samples (pH-stat digestion) to a Caco-2 Transwell® model and quantified FA concentrations in the basolateral medium (SI Table S3). We measured TEER at 0, 1, 3, 6, and 24 h and observed no effect on membrane integrity (SI Fig. S5a and b). To calculate the percentage of total FA and DHA basolateral recovery by the cells, we first calculated basolateral FA and DHA concentrations. Here, we assume complete translocation from the bioaccessible part of the digest to the basolateral side. If all bioaccessible FAs were transported to the basolateral side, this would be measured as 225 μg total FA and 139 μg DHA for bulk oil, and 37 μg total FA and 21 μg DHA for powdered oil (Fig. 4a). Total basolateral FA recovery was significantly higher in bulk oil (3.8 ± 0.7 μg) than in powdered oil (2.6 ± 2.1 μg). In contrast, DHA concentrations did not differ significantly between the two formulations (1.6 ± 2.5 μg *vs.* 1.0 ± 2.0 μg, respectively; Fig. 4b), which better represents the biological situation. However, when expressed as a percentage of the theoretical maximum, basolateral recovery of total FA and DHA was significantly higher for the powdered oil (7.2 ± 0.6% and 4.8 ± 0.9%, respectively) compared to bulk oil (1.7 ± 0.3% and 1.2 ± 0.2%, respectively; Fig. 4c); these normalized values account for the differences in initial FA and DHA content. These findings highlight the matrix's influence on DHA digestion efficiency, as the powdered formulation showed greater bioaccessibility than bulk oil. Of note, because FAs stuck to the plastic, as indicated previously, these *in vitro* results cannot be extrapolated to the *in vivo* bioavailability of these specific samples.

Lupin protein outperforms soy and wheat protein in forming lipid droplets in a vegan cheese-like matrix

To further investigate the effect of oil incorporated into a matrix, we formulated three VCMs based on soy (SPI), lupin

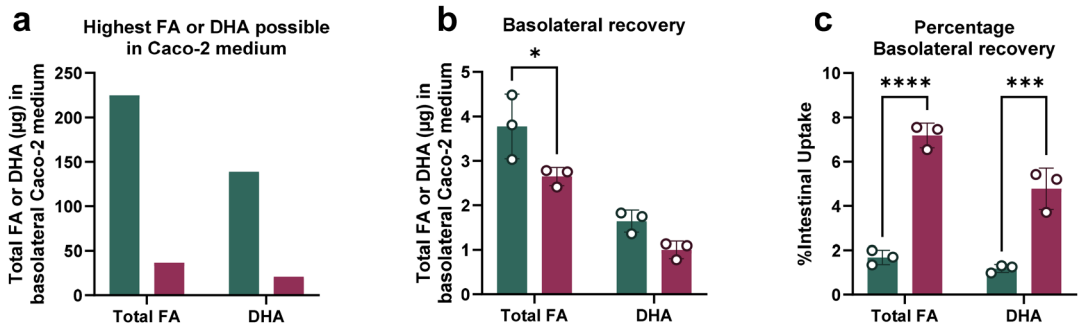

Fig. 4 (a) Calculation of the highest possible μg of total fatty acid (FA) and docosahexaenoic acid (DHA) in basolateral Caco-2 medium after 24 h incubation with digested bulk oil (green) and powder (red), illustrating differences in the initial total FA and DHA content. (b) Basolateral recovery: μg total FA and DHA in basolateral media (n = 3), representing a more biological situation. (c) Percentage basolateral recovery of total FA and DHA (n = 3); these normalized values account for the differences in initial FA and DHA content. Error bars represent SD. Data were analyzed using an ordinary two-way ANOVA followed by Sidak's multiple comparisons test. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$.

