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Buttermilk as a Potential Wall Material for Delivering Algal Oil: an *In Vitro* and *In Vivo* Study

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ABSTRACT: The buttermilk was selected as a wall material to deliver the docosahexaenoic acid (DHA) in this current research, including algal oil encapsulating with pure buttermilk (BMO) and its mixture (buttermilk and maltodextrin (BMMO)). The results showed that the particle growth rate of BMO and BMMO was slower than that of commercial algal oil powders (CMOs) during simulated digestion *in vitro*. Moreover, in the Morris water maze experiment, the mice in BMO and BMMO groups took less time to find the platform compared to that in CMO group, and their DHA content in the brain was significantly higher. The immunoglobulin detection revealed that feeding BMO and BMMO could improve the immune function of rats. Therefore, buttermilk will be a potential wall material which are able to improve the digestion characteristics of algal oil and the DHA bioavailability, and these results also promote the value-added utilization of by-products in the dairy industry.

Keywords: Buttermilk, DHA, *In vitro* digestion, *In vivo* experiment, Bioavailability

1. Introduction

Buttermilk (BM) is the by-product released by whipped cream in butter production and processing, which is mainly classified into sweet BM and whey BM. The composition of BM is similar to skim milk, such as protein, lactose, ash, etc., while BM is rich in polar lipids due to the large amount of milk fat globules membrane that enter BM during its production process [1]. The polar lipids in BM is mainly composed of glycerophospholipids (phosphatidylcholine, phosphatidyl ethanolamine, phosphatidylinositol, and phosphatidylserine), sphingomyelins, and gangliosides, whose concentration is about 6 times that of skim milk [2]. Some studies have shown that these polar lipids are rich in polyunsaturated fatty acids, such as linoleic acid, linolenic acid and docosahexaenoic acid [3]. They play an indispensable role in the development of infants, with the effect of improving cognitive ability, promoting neurodevelopment, anti-inflammatory, and so on. In addition, phosphatidylcholine and sphingomyelins of BM can provide choline, resulting in that

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BM can regard as a potential source of choline for infant formula [4]. Despite the economic slowdown in recent years, the annual BM output is high: according to statistics, a total of 122,000 metric tons of BM was produced in Canada in 2021, and 10.98 thousand tons of BM was produced in Hungary in 2023 [5,6]. The yield of butter in China reached 10.9 thousand tons in 2022. The BM yield during the churning process can reach about 35%, indicating that the BM industry can be further exploited in China [7]. Due to the higher yield of BM, it had formerly been often used as waste or animal feed. However, it is often used as an important source of separation of milk fat globules membrane in recent years. In addition, BM is used in dairy products such as cheese and low-fat yogurt to improve the emulsification effect, the texture characteristics, and nutritional value of the product. The BM can be used as a potential encapsulation material for the delivery of bioactive substances, some studies have reported that BM can stably deliver beta-carotene, moreover, some researchers have reported that omega-3 unsaturated fatty acids encapsulated by BM has a good encapsulation effect [8].

Docosahexaenoic acid (DHA, C22:6), a beneficial fatty acid (FA) for the brain development of infants, exists widely in marine organisms, and it is predominantly sold as microalgal oil or fish oil [9]. In the human brain, DHA is the main component of the brain grey matter, accounting for about 30% of the polyunsaturated FAs, which is an essential material basis to ensure brain normal function. Some studies have shown that the addition of DHA to the diet of pregnant women improves the cognitive ability of infants. However, when the DHA is not supplied at the correct time, it may lead to fetal brain growth and development disorders when the DHA is not supplied timely, resulting in fetal brain development disorders and low intelligence. Judge *et al.* (2007) reported that 9-month-old infants showed a significant increase in problem-solving ability after DHA supplementation from 24 weeks of gestation to delivery (covering the most critical last trimester), with no effect on recognition memory [10]. Thus, DHA supplementation is an important method to promote the early neurological development of infants and young children. The DHA molecule contains six double bonds, resulting in the easily oxidizing and producing hydroperoxide, so some wall materials, such as whey protein and maltodextrin, are used to protect the DHA and further increase its bioavailability[11]. However, there are some disadvantages for the commercial DHA microencapsulated powder, such as the low digestibility and availability. It is very important to find new wall materials and establish a new delivery system to increase the effective use of DHA in the body.

Simulated *in vivo* digestion allows researchers to study the digestion of specific foods in a laboratory environment, in which biological digestive juices are added with commercial enzymes as digestive juices, and the human gastrointestinal digestive environment is simulated [12]. These experiments are simple and low-cost and have been widely used in recent years. For the DHA digested *in vitro*, Hu *et al.* (2022) reported that the releasing rate of DHA obtained from algal oil was faster than that in tuna oil, while the lower lipid digestibility was showed in algal oil due to the structural differences [13]. Another study found that that the DHA microcapsules, fabricated with sunflower phospholipid, protein, and maltodextrin, had a higher

hydrolytic efficiency of DHA in comparison with commercially available products, and effectively resisted gastric proteolysis and protected its biological activity on the way to the intestine [14].

Although the DHA is successfully encapsulated by BM, its digestion and bioavailability are unclear. In this current research, the physical property of the algal oil powder coated with BM (BMO), algal oil powder coated with BM and maltodextrin (BMMO), and two kinds of commercial algal oil powder (CMO 1 and 2) were characterized through an *in vitro* digestion experiment of infant gastrointestinal tract. Furthermore, the animal experiment was used to evaluated the DHA bioavailability by different wall materials on the growth, development, learning, and memory ability of rats (3-week-old). These results will verify the potential of BM as the wall material in the DHA delivering, and provide scientific data for preparing microencapsulated DHA powder with a high utilization rate and activity. In addition, these results will improve the value of BM utilization and further develop a new utilizing direction for the dairy processing by-products in the food industry.

