Food Hydrocolloids for Health 2 (2022) 100098

Contents lists available at ScienceDirect

Food Hydrocolloids for Health

journal homepage: www.elsevier.com/locate/fhfh

Investigation of the effect of nanocellulose on delaying the *in vitro* digestion of protein, lipid, and starch

Ling Chen [a,b,c], Ruoxuan Deng [d,e], Wallace Yokoyama [f], Fang Zhong [a,b,c,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[c] International Joint Laboratory on Food Safety, Jiangnan University, Wuxi 214122, China
[d] Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, the Netherlands
[e] Laboratory of Food Process Engineering, Wageningen University & Research, Wageningen, the Netherlands
[f] Western Regional Research Center, ARS, USDA, Albany, CA 94710, USA

ARTICLE INFO

Keywords:
Fluorescence
In vitro digestion
Macronutrients digestion
Nanocellulose
Viscosity

ABSTRACT

Nanocellulose (NC) has the potential to be used as a dietary fiber supplementation in functional foods that can help to improve overall health. However, the effect of NC on macronutrients and the precise mechanisms still remain unclear. An *in vitro* digestion model was used to investigate the effect of NC on the digestion of proteins, lipids and starch. It was found that NC at low concentration (0.5 wt.%) has a significant inhibitory effect on starch digestion with the inhibition ratio of 14.3%, but no obvious effect on the digestion of lipids and protein. The delay of starch digestion was attributed to the interaction of NC with the pancreatic amylase through static quenching, examined by the fluorescence spectroscopy. NC exhibited a great adsorption capacity on α-amylase with the enzyme activity inhibition ratio of 14.6%, and the number of NC binding sites on α-amylase was 1-2. This study indicate dietary fibers like NC could delay starch digestion and be used in functional foods to help people with obesity and other specialized needs.

1. Introduction

Dietary fiber refers to the portion of plant-derived food which is hardly hydrolyzed by digestive enzymes and absorbed in the gastrointestinal tract. Studies have shown that dietary fiber has distinct functional properties (Chassaing et al., 2015; Zhong et al., 2015), such as suppressing postprandial serum glucose (Maćkowiak et al., 2016), reducing serum cholesterol levels (Mackie et al., 2016), decreasing protein digestibility (Hughes et al., 1996), promoting satiation and improving satiety and preventing obesity (Kendall, Esfahani, & Jenkins, 2010). Dietary fibers can act by changing the nature of the contents in the gastrointestinal tract and altering the manner of the nutrients and chemicals absorbed (Eastwood & Kritchevsky, 2005). According to previous studies, the physical function of dietary fiber contributing to the delay of macronutrients digestion was based on two main mechanisms. One is the bulking effects that can delay gastric emptying rate and hinder intestinal absorption. After the dietary fibers enter the digestive tract, they would absorb water and swell significantly, increasing the viscosity of the digestive system, and delaying the transition rate of food matrix in a physical restraint way (Takahashi et al., 2005). Thereby, the digestion of food would be inhibited, and the diffusion of released small nutrient molecules could be restricted through physical barriers, reducing the absorption of nutrients. It was reported by Goff et al. that dietary fiber which maintained high viscosity during digestion can delay starch digestion and the diffusion and absorption of glucose (Fabek & Goff, 2015; Fabek et al., 2014). Another mechanism is the binding effects of dietary fibers that can inhibit the activity of enzymes and reduce the digestion of macronutrients. The study of Tsujita et al. has reported that citrus pectin could delay the lipolysis through the inhibition of the activity of lipase in the small intestine (Tsujita et al., 2003).

Dietary fibers can be divided into soluble fiber and insoluble fiber based on their solubility and gelling behaviors. Among which viscous soluble fibers, like xanthan gum, pectin and carrageenan, have been of great interest due to their gastric emptying delay properties (Liu & Kong, 2019c). Insoluble fibers like cellulose were widely reported to promote the regularity of intestine (Phillips et al., 1995). Presently, there are also several researches demonstrated the insulin and glycemic modulation effect of cellulose. Chau et al. reported that insoluble dietary fiber derived from carrots can reduce their serum cholesterol of hamster, and a stronger functionality was found along with the reduced cellulose particle size (Hsu et al., 2006). The functional properties of microcrystalline cellulose (MCC) as a dietary fiber have been studied.