(LPC), and wheat (WPI) protein. Macroscopically, all three matrices showed a cheese-like structure. We investigated lipid droplet size and structure by staining the matrices with Bodipy (green) to visualize lipids and Rhodamine B (red) to localize protein, as shown in Fig. 5. Notably, the LPC-based matrix exhibited the smallest lipid droplets with a consistent protein structure. In contrast, SPI displayed lipid droplet formation to a lesser extent, with protein clustering. WPI showed network formation, preventing lipid droplet formation. In summary, LPC emerged as the most suitable protein source for generating small lipid droplets. For this reason, we used lupin protein concentrate for the rest of this study.

Microalgae-derived DHA is more bioaccessible in a VCM than in bulk oil

To compare DHA bioaccessibility between VCM and bulk oil, we first determined the FA composition of the algae oil and coconut oil used in the formulation (SI Table S4). In line with manufacturer specifications, DHA was the only ω-3 LC-PUFA detected in the algae oil, constituting 46% of total fatty acids. As expected, coconut oil primarily consisted of medium-chain FAs, including lauric, myristic, and palmitic acids.[34] Both the VCM and bulk oil contained the same amount of lipids and DHA. Titration during digestion showed a significantly higher percentage of FFA released at the end of digestion in VCM (76.6 ± 5.7%) than in bulk oil (53.8 ± 0.6%) (p = 0.0024) (Fig. 6a and SI Fig. S6a–c), indicating enhanced lipid hydrolysis. This finding was corroborated by analysis of the micellar phase, where we assessed bioaccessibility by the concentration of free DHA and monoacylglycerol-DHA (MAG-DHA) after digestion. The total FA concentration in the micellar layer was 21.0 ± 1.6 mg FA per g in the VCM, compared with 16.4 ± 0.9 mg FA per g in the bulk oil (Fig. 6b). Specifically, DHA bioaccessibility was 2.28 ± 0.10 mg DHA per g in the VCM micellar layer, compared with 1.39 ± 0.11 mg DHA per g in the bulk oil (Fig. 6c). Overall, DHA bioaccessibility was 38% higher in the VCM than in the bulk oil, while total FA bioaccessibility increased by 20% (Fig. 5d).

Basolateral DHA recovery across Caco-2 monolayers is comparable between VCM and bulk oil

Finally, we investigated basolateral recovery from VCM and bulk algae oil using the Caco-2 Transwell® model. TEER measurements over time indicated low cytotoxicity (SI Fig. S7a and b). Notably, consistent trends were observed across empty digesta, VCM, and oil samples, indicating that changes in TEER relative to the media control were primarily enzymatic in nature rather than sample-induced. Analysis of the basolateral