2. Materials and methods

2.1 Materials

Pepsase (> 250 units/mg), *Rhizopus oryzae* lipase (> 30 units/mg), pancrelipase (100-400 units/mg), and Nile blue A were purchased from Sigma–Aldrich. Hydrochloric acid, potassium chloride, sodium chloride, calcium chloride, cholate, cholic acid sodium salt, chloroform, methanol, and sodium thiosulfate were procured from Sinopharm Chemical Reagent Co., Ltd. IgG and IgM immunoglobulin kits were provided by Jiangsu Maisha Biological Co. LTD. Based our previous study [15], there were four types of samples: BMO (the oil content was 33.3%), BMMO (BM : maltodextrin = 1:1; the oil content was 33.3%), CMO 1 and 2. The main components of the wall material of CMO 1 and 2 were lactose, solid corn syrup, and whey protein powder.

2.2 Microencapsulated emulsion and preparation of simulated digestion fluid

The microencapsulated powders were weighed and dissolved into the microencapsulated emulsion, whose fat content was 2%. To prepare simulated gastric fluid (SGF), NaCl (2 g) and HCl (1 mol/L, 13 mL) were dissolved in deionized water (800 mL). The pH was adjusted to 5.5 with 0.1 mol/L HCl and the volume adjusted to 1000 mL. Subsequently, gastric lipase (1.3 mg/mL) and pepsin (1.5 mg/mL) were added, and the solution was stirred for 20 min at room temperature. Simulated intestinal fluid (SIF) included 164 mmol/L NaCl, 10 mmol/L KCl, and 119 mmol/L $CaCl_2$. The pH was adjusted to 7 with 0.2 mol/L NaOH, cholate (0.5 mg/mL), and pancreas lipase (1 mg/mL) added, and the solution was stirred at 37°C for 20 min.

2.3 Dynamic digestion

The microencapsulated emulsion (10 mL) was incubated in a water bath (37°C) for 10 min. Then, 5 mL of SGF was added and the solution was stirred at 37°C for 1 h. The pH was maintained at 5.5 by using 0.1 mol/L NaOH solution. After gastric digestion, the pH of the digestive fluid was adjusted to 6.5 with 0.2 mol/L NaOH, and 10 mL of SIF was added to simulate intestinal digestion for 2 h. During intestinal digestion, the pH

and temperature of the solution were kept stable at 6.5 and 37℃, respectively. Samples were taken from the initial emulsion, the gastric digestion at 30 min and 60 min, and the intestinal digestion at 30 min, 60 min, 90 min, and 120 min.

2.4 Particle size determination

The wet method of a dynamic light scattering instrument (Mastersizer 3000 Malvern, UK) was used to determine the particle size of microcapsule. The refractive index of the sample and dispersion medium (water) were set as 1.456 and 1.33, respectively. The absorbance of the oil droplets was 0.001.

2.5 Zeta potential determination

The sample was diluted 400 times with buffer solution (20 mmol/L imidazole, 50 mmol/L NaCl, and 5 mmol/L $CaCl_2$, pH =7.0), and the temperature was 25℃. The refraction coefficient was 1.458, the viscosity was 0.89 cp, and the dielectric constant was 79.

2.6 Microstructural evaluation with laser confocal microscopy

The microstructure of the microencapsulated emulsion during gastrointestinal digestion was examined with a laser confocal scanning microscope (with a Kr/Ar laser). The sample was stained with 0.01% (w/v) Nile Blue dye with a sample-to-dye ratio of 1:1, and samples were incubated at room temperature for 20 min before examining the microstructure. An oil-immersion objective lens (63×) was used to observe the samples. The signal at 568 nm corresponded to both the protein phase and fat phase, while the signal at 488 nm corresponded only to the fat phase.

2.7 Free fatty acid released

The free fatty acid (FFA) released (%) was detected by pH-Stat method [16], which was calculated by recording the volume of NaOH solution to maintain the system at pH 7.0 during intestinal digestion

$$FFA\ \ released\ \ (\%) = 100 \times (\ \frac{V_{NaOH} \times m_{NaOH}\ \times\ M_{lipid}}{W_{lipid}\ \times 2})$$

V_{NaOH} was the volume of NaOH solution required for neutralization, m_{NaOH} was the molar concentration of NaOH solution, W_{lipid} was the total weight of the lipid initially present in the reaction, and M_{lipid} is the average molecular weight of the lipid (g/mol).

2.8 In vivo experiment

2.8.1 Animals, experimental design, and treatments

Forty rats (male, 21–28 days old at the start of experiment, from Beijing Huafukang Bioscience Co., Ltd., Beijing, China) were housed under a 12-h photoperiod at (22 ± 2) ℃ with 55% ± 5% relative humidity. These mice were randomly divided into five groups: (1) the control group (C group, n=8); (2) the rats fed with uncovered algal oil (Oil group, n=8); (3) the rats fed with BMO (BMO group, n=8); (4) the rats fed with BMMO (BMMO group, n=8); (5) the rats fed with CMO (CMO group, n=8). The rats were given emulsion with the DHA 200mg/kg/day at 9:00 a.m. every day. The gavage volume was 0.1 mL/10g body weight. Prior

to the experiment, the rats were adapted to the environment for 1 week, during which time they were fed the AIN-93 diet. Subsequently, the rats were fed with the above-mentioned algal oil for 28 days, during which time they were free to eat and drink. Their body weight and food intake were recorded every 3 days. The Morris water maze experiment was conducted during the last five days at the end of feeding. On the 28th day, all rats were sacrificed by cervical dislocation. Brain tissue and spleen tissue were immediately collected in sterile freezer tubes and frozen in liquid nitrogen for further analysis. All animal-related experiments were approved by the Animal Care and Use Committee of the Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences.

2.8.2 Determination of routine blood and serum biochemical indicators

The rats were fasted for 12 h on the last day of feeding, and rat blood was collected and evaluated with a blood cell counter for routine blood indexes, including red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean red blood cell hemoglobin (MCH), mean red blood cell volume (MCV), mean red blood cell hemoglobin concentration (MCHC), platelets (PLT), white blood cells (WBC), lymphocytes (LYM), neutrophils (NEUT), monocytes (MONO), eosinophils (EO), basophils (BAEO), and reticulocytes (RET). Serum biochemical indicators were determined with an automatic blood biochemical analyzer, including serum total cholesterol (CHO), total triglycerides (TG), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol level (HDL-c).