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China.
E-mail address: fzhong@jiangnan.edu.cn (F. Zhong).

https://doi.org/10.1016/j.fhfh.2022.100098
Received 10 May 2022; Received in revised form 16 September 2022; Accepted 7 October 2022

A rat study carried by Vainshtein et al. stated the beneficial effect of MCC on diabetes without any adverse effects (Vaĭnshteĭn et al., 1987). MCC and nanocellulose (NC) with different particle sizes fabricated from sweet potato residues were fed with rat in Lu et al.'s study, and it was found that NC showed a better effect on regulating serum cholesterol and glucose (Lu et al., 2015). Compared with MCC, NC as a small-size cellulose degradation product, possessed a larger specific surface area and adsorption capacity (Jiang & Hsieh, 2015), and has great potential as a dietary fiber supplementation. However, the current research was mostly focused on the microstructure, crystalline structure, and thermal stability of NC. Few reports investigated the functionality of NC as a dietary fiber and its mechanism of action.

Compared with animal or clinical study, *in vitro* models have been widely applied to carry out studies on the effect of food structure or food composition upon nutrient bioaccessibility, due to their great properties of low-cost, time-saving, and high reproducibility. Among all the *in vitro* models, static methods have been the simplest but with highest consistency. Therefore, they are primarily used to study digestion of simple foods and conduct preliminary trials for nutritional and health claims (Ng & Rosman, 2019; Ng & See, 2019).

Hence, in this study, the effects of NC on the digestion of macronutrients were studied, MCC was used as a control. An *in vitro* digestion model was used to characterize the digestion behavior of food matrix consisting of proteins, lipids and starch at the presence of NC/MCC. The systematic viscosity, glucose diffusion and adsorption, enzyme activity inhibition ratio and the interactions between NC and enzymes were also investigated.

2. Materials and methods

2.1. Materials

Nanocellulose (NC) and microcrystalline cellulose (MCC) were obtained from Hangzhou Yuhan Technology Co., Ltd (Hangzhou, China). Peanut oil was bought from Shandong Luhua Group Co., Ltd (Shandong, China). Glucose assay kit (F006-1-1) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Digestive components including α-amylase from porcine pancreas (A3176, ≥5 units/mg solid), pepsin from porcine gastric mucosa (P7000, ≥250 units/mg solid), pancreatin from porcine pancreas (P7545, 8 × usp), lipase from porcine pancreas (Type II, L3126, ≥125 units/mg protein), amyloglucosidase (*Aspergillus niger*, A7095, activity: 260 U/mL) and bile extract porcine (B8631) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Waxy corn starch was purchased from Ingredian Co., Ltd (Shandong, China). Ninhydrin, dimethyl sulfoxide, acetic acid, sodium acetate, hydrochloric acid, sodium hydroxide and other reagents of analytical grade were from China Medicine (Group) Shanghai Chemical Reagent Co. (Shanghai, China).

2.2. Food mixture preparation

Food mixture was prepared according to the method of Goff et al. and Wooster et al. with modification (Fabek et al., 2014; Wooster et al., 2014). Briefly, 6% of sodium caseinate and 4% of waxy corn starch were dissolved in the deionized water. 10% peanut oil was then mixed with the aqueous phase using a homogenizer at 15,000 rpm for 5 min. NC and MCC were added into the food mixture in the following studies on demand.

2.3. In vitro *simulated gastro-intestinal digestion*

An *in vitro* digestion model with three-stage that mimic the mouth, stomach and small intestine phase was used to simulate the human gastro-intestinal tract according to our previous method and the method of Minekus et al with modification (Lin et al., 2017; Minekus et al., 2014).