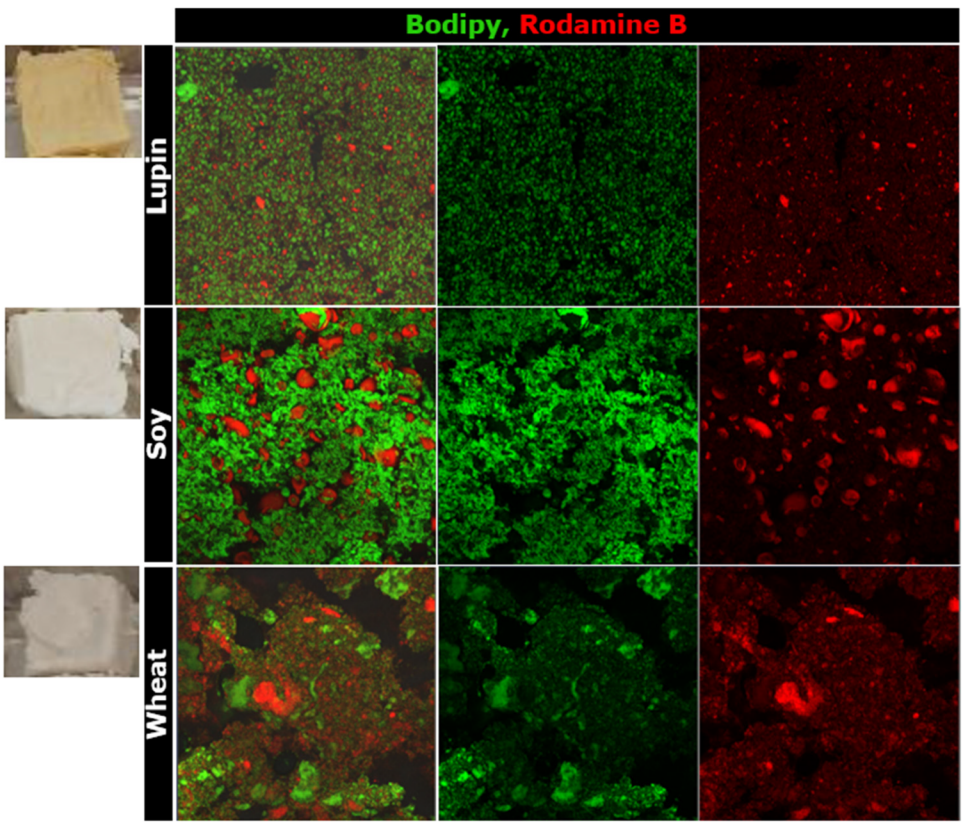

Fig. 5 The vegan cheese matrix with different protein sources: lupin protein concentrate (LPC), which forms small lipid droplets; soy protein isolate (SPI), which forms lipid droplets to a lesser extent; and wheat protein isolate (WPI), which shows no droplet formation. The photo on the right shows the macroscopic structure of the vegan cheese matrix; the images on the left are confocal images with Bodipy (green) for lipids and Rhodamine B (red) for proteins; the image size is 1.76 μm × 1.76 μm.

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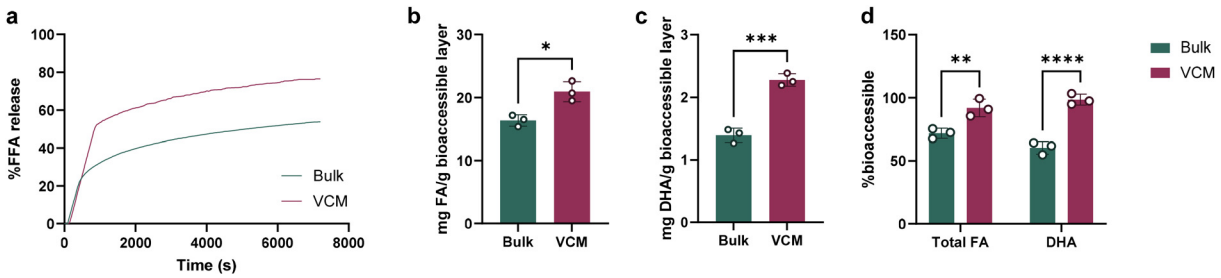

Fig. 6 Bioaccessibility of the bulk oil (green) and VCM (red) (n = 3). (a) Percentage free fatty acid (% FFA) release over time (s) during the intestinal phase of digestion. Content of fatty acid (FA) (mg FA per g micellar layer) for (b) total FA, and (c) Docosahexaenoic acid (DHA) after digestion, representing the more biological situation (n = 3). (d) % Bioaccessible total FA and DHA compared with the starting material, representing relative digestion efficiency for each form independent of differences in initial lipid content (n = 3). Error bars represent SD (b and c). Data were analyzed using a two-tailed unpaired t-test after assessing normality with the Shapiro–Wilk test. (d) Data were analyzed using an ordinary two-way ANOVA followed by Sidak's multiple comparisons test. *:$p < 0.05$, **:$p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

medium revealed comparable total FA and DHA concentrations, as well as chylomicron production, after 24 h incubation, suggesting no significant difference (Fig. 7a–c). These findings suggest that, despite increased DHA bioaccessibility in VCM compared with pure oil, DHA absorptive capacity remains unaltered *in vitro*.