2.8.3 Morris water maze

The water maze comprised a circular flume, an automatic video recording device, a computer analysis, and a processing system. The diameter of the tank was 1.5 m, and the water temperature was maintained at (23 ± 2) ℃. The circular flume was divided into four different quadrants; the entry point was marked in the middle of the quadrants. The platform was placed underwater in the second quadrant. The water maze experiment was conducted according to a previous study [17].

For the positioning navigation experiment, each rat was trained for 4 times and entered the water facing the pool wall from a fixed position in the fourth, third, second, and first quadrants successively. The escape latency time from entering the water to find the platform was recorded. After the rats climbed onto the platform, they were allowed to stay on it for 15 seconds. If the rats did not find the platform within 90 s, then the experimenter would guide them to the platform and allow them to stay on it for 15 s; for these trials, the escape latency of the incubation period was recorded as 90 s. The interval between the two training sessions was 30 s, and the average escape latency of the four training sessions was recorded as the escape latency for the day. The training lasted for 5 days, and the escape latency on day 5 was considered the final result of accessing the learning ability of the rats.

For the spatial search experiment, the platform was removed and the rats were put into water from the fourth quadrant. The number of times of the rats crossed the original platform location within 90 s was recorded to evaluate the memory of the rats.

2.8.4 Fatty acid profile in the rat brain

Lipid extraction and FA methyl esterification were performed as described previously [18]. Gas chromatography equipped with a flame ion detector (GC-FID) was used to detect the FAs, and a capillary column was DB-23 (60 m × 250 mm × 0.32 mm). The detailed protocol has been published in a previous study [19].

2.8.5 Determination of immunoglobulin

A double-antibody, one-step sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the immunoglobulin level. Briefly, samples, standard products, and detection antibodies labeled with horseradish peroxidase (HRP) were transferred to the micropores pre-coated with a rat immunoglobulin-captured antibody; then the plate was incubated and thoroughly washed. A blue product was produced with tetramethylbenzidine; the reaction was stopped with acid, which changed the color to yellow. The absorbance of the product at 450 nm was measured; it correlated positively with the concentration of rat immunoglobulin.

2.9 Data analysis

The data are represented as the mean ± standard deviation of at least three experiments. One-way analysis of variance (ANOVA) followed by Duncan's method was used for multiple comparisons between the groups. Different lowercase letters indicate significant differences ($P < 0.05$). SPSS Statistics version 22.0 was used for the statistical analysis.

3. Results and discussion

3.1 Particle size during simulated digestion

The BM, a natural emulsifying material, is rich in protein, phospholipids, and gangliosides [15,20]. The particle size distribution is an important indicator during the gastrointestinal digestion period. It was evident that the BMO particles were 1 - 100 μm in diameter during the initial stage and after gastric digestion for 30 and 60 min (Figure 1A). The BMO emulsion presented an unimodal distribution, and there were multiple peaks during intestinal digestion for 30, 60, 90, and 120 min. The BMO particle size decreased after 30 and 60 min of intestinal digestion, which was caused by the fat being digested by lipase. However, there was a slight accumulation of fat after 90 and 120 min of intestinal digestion, resulting in larger particles. For the BMMO, its particle size ranged from 1 to 100 μm in the initial stage and showed a bimodal distribution (Figure 1B). The possible reason for this phenomenon was that the maltodextrin was dispersed in the emulsion, thus causing the appearance of small particles. The BMMO particle increased in size during gastric digestion (30 and 60 min) as the fat accumulated, and then decreased after 30 and 60 min of intestinal digestion. However, there was an increasing trend was observed after 90 and 120 min of intestinal digestion, during which fat slightly accumulated. In addition, the particle size distributions of CMO 1 and CMO 2 both were unimodal (0.01 - 1 μm), and they increased during whole gastric digestion (Figure 1C and D). During intestinal digestion, fat accumulated and the CMO 2 particle size decreased after 30 and 60 min, and then it increased

after 90 and 120 min, while the CMO 1 particle size increased during intestinal digestion. This was likely due to the marked fat accumulation in intestinal digestion.