2.3.1. Mouth stage

Simulated saliva fluid (SSF, containing 1.1 mg/mL KCl, 0.5 mg/mL KH_2PO_4, 1.1 mg/mL $NaHCO_3$, 0.03 mg/mL $MgCl_2(H_2O)_6$, 0.006 mg/mL $(NH_4)_2CO_3$, 0.2 mg/mL $CaCl_2(H_2O)_2$ and 150 U/mL α-amylase) was freshly prepared according to the method of Minekus et al. (Minekus et al., 2014) 5 mL of food matrix was mixed with 5 mL of SSF in a water-jacketed beaker and prewarmed at 37 ˚C. The mixture was adjusted to pH 7.0±0.2 and kept 37 ˚C with continuous agitation at 100 rpm for 2 min.

2.3.2. Stomach stage

10 mL simulated gastric fluid (SGF, containing 0.5 mg/mL KCl, 0.1 mg/mL H_2PO_4, 2.1 mg/mL $NaHCO_3$, 2.5 mg/mL NaCl, 0.02 mg/mL $MgCl_2(H_2O)_6$, 0.04 mg/mL $(NH_4)_2CO_3$, 0.02 mg/mL $CaCl_2(H_2O)_2$ and 4000 U/mL pepsin) was immediately added into the digesta after SSF incubation. The mixture was adjusted to pH 3.0±0.2, and kept stirring at 100 rpm for 1 h at 37 ˚C.

2.3.3. Small intestine stage

Simulated small intestinal fluid (SIF), containing 0.5 mg/mL KCl, 0.1 mg/mL KH_2PO_4, 7.1 mg/mL $NaHCO_3$, 2.2 mg/mL NaCl, 0.07 mg/mL $MgCl_2(H_2O)_6$, 3 mg/mL $CaCl_2(H_2O)_2$, 2 mg/mL pancreatin, 5 mg/mL bile salts and 342 μL amyloglucosidase, was previously prepared. After the SGF incubation, the digesta was immediately adjusted to pH 7.0 with 1.0 M NaOH, and 15 mL of SIF was added into the system. The mixture was kept pH at 7.0±0.2 with stirring at 100 rpm for 2 h at 37 ˚C.

2.4. Free fatty acids analysis

The lipolysis activity, refers to the percentage of the released free fatty acids (FFAs), was determined by pH-stat titration method, according to our previous study (Lin et al., 2018). 0.25 M NaOH was used to maintain the pH at 7.0 during simulated intestinal digestion. The volume of NaOH consumed over time was based on ionized fatty acids and recorded. The released FFAs was calculated as the following equation:

$$\text{FFA}\ (\%) = \frac{V_{NaOH} \times C_{NaOH}}{2n_{triglycerides}} \times 100$$

where $n_{triglycerides}$ was the amount of lipids in the whole digestive system (mol); V_{NaOH} was the volume (L) of NaOH solution consumed during the simulated intestinal digestion; C_{NaOH} was the concentration of the standard NaOH solution (M).

2.5. In vitro *digestion of starch*

The hydrolysis of starch (waxy corn starch) could be characterized by determining the release of glucose during digestion. According to the method of Fabek et al. (2014), the digesta was pipetted after SSF incubation, and at 10, 20, 30, 60, 90 and 120 min timepoint during SIF incubation. The collected samples were boiled at 100 ˚C to stop the enzyme reaction, and centrifuged at 5000 g. Then, the supernatant was used for determining the released glucose with a glucose assay kit (F006-1-1) following the manufacturer's instructions. Briefly, samples were incubated with the working solution at 37 ˚C for 15 min. Absorbance of the standard, blank and sample were measured using a spectrophotometer at 505 nm. The hydrolyzed starch was calculated as the following equation:

$$\text{Starch hydrolyzed}\ (\%) = \frac{A_i \times V_i \times C_0 \times 0.9}{A_0 \times W} \times 100$$

where A_i was the absorbance of the sample; V_i was volume of the sample (mL); C_0 was the concentration of the standard (g/mL); A_0 was the absorbance of the standard; W was the total mass of starch in the system (g); 0.9 is the stoichiometric constant for the conversion of glucose to starch.