Discussion

This study shows that incorporating algae oil into protein matrices significantly enhanced DHA bioaccessibility compared with bulk oil. However, this higher bioaccessibility did not consistently translate into increased basolateral recovery in the Caco-2 Transwell® model, likely reflecting inherent limitations of the *in vitro* system. Nonetheless, models such as the lipid INFOGEST protocol coupled to a Caco-2 Transwell® system, as optimized here, remain valuable tools for early-stage screening of lipid bioaccessibility between different samples. They provide a controlled, reproducible environment to compare lipid sources and predict relative *in vitro* basolateral recovery trends. This study demonstrates how such a model can be refined to maximize its translational relevance while minimizing reliance on animal or human studies in the initial phases of research.

Lipid digestion, unlike protein digestion, is not yet fully standardized, although Tormási *et al*. showed that the addition of rabbit gastric lipase to the INFOGEST 2.0 protocol significantly improved FA digestion compared to the 1.0 protocol.[26,35] We tested this lipid-optimized INFOGEST protocol,[26] both with bovine bile and individual bile salts, against our in-house protocol,[28] and found that the digestion protocol strongly influenced lipid release. Compared with our in-house method,[28] the lipid-optimized INFOGEST protocol,[26] including gastric lipase and higher lipase activity, yielded greater total FA and DHA release. Bovine bile extract slightly outperformed individual bile salts, likely due to additional emulsifying components.[26] Based on these findings, we strongly recommend using the lipid-optimized INFOGEST protocol, including gastric lipase, for studies assessing lipid digestion. We prefer bovine bile extract when the primary outcome is lipid digestion, whereas individual bile salts may be more suitable for downstream cell culture experiments where bovine bile extract could interfere with cellular responses.

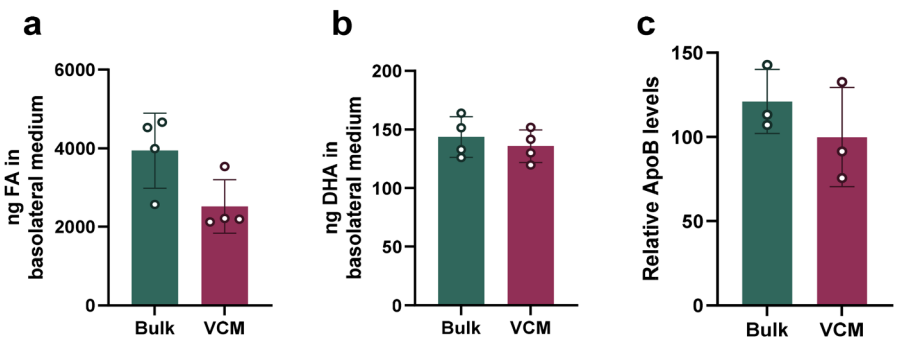

Fig. 7 Basolateral recovery of *in vitro* digested bulk oil and Vegan Cheese Matrix (VCM) across a Caco-2 Transwell® model (24 h incubation) and chylomicron formation. (a) Basolateral recovery of total fatty acids (FA) in basolateral medium (n = 4) and (b) ng of docosahexaenoic acid (DHA) in basolateral medium (n = 4); (c) chylomicron formation by ApoB ELISA of the VCM and algae oil, normalized to the empty digest (n = 3). Error bars represent SD. We analyzed data using a two-tailed unpaired t-test after assessing normality with the Shapiro–Wilk test. Differences were statistically non-significant.

To assess the bioaccessible percentage of the digested samples, we used both back-titration and GC-FID. Although back-titration is suitable for some experiments and relatively easy, GC is needed for complex matrices. Back-titration of % FFA requires proper controls, which are not feasible for complex products. This limitation arose with commercial vegan omega-3 matrices, so we assessed digestion efficiency *via* micellar fraction analysis instead.[36–38] We therefore recommend GC-FID for assessing bioaccessibility, as it can be applied to a wide range of sample matrices, including complex formulations, facilitating more robust comparisons between studies and laboratories.