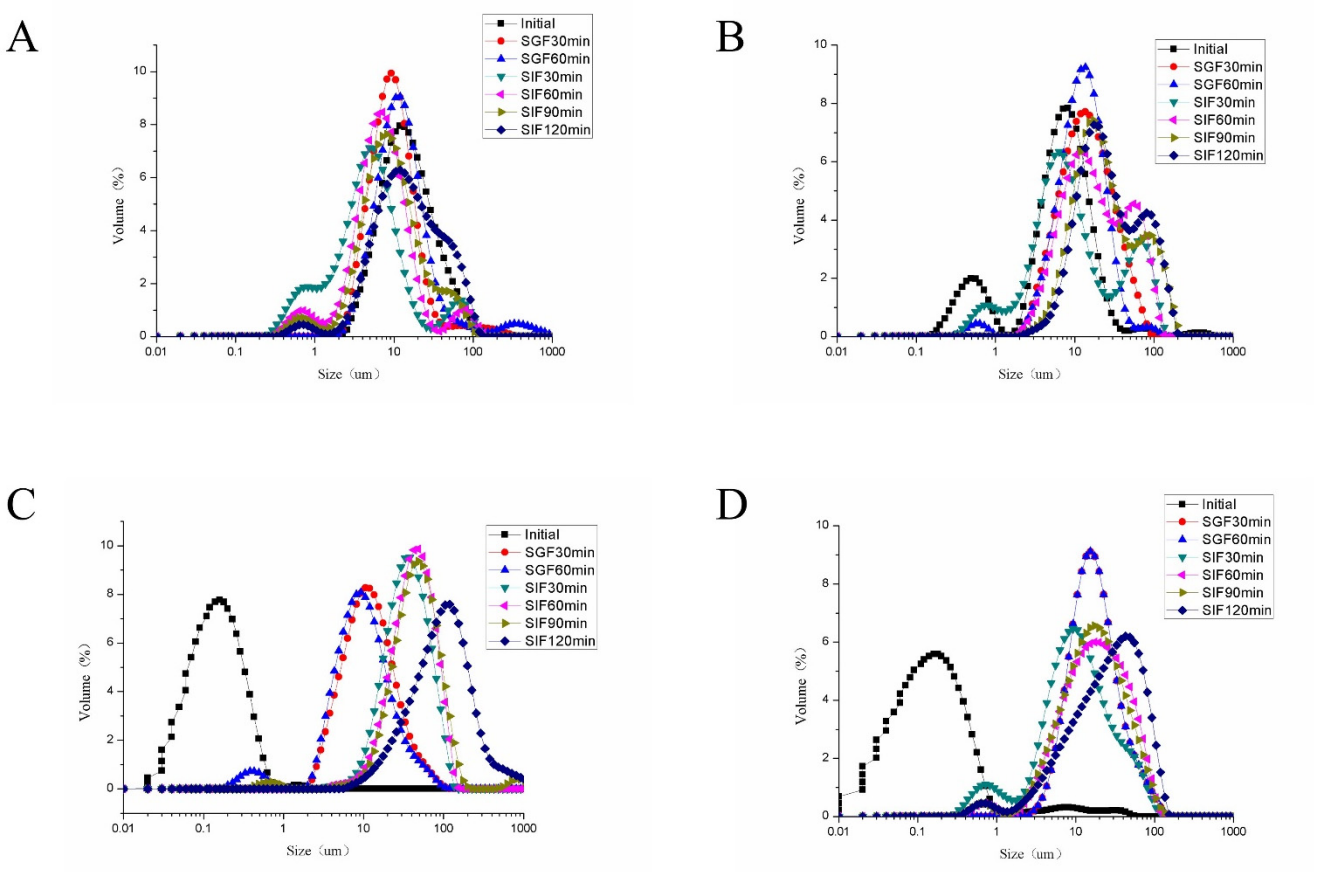

Figure 1 Particle size distribution of four a galoilmicrocapsules at diferent digestion periods,A:agal oilcovered with buttermilk; B: algal oil embedded in buttermilk and maltodextrin; C: commercially microcapsulealoal oil nowder 1 D' commercially microcansule algal oil nowder: nitial, initial emulsion: SGE 30 min.gastric digestion for 30 min: SGF 60 min: gastric digestion for 60 min: SIF 30 min: intestinal digestion for 30min: SIF 60 min: intestinal digestion for 60 min: SF 90 min : intestinal digestion for 90 min: SIF 120 min:intestinal digestion for 120 min

The Graph A of Figure 2 showed the variation in average particle size of BMO, BMMO, CMO 1, and CMO 2 during different digestion periods. We could see that the initial BMO and BMMO particle sizes were 100 times larger than those of CMO 1 and CMO 2. This result was likely caused by the higher protein content of BMO and BMMO wall materials, leading to a lot of microcapsules with larger particle sizes [21,22]. The BMO particle size decreased and then increased during incubation with SGF, while BMMO particles showed the reverse trend. For CMOs 1 and 2, their particle sizes all increased during gastric digestion. Moreover, the particle size of BMO, BMMO, CMO 1, and CMO 2 all showed a trend to increase during intestinal digestion. The CMO 1 (140 μm) and CMO 2 (60 μm) particles were significantly larger than the BMO (25 μm) and BMMO (40 μm) particles after 120 min of intestinal digestion, indicating that BMO and BMMO were more easily digested than CMO 1 and 2. This phenomenon was might be the result of oil accumulation from CMO 1 and 2 when the microencapsulated wall materials were broken, and the oil could have further accumulated as digestion proceeded.

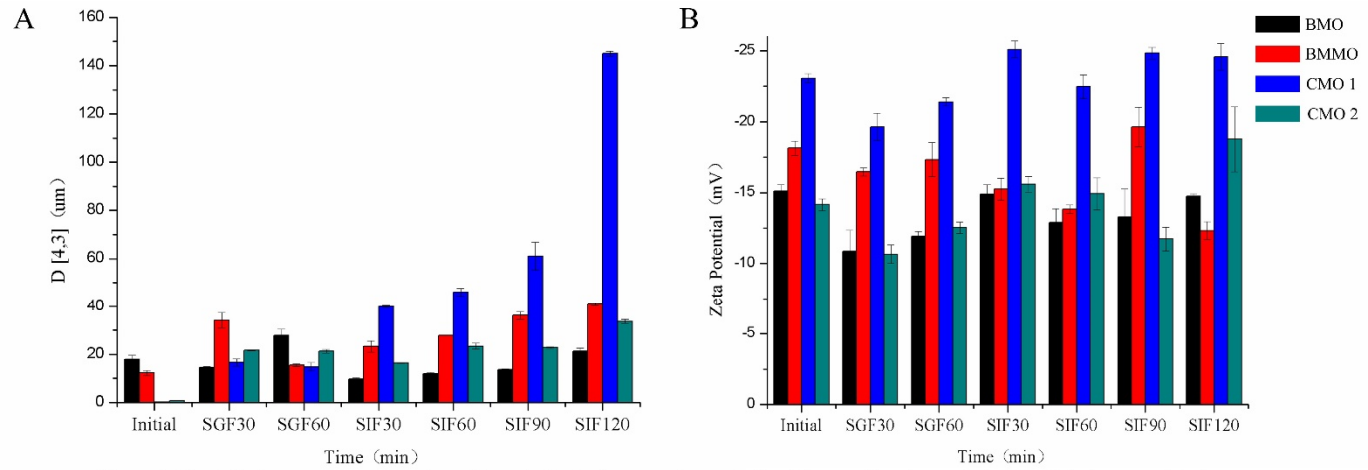

Figure 2 Plot A is the average particle size of four algal oil microcapsules varied in different digestion periods. Plot B is the potential changes of four algal oil microcapsules in different digestion periods. BMO: algal oil covered with buttermilk; BMMO: algal oil embedded in buttermilk and maltodextrin; CMO1 and 2: commercially microcapsule algal oil powder 1 and 2; Initial: initial emulsion; SGF 30 min: gastric digestion for 30 min; SGF 60 min: gastric digestion for 60 min; SIF 30 min: intestinal digestion for 30 min; SIF 60 min: intestinal digestion for 60 min; SIF 90 min: intestinal digestion for 90 min; SIF 120 min: intestinal digestion for 120 min