It should be noted that lipid bioaccessibility was operationally defined in this study as the amount of FAs or DHA recovered in the aqueous fraction following *in vitro* digestion and transmethylation. This aqueous fraction, commonly referred to as the bioaccessible fraction, predominantly contains mixed micelles and vesicles composed of bile salts, phospholipids, and lipid digestion products, and is therefore widely used to assess lipid bioaccessibility in static *in vitro* digestion models.[39] However, recent methodological studies by Rivera *et al.* (2026) have demonstrated that different analytical approaches, such as selective FFA quantification *versus* total FAME analysis of the bioaccessible fraction, can yield substantially different quantitative estimates of lipid bioaccessibility.[40] Moreover, the recent consensus paper by Grundy *et al.* (2025) recommends distinguishing the biological concept of bioaccessibility from the analytical method used to quantify it.[41] Therefore, the values reported here should be interpreted as an operational measure of lipid bioaccessibility, while the relative differences between samples remain directly comparable because all formulations were analyzed using the same digestion and analytical workflow.

Importantly, favorable outcomes from *in vitro* digestion alone do not necessarily translate to improved basolateral recovery. Therefore, to further assess the samples, we coupled the digested samples to a basolateral recovery protocol. For this, we used the Caco-2 Transwell® model, selected an appropriate digestion protocol, and established a reliable quantification method. First, coupling lipid digesta to basolateral recovery assays, such as the Caco-2 Transwell® model, is challenging because lipids are not always measurable basolaterally.[42] In this study, a 50× dilution of digested samples was necessary to minimize cytotoxic effects on Caco-2 cells; this dilution is used before[28] and this dilution likely further reduced detectable basolateral lipid levels. Although using individual bile salts, as applied here, can improve cell compatibility, additional measures such as purified digestive enzymes may further reduce cytotoxicity, albeit at a substantially higher cost.[26]

Despite using an optimized protocol, basolateral DHA levels remained low, with substantial lipid loss to the plastic (up to 80%) and retention in the apical compartment. Further optimization of digestion and recovery is therefore essential. Furthermore, performing a full mass balance is essential, as lipid losses (*e.g.*, adsorption to plastic) can otherwise lead to underestimation of basolateral recovery. This strategy should be carefully optimized to ensure accurate interpretation of lipid uptake. GC-FID quantification of DHA in the basolateral medium also required optimization. Low FA levels and high water content interfered with FAME preparation.[43] Freeze-drying before derivatization resolved this and improved analytical reliability. Although these methodological limitations may affect the absolute quantification of basolateral recovery and preclude direct comparison with *in vivo* uptake, the model remains valuable for comparative assessment of lipid formulations under standardized experimental conditions.

The basolateral recovery results should therefore be interpreted as comparative outcomes within the Caco-2 Transwell® system rather than as quantitative estimates of intestinal DHA uptake *in vivo*. The model enables direct comparison of relative basolateral recovery between formulations under standardized experimental conditions but cannot predict physiological uptake. Therefore, the Caco-2 Transwell® system should primarily be regarded as a screening tool for selecting promising lipid formulations for subsequent *in vivo* validation.