Figure 2 Plot A is the average particle size of fou algal oil microcasules vared in diferent dipestion nerods plot B is the notential changesof four algal oil microcapsules in diferent digestion periods. BO: algal oil covered with buttermilk: BMMO: algal oil embedded inbutenmilk and maltodextrin: COl and 2: commercially microcansule alsal oi powder 1 and 2: nitial: intial emulsion: SGF 30 min: gastricdigestion for 30 min: $GF 60 min: gastric digestion for 60 min: SIF 30 min: intestinal digestion for 30 min: SIF 60 min: intestinal digestion fo60 min: SIF 90 min: intestinal digestion for 90 min: SIF 120 min: intestinal digestion for 120 min

3.2 Zeta potential during the simulated digestion

The size and charge of the droplet surface indicate their stability and ability to interact with other components [23], so the zeta potential of samples were measured during simulated digestion. As shown in Figure 2B, the zeta potentials of all samples were negative at all digestive stages, and they were influenced significantly by the different emulsion states. In the initial stage, the potential of CMO 2 was the highest (-14.17 mV), followed by BMO (15.10 mV), BMMO (18.13 mV), and CMO 1 (23.07 Mv), which might be induced by the phospholipid was contained in the buttermilk and it would cause the decrease of zeta potential in BMO and BMMO [14,24]. The zeta potential of all samples increased after 30 min of gastric digestion, and the zeta potential of BMMO continued to increase as digestion proceeded. However, there was a trend for a decrease in the zeta potentials of BMO, CMO 1, and CMO 2. After 120 min of intestinal digestion, the zeta potential of BMO, BMMO, CMO 1, and CMO 2 was -14.73 mV, -12.33 mV, -24.57 mV, and -18.77 mV, respectively. Moreover, the zeta potential of each group was higher than that of the initial emulsion during the simulated gastric digestion. These changes were likely due to the pH: it was maintained at 5.5 after the addition of SGF, during which time the negative zeta potential decreased. However, the pH was maintained at 7 after adding SIF, and the negative potential increased to a value that was similar to what was measured prior to simulated digestion.

3.3 Microstructural evaluation during simulated digestion

It is important for the microcapsule wall material to break down during digestion, so we used laser confocal microscopy to observe changes in the microstructure during digestion. The BMO wall material mainly contained casein, whey protein, lactose, and milk fat global membrane. For BMMO, its wall material

was same as BMO plus maltodextrin. Prior to simulated digestion, BMO and BMMO presented dense algal oil particles surrounded by protein. The wall materials of microcapsule were digested after adding the gastric fluid, and there was a decrease in fat density after 60 min of simulated gastric digestion, and the algal oil was exposed. Moreover, after 60 min of simulated intestinal digestion, most of the algal oil in BMO and BMMO had been digested into small droplets. The algal oil droplets were further digested as the simulated intestinal digestion time increased to 120 min, and there was some accumulation at SIF 120 min (Figure 3).

For CMO 1 and 2, they all contained lactase, and CMO 1 and CMO 2 consisted of corn syrup and little whey protein, respectively. Compared with the BMO and BMMO, the CMO microstructures all had no green color in the initial emulsion period, indicating the lack of protein particles covering the algal oil in CMOs. Furthermore, there was marked algal oil aggregation after 60 min of simulated gastric digestion, and this aggregation gradually increased during simulated intestinal digestion, which was consistent with the results of particle size. The oil accumulation was not conducive to lipase-mediated hydrolysis of oil, which would prevent the digestion and absorption of algal oil. These results showed that the BMO and BMMO were easier to digest than CMO 1 and 2.

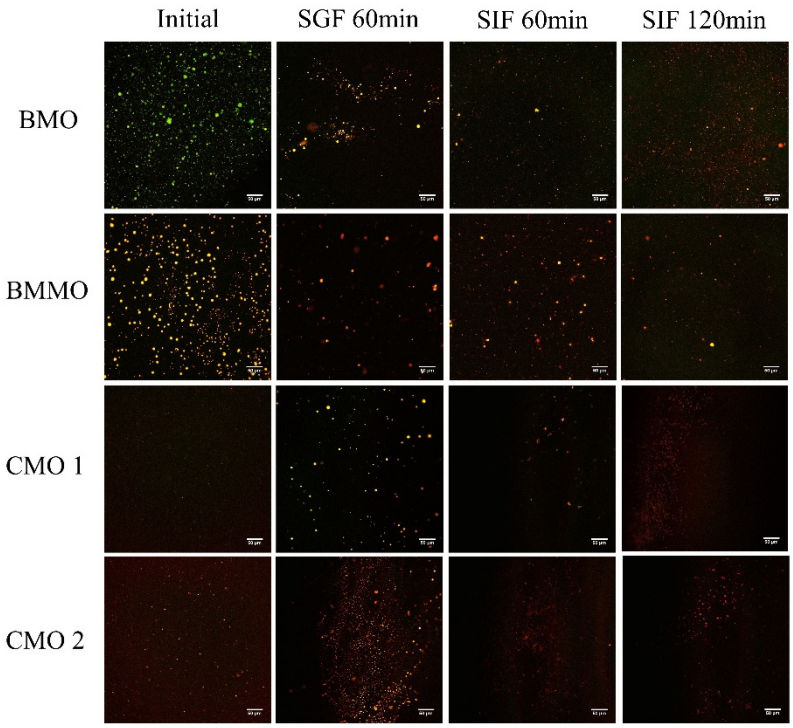

Figure 3 Microstructures of fourkinds of microcapsule algal oilpowder at diferent digestionperiods.BMO: algal oil coyeredwith buttermilk:BMMO: algal oilembedded in buttermilk andmaltodextrin: CMOl and 2:commercially microcapsule algaloil powder 1 and 2; Initial: initialemulsion: SGF 60 min: gastricdigestion for 60 min; SIF 60 min:intestinal digestion for 60 min:SIF 120 min: intestinal digestionfor 120 min.