The optimized protocol allowed us to compare the relative efficiency of different lipid formulations. With this in place, we assessed the bioaccessibility of bulk, encapsulated, and powdered oils. When comparing bulk oil to encapsulated oil, we observed no relevant differences in digestion efficiency, supporting previous findings that capsule materials effectively carry DHA.[44] As expected, the powder showed higher DHA bioaccessibility than the bulk oil when normalized to the amount of starting material. Improved emulsification likely improves access for digestive enzymes that attach to the surfaces of smaller lipid droplets. This results in a greater surface area for binding in the emulsified samples.[21,22,43]

Because we observed differences in the bioaccessibility of bulk and powdered oils, we used these samples to mimic basolateral recovery in the Caco-2 Transwell® model. Although total FA levels in the basolateral compartment were significantly higher in the bulk oil compared to the powdered oil, DHA levels were comparable between the two. Because the starting lipid and DHA concentrations in the bulk oil were approximately five times higher than in the powdered oil, this suggests that the powdered oil was markedly more efficient at delivering DHA across the Caco-2 monolayer. These findings support the idea that incorporating DHA into a matrix enhances its basolateral recovery, likely due to improved emulsification and enzyme accessibility during digestion.[21,22]

We would like to highlight that the differences we saw are mainly in the normalized results. We used this normalization because the initial FA and DHA levels were not the same. However, we should note that normalization is not completely fair because the enzyme-to-oil ratio differs, so it does not reflect the biological situation as the absolute values do. Therefore, it would be better to use the same amount of FAs and DHA in the bulk oil and matrix samples. Based on this rationale, we developed a custom food matrix enriched in vegan omega-3: the vegan cheese matrix. In this matrix, we

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could standardize DHA content, which is not possible with the commercial powdered formulation.

Results show that incorporating algae oil into the vegan cheese-like matrix significantly increased DHA bioaccessibility compared with bulk algae oil. This was also true for total FAs, although the matrix effect on DHA was slightly more pronounced. The literature strongly supports higher ω-3 LC-PUFA digestion in a lipid-containing matrix in the form of a nanoemulsion or gelled emulsion, which we also show in an emulsion-based real food product. Studies show that incorporating algae oil-derived ω-3 LC-PUFAs in a nanoemulsion[7] and fish oil-derived ω-3 LC-PUFAs in a gelled emulsion[45,46] enhanced their bioavailability compared with bulk algae oil and gelatine-encapsulated fish oil, respectively. This effect likely results from reduced lipid droplet size, enhanced lipid dispersion, and stabilization of the oil–water interface, all of which increase triglyceride accessibility to digestive lipases and promote efficient micelle formation. Smaller lipid droplet size increases the surface area-to-volume ratio and thereby enhances the accessibility of triglyceride ester bonds at the oil–water interface to lipases (*e.g.*, pancreatic lipase).[7,23] This emulsion of ω-3 LC-PUFAs more effectively assists with lipid digestion as compared to the bulk oil in capsules.

Importantly, these findings suggest that enhanced DHA bioaccessibility is not attributable to the food matrix alone, but to the formulation's physicochemical characteristics. During matrix development, we screened different protein sources and selected the formulation that produced the smallest lipid droplets for subsequent experiments. Besides droplet size, the protein matrix may also contribute by stabilizing the oil–water interface and limiting droplet coalescence during digestion,[47,48] thereby facilitating lipase access and ultimately promoting lipid digestion. Although the present study was not designed to distinguish the relative contribution of these mechanisms, the results highlight that lipid dispersion, interfacial properties, and matrix microstructure together determine the digestion efficiency of DHA-containing food matrices.[20,49]

Recent work on structured fish oil emulsions further demonstrated that interfacial properties influence lipid digestion. Specifically, protein conformation and interfacial stability affected FFA release, with a more stable interface delaying intestinal digestion.[50] This supports the importance of interfacial properties in determining the digestion of ω-3 LC-PUFA-containing systems. However, the direction of this effect may depend on the specific formulation and interfacial characteristics.

Unexpectedly, despite the higher DHA bioaccessibility observed in the VCM digests, basolateral DHA recovery in the Caco-2 Transwell® model remained similar between the VCM and bulk oil. This finding challenges the implicit assumption that increased micellar DHA on the apical side will translate proportionally into basolateral recovery. Such a relationship likely holds only at low concentrations; beyond a certain threshold, uptake mechanisms may become saturated, limiting further translocation.