3.4 Free fatty acid (FFA) release rate during the in vitro experiment

In the stomach, the lipids are first digested via acid-resistant gastric lipase, and then most digestion of lipids occurs in the small intestine through pancreatic lipase. After emulsification and lipase hydrolysis, triacylglycerol is converted to FFAs, and the quality of the wall material influences the FFA release rate [25]. The lipids released first are from food matrixes through the digestive system, they are then absorbed by the

human body. Hence, the bioavailability of lipids is mainly depended on the FFA release rate (Pan et al., 2021). In this experiment, the FFA release rate in all samples increased slowly during gastric digestion (Figure 4), and it was 7.97%, 7.80%, 3.69%, and 4.27% for BMO, BMMO, CMO 1, and CMO 2, respectively, at 60 min of simulated gastric digestion. As digestion proceeded, the releasing rate of FFA in four groups all increased significantly at first, and then the increasing rate was gradually slowed during simulated intestinal digestion. Among the four groups, CMO 2 showed the fastest FFA release rate, and it could reach 31.51% after 30 min of simulated intestinal digestion, which was significantly higher than that of BMO (25.53%), BMMO (23.49%), and CMO 1 (21.92%). The FFA release rate was 29.79%, 30.80%, 30.63%, and 36.50% for BMO, BMMO, CMO 1, and CMO 2, respectively, after 60 min of simulated intestinal digestion. and they all gradually slowed down at this time. By the end of simulated intestinal digestion (120 min), the FFA release rate of FFA in BMO, BMMO, CMO 1, and CMO 2 was 36.71%, 36.70%, 37.24%, and 42.44%, respectively.

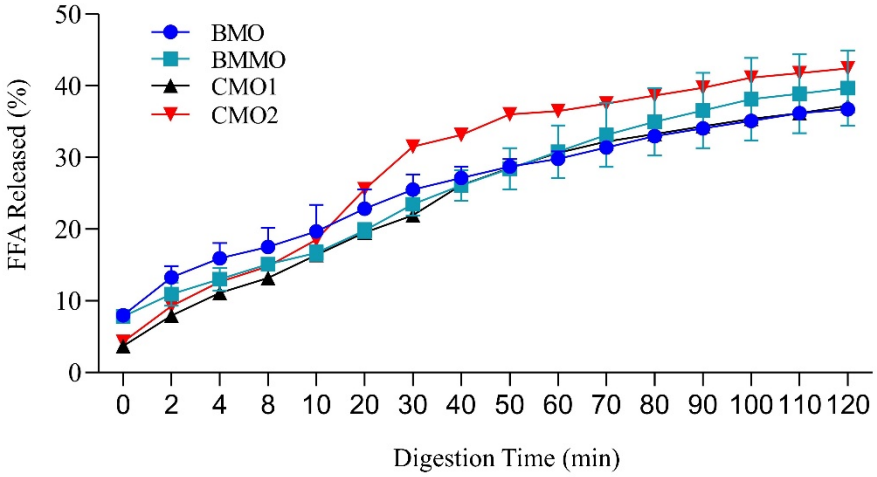

Figure 4 The FFA released of four kinds of microcapsule algal oil powder at intestinal digestion period. FFA: freefatty acid: BMO: algal oil covered with buttermilk; BMMO: algal oil embedded in butermilk and maltodextrin:CMO1 and 2: commercially microcapsule algal oil powder 1 and 2

3.5 Rat weight, routine blood, and serum biochemical indicators

We conducted an animal experiment to determine the functional characteristics of algal oil encapsulated with different wall materials. The mental state and physical condition of the rats in each group were good during the 4-week feeding period (Table S1), with no abnormalities. The body weight of all rats in the five groups increased steadily as the experiment proceeded. Compared with the control group, the body weight of rats in the Oil group was significantly higher on day 10 ($P < 0.05$). However, the body weights of rats in the BMO, BMMO, and CMO were higher than that of the control group and Oil groups on day 4 ($P < 0.05$). These phenomena indicated that the dietary addition of DHA could promote the growth and development of rats, which was consistent with the previous results [26]. Both the blood routine index and serum biochemical index reflect the physiological and nutritional status of anima. As shown in Table S2, the values of RBC, HGB, HCT, MCH, MCV, MCHC, PLT, WBC, LYM, NEUT, MONO, EO, BAEO, RET, CHO, TG, HDL-c, and HDL-c levels were not different between the experiment groups and the control group ($P > 0.05$). Therefore, algal oil, regardless of the wall material, did not affect the blood composition.

3.6 Morris water maze

The Morris water maze experiment is an experiment that forces experimental animals (mice) to swim and learn to find hidden platforms in water, mainly used to evaluate the spatial position learning, memory ability, and sense of direction (spatial positioning) in experimental animals [27,28]. We used this test to access the influence of microencapsulated algal DHA on the rat brain. The escape latency of the four experimental groups showed a decreasing trend compared with the control group (Figure 5A). Among the test groups, the escape latency in the Oil and CMO groups was not significantly different from the control group ($P > 0.05$), a finding was consistent with previous studies [29,30]. It was clear that the number of escape latency in the BMO and BMMO groups was higher significantly than that of the control and Oil groups. Moreover, the incubation period of all experimental groups was significantly shortened in comparison with the control group ($P < 0.05$), indicating that the learning of these rats was enhanced. A previous study reported that adequate DHA supplementation improved the growth, spatial learning, and memory impairments of Sprague Dawley rats, and upregulated the mammalian target of the rapamycin (mTOR) pathway, although excessive DHA supplementation did not exert a more beneficial effect [26].

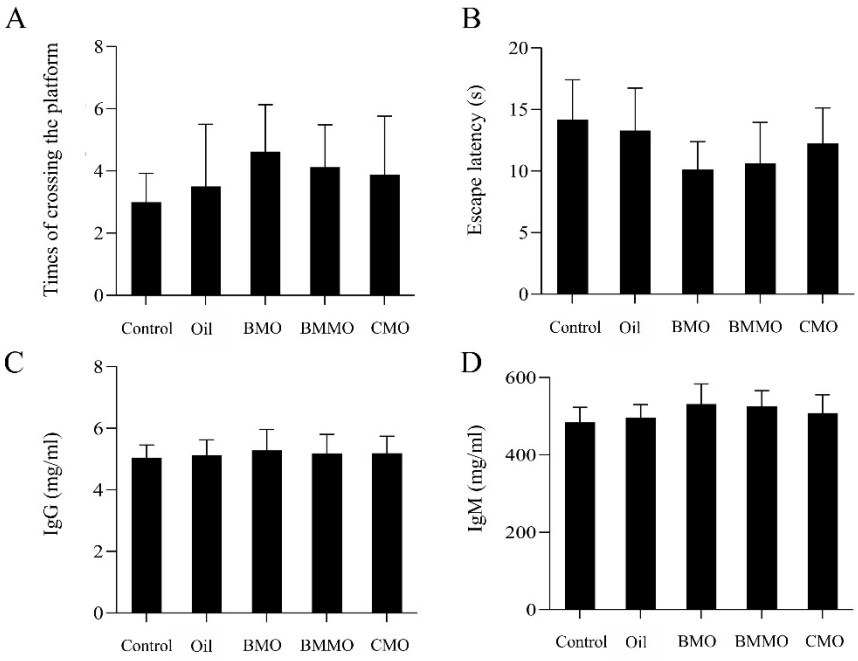

Figure 5 Plot A is the times of crossing the platform of rats in different groups. Plot B is theescape latency of rats in different groups. Plot C is the concentration oflgG of rats indifferent groups. Plot D is the concentration ofIgM of rats in different groups, Control.control group: Oil: the rats fed with algae oil which coyered no wall materials: BO: therats fed with algal oil covered with buttermilk: BMO: the rats fed with algal oilembedded in buttermilk and maltodextrin: CO: the rats fed with commerciallymicrocapsule algal oil powder.

In the spatial search experiment, the number of platform crossing was shown in Figure 5B. Compared with the control group, there was a trend for an increase in the number of platform crossings in the four experimental groups. There was no significant difference between the Oil, BMMO, CMO, and control groups ($P > 0.05$). However, the number of platform crossing in the BMO group was significantly higher than that in the control group ($P < 0.05$), indicating that BMO could enhance the memory of rats. These results were likely caused by the unique components of BM, such as abundant phospholipid and MFGM protein. It was reported

that the phospholipid played an indispensable role in the establishment of the infant brain nervous, intestinal immune, and gastrointestinal digestive systems [31,32].

3.7 Brain FA profile

To determine the bioavailability of microencapsulated DHA, the rat brain FA profile was determined (Table 1). Twenty-four FAs were detected in rat brains. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9c), scolic acid (C20:3n3), and C22:6n6 were the main FAs, accounting for > 90% of total FAs. After feeding algal oil, the proportion of C16:0, C18:0, C18:1n9c, and C20:3n3 decreased significantly in CMO, BMO, and BMMO groups in comparison with control and Oil groups ($P < 0.05$). For the DHA, furthermore, its percentage was the highest in the BMMO group, accounting for 17.86% of total FAs, followed by the BMO group (17.54% of total FAs), the CMO group (16.84% of total FAs), the Oil group (15.86% of total FAs), and the control group (15.67% of total FAs). The percentage of DHA in the BMMO, BM, and CMO groups was significantly higher than that in the control and Oil groups, indicating that the DHA content in the rat brains increased significantly after feeding microencapsulated powder containing DHA. A previous study showed that the DHA content in rat brains was closely related to enhanced memory function, and the learning and memory ability of rats was improved by increasing the brain DHA concentration [33]. This finding corresponded to the Morris water maze results, and it also showed that the algal oil encapsulated in BM led to better DHA bioavailability compared with pure algal oil or CMO.