DHA transport across enterocytes is not strictly passive. LCFAs can be absorbed *via* multiple pathways, including passive diffusion, transporter-mediated uptake (*e.g.*, CD36/FAT, FATP), and intracellular trafficking to the ER and Golgi prior to chylomicron assembly. Thus, a higher apical concentration does not necessarily yield greater basolateral DHA levels, particularly when transporters are saturated or intracellular processing capacity is exceeded. These findings indicate that apical micellarization alone does not determine how much DHA ultimately reaches the basolateral compartment. One possible explanation is that transport and intracellular processing become limiting, so higher micellar DHA concentrations do not necessarily result in greater basolateral export. In addition, limitations of the Caco-2 model, including reduced chylomicron secretion and basolateral lipid release compared with the human intestine, may contribute to the discrepancy between micellarization and basolateral recovery. Furthermore, our mass balance experiments demonstrated substantial adsorption of fatty acids to the Transwell® inserts, which may have further reduced the apparent recovery of DHA.

Given the diversity of lipid digestion methods currently in use, future research should prioritize developing a standardized lipid bioaccessibility and basolateral recovery protocol, analogous to INFOGEST 2.0 for protein, coupled with physiologically relevant models such as the Caco-2 Transwell® system. The experimental results presented here provide a foundation for such efforts, highlighting how model refinements can improve comparability and translational relevance across studies. Optimizing *in vitro* models for early-stage screening also reduces reliance on animal and human studies, accelerating the development of safe, effective, and sustainable ω-3-enriched products for public health.

Conclusion

This study shows that incorporating algae oil into a protein matrix significantly enhances DHA bioaccessibility compared with bulk oil, although this improvement did not always translate into higher basolateral recovery in the Caco-2 Transwell® model. These findings indicate that while matrix incorporation optimizes lipid digestion through improved emulsification and enzyme accessibility, intestinal uptake mechanisms or limitations of the *in vitro* model may ultimately constrain apparent basolateral recovery. By optimizing both the lipid digestion protocol and the Caco-2 assay, this study provides a robust, reproducible framework for assessing lipid bioaccessibility and basolateral recovery between samples. Although the Caco-2 assay enables standardized comparison between lipid formulations, it should be regarded as a comparative *in vitro* screening tool rather than a quantitative predictor of intestinal DHA uptake *in vivo*. Future efforts should focus on further standardizing bioaccessibility/uptake assays, refining mass balance approaches, and integrating physiologically relevant conditions to better predict *in vivo* outcomes. Ultimately, improving ω-3 delivery in plant-based food products could

help close nutritional gaps in populations with low fish intake, contributing to better long-term health.

Author contributions

Daniëlle Wessels: conceptualization, methodology, data collection & curation, formal analysis, visualization, writing – original draft, review & editing. Laura Leduc: methodology, data collection, formal analysis, review. Marie Hennebelle: conceptualization, methodology, data curation, review. Shanna Bastiaan-Net: conceptualization, methodology, review. Paula Perez Pardo: review. Lonneke Janssen Duijghuijsen: conceptualization, methodology, review. Vincenzo Fogliano: conceptualization, methodology, review. Nicoletta Pellegrini: conceptualization, methodology, data curation, review. Tamara Hoppenbrouwers: conceptualization, methodology, data curation, review.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data supporting this article have been included as part of the supplementary information.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6fo02980a.

Acknowledgements

This work was supported by the Dutch Ministry of Economic Affairs Top Sector Agri&Food [grant number LWV21.160. It takes guts to BBB: Using sustainable omega-3 rich oils to improve gut-brain health]. The authors gratefully acknowledge Renata Ariens for introducing them to the INFOGEST protocol, Monic Tomassen for her valuable expertise in Caco-2 cell culture, Peter de Gijsel for his assistance with GC-FID analysis, and Ghina Kotob for her help with the microscopy pictures of the different cheeses.

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