Table 1 The proportion of fatty acid of rats' brain in different groups

Fatty acids	Control	Oil	CMO [a]	BMO	BMMO
C14:0	0.15±0.01	0.15±0.01	0.14±0.01	0.15±0.01	0.15±0.01
C15:0	0.07±0.00^{a}	0.06±0.00^{b}	0.05±0.01^{b}	0.06±0.00^{b}	0.06±0.00^{b}
C15:1	0.21±0.03^{a}	0.25±0.01^{b}	0.34±0.04^{c}	0.29±0.02^{d}	0.28±0.03^{bd}
C16:0	23.21±1.16^{a}	23.22±2.03^{a}	21.44±1.13^{b}	21.45±1.5^{b}	22.35±1.21^{ab}
C16:1	0.38±0.04^{a}	0.39±0.04^{a}	0.28±0.03^{b}	0.33±0.03^{c}	0.35±0.04^{bc}
C17:0	0.22±0.01^{a}	0.18±0.01^{b}	0.17±0.01^{b}	0.18±0.01^{b}	0.18±0.01^{b}
C17:1	0.06±0.01^{a}	0.05±0.00^{b}	0.03±0.00^{c}	0.05±0.01^{b}	0.04±0.00^{b}
C18:0	22.28±0.84^{ab}	23.11±1.65^{b}	22.61±1.04^{ab}	21.4±0.86^{a}	21.64±0.78^{a}
C18:1n9c	21.44±0.6^{ab}	20.58±1.08^{a}	22.03±1.08^{b}	22.55±1.56^{b}	21.39±0.84^{ab}
C18:2n6c	0.97±0.07^{a}	0.81±0.07^{b}	0.93±0.08^{a}	0.92±0.05^{a}	0.96±0.07^{a}
C18:3n6	0.02±0.00	0.01±0.00	0.02±0.00	0.03±0.00	0.01±0.00
C18:3n3	0.03±0.00^{a}	0.03±0.00^{a}	0.04±0.01^{a}	0.03±0.01^{a}	0.02±0.01^{b}
C20:0	0.39±0.06^{ab}	0.31±0.01^{c}	0.35±0.09^{bc}	0.43±0.07^{a}	0.35±0.05^{bc}
C20:1n9	2.00±0.32^{ab}	1.64±0.12^{a}	2.06±0.62^{ab}	2.33±0.48^{b}	1.81±0.30^{a}
C20:2	0.31±0.04^{a}	0.25±0.02^{b}	0.29±0.07^{ab}	0.32±0.04^{a}	0.28±0.02^{ab}
C20:4n6	0.56±0.04	0.50±0.04	0.53±0.04	0.55±0.05	0.56±0.04
C20:3n3	11.10±1.03^{a}	11.89±0.71^{b}	11.06±0.67^{ab}	10.61±1.2^{c}	10.95±1.06^{ac}
C20:5n3	0.28±0.03^{ab}	0.25±0.02^{a}	0.25±0.06^{ab}	0.30±0.04^{b}	0.26±0.04^{ab}
C22:1	0.15±0.02^{ab}	0.11±0.01^{a}	0.23±0.14^{ab}	0.16±0.03^{b}	0.16±0.02^{b}
C22:2	0.01±0.00^{a}	0.02±0.00^{ab}	0.03±0.01^{b}	0.03±0.01^{b}	0.03±0.01^{b}
C23:0	0.02±0.00^{a}	0.03±0.00^{ab}	0.04±0.00^{b}	0.04±0.01^{b}	0.04±0.01^{b}
C24:0	0.12±0.03^{a}	0.10±0.00^{b}	0.04±0.01^{c}	0.09±0.01^{b}	0.06±0.02^{d}
C24:1	0.29±0.08^{a}	0.12±0.01^{bc}	0.09±0.02^{c}	0.11±0.01^{bc}	0.14±0.01^{b}
C22:6n6	15.67±0.72^{a}	15.89±0.29^{a}	16.84±0.75^{b}	17.54±0.36^{c}	17.86±1.00^{c}
SFA	46.52±0.39^{a}	47.20±0.8^{b}	44.92±1.00^{b}	43.86±1.10^{b}	44.89±0.57^{b}
MUFA	24.52±1.08^{ab}	23.14±1.28^{a}	25.07±1.93^{b}	25.82±2.13^{b}	24.17±1.23^{ab}

PUFA	28.96±1.93[a]	29.66±1.15[ab]	30.00±1.68[bc]	30.32±1.75[bc]	30.93±2.26[c]
UFA	53.48±3.02[a]	52.8±2.43[a]	55.08±3.61[b]	56.14±3.88[c]	55.11±3.48[b]

[a]: CMO = commercial microcapsule algal oil; BMO = algal oil embedded with buttermilk; BMMO = algal oil embedded with the mixture (buttermilk : maltodextrin = 1:1) SFAs (saturated fatty acids) = ∑(C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C23:0, C24:0); MUFA (monounsaturated fatty acid) = ∑(C15:1, C16:1, C17:1, C18:1n9c, C20:1, C22:1, C24:1); PUFA(polyunsaturated fatty acids) = ∑(C18:2n6c, C18:3n6, C18:3n3, C20:2, C20:3n6, C20:4n6, C20:3n3, C20:4n6, C20:5n3, C22:2, C22:6n3); UFA (unsaturated fatty acids)= ∑(MUFA, PUFA)

3.8 Immunoglobulin levels

We determined immunoglobulin G (IgG) and immunoglobulin M (IgM) levels in rat liver by using a double-antibody, one-step sandwich ELISA kit. The IgG concentration was similar in all groups, indicating that there was no significant difference in IgG among the five groups ($P > 0.05$). The IgM concentration in the BMO and BMMO groups was 525 μg/mL and 515 μg/mL, respectively, higher compared with the control group (485 μg/mL) ($P < 0.05$) (Figure 5C and D), indicating that the DHA microencapsulated in BMO and BMMO could improve the immunity of rats. In a previous study, the researchers found that the rats fed with eicosapentaenoic acid (EPA) or DHA had a significant higher IgM level than rats fed with saturated FAs, this result showed that dietary fat could affect the IgM production of rat lymphocytes (HUNG et al., 1999). Another study reported that the leptin signaling inhibitor SOCS3 s was reduced after DHA supplementation, and the leptin JAK2-Akt signaling pathway in the hypothalamus was activated. Moreover, the researchers also found that DHA not only reduced energy intake and weight gain but also corrected diet-induced hypothalamic inflammation [34].

4. Conclusion

Although the particles of BMO and BMMO were larger than those of CMO 1 and 2, the BMO and BMMO particle size increased more slowly compared with CMO 1 and 2 during simulated digestion. Moreover, the microstructure of CMO 1 and CMO 2 showed apparent accumulation of fat, it was in accordance with the particle size results, indicating that the digestion of fat by lipase was absorbed more easily by BMO and BMMO. These phenomena indicated that algal oil embedded in BM had better *in vitro* characteristics in comparison with other wall materials. In addition, the animal experiment demonstrated that adding BMO and BMMO to the diet could promote the growth of rats without altering routine blood and serum biochemical indicators. In the Morris water maze, the rats in BMO and BMMO groups had a shorter escape latency to find the platform compared to that in control, Oil, and CMO groups, and the BMO group showed the highest number of platform crossings. Moreover, the brain DHA content and immunity of rats was significantly improved in BMO and BMMO groups compared that in control, Oil, and CMO groups. In summary, compared with CMOs, the algal oil encapsulated with buttermilk could obtain the better digestive characteristics, and it could effectively deliver DHA *in vivo* and significantly enhance the digestion, growth, development, and learning ability of rats. These findings will confirm the possibility of buttermilk as the DHA embedded wall material, and explore a new way for the recycling of by-products of dairy processing and further promote the development of the food nutrition industry.

Interest statement

All authors declared that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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