2.6. In vitro *digestion of protein*

The hydrolysis of protein (sodium caseinate) could be characterized by determining the amounts of amino acids produced by digestion, using the ninhydrin reaction according to the method of Jarunglumlert et al. (Jarunglumlert et al., 2015) 29.5 mg of $NaBH_4$ was dissolved in 300 mL of DMSO (containing 0.15 mol/L ninhydrin) to prepare the ninhydrin solution. Digesta was collected after 1 h SGF incubation and 2 h SIF incubation, and the enzyme activity was stopped by 1 M NaOH to pH 7 and 1 M HCl to pH 2 separately. 0.5 mL of collected sample and 0.5 mL of 5% (w/v) TCA were mixed at 25 ˚C and incubated for 10 min to precipitate large peptides and undigested proteins. 200 μL of supernatant were gathered after centrifuging at 5000 g for 10 min, and then added to a mixture of 600 μL of ninhydrin solution and 200 μL of 0.4 mol/L sodium acetate buffer (pH 5.0). The mixture was incubated in a thermostatic bath at 80 ˚C for 30 min. Absorbance of the amino acid concentration of standard (leucine solution) and sample were measured using a spectrophotometer at 570 nm. The total amount of sodium caseinate was determined using the same method as mentioned. The complete hydrolyzed amino acids of sodium caseinate was obtained by adding 6 M HCl to casein protein and incubating for 24 h at 105 ˚C. The percentage of digested casein and the hydrolyzed protein were calculated using the following equation:

$$\text{Released amino acid(mmol)} = \frac{2 \times A_i \times V_i \times C_0}{A_0} \times 100$$

$$\text{Protein hydrolyzed}(\%) = \frac{\text{Amount of amino acids produced during digestion}}{\text{Amount of amino acid produced by complete hydrolysis of sodium caseinate}} \times 100$$

where A_i was the absorbance of the sample; V_i was the volume of the sample (mL); C_0 was the concentration of the standard (mmol/mL); A_0 was the absorbance of the standard.

2.7. Effect of cellulose on diffusion of glucose

10 mL of glucose solution (100 mmol L^{-1}) containing 0.5 wt.% or 1.5 wt.% NC or MCC was placed into a dialysis bag and immersed in 200 mL deionized water at 37 ˚C. 10 μL of the dialysate was sampled at 20, 30, 60, 90, 120, 180, and 300 min timepoint during dialysis. The released glucose was determined by the glucose assay kit as mentioned. Glucose solution without NC or MCC was used as a control, and the glucose retardation index was calculated using the following equation:

$$\text{Glucose retardation index(mmol/g)} = 100 - \frac{\text{Glucose content with the addition of cellulose}}{\text{Glucose content of control}} \times 100$$

2.8. Effect of cellulose on adsorption of glucose

The glucose solution (5-200 mM) was mixed with 0.5 wt.% NC or MCC in a water-jacketed beaker, and reacted for 6 h at 37 ˚C. The supernatant was taken after centrifuging and determined by the glucose assay kit. 100 mL of glucose solution was used as a positive control, and phosphate buffer (0.1 M, pH=6.5) containing 0.5 wt.% NC was used as the negative control. The adsorbed glucose was calculated using the following equation:

$$\text{Adsorbed glucose(mmol/g)} = \frac{A - (B - C)}{W}$$

where A was the glucose content of positive control (mmol), B was the glucose content of the sample group (mmol), C was the glucose content of the negative control (mmol), and W was the sample mass (g).

2.9. Effect of cellulose on adsorption of trypsin

0.5 wt.% NC or MCC was incubated with 5 mL of trypsin solution (3.2 mg/mL) at 37 ˚C and kept stirring. Samples were taken at timepoint of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 h during incubation, and filtered through a 160-mesh filter cloth (Canspec Scientific, Shanghai, China). The residue was washed for three times with 2 mL PBS. The filtrate was collected and quantified to 10 mL. The enzyme amount was determined using the Bradford method. The adsorbed trypsin was calculated using the following equation:

$$\text{Adsorbed trypsin}(\%) = \frac{\text{The enzyme amount before adsorption} - \text{The enzyme amount in the filtrate}}{\text{The enzyme amount before adsorption}} \times 100$$

2.10. Effect of cellulose on enzymatic activity

2.10.1. Effect of cellulose on the activity of pancreatic α-amylase

40 mL waxy corn starch solution (4 wt.%) containing 0.5 wt.% NC or MCC were mixed with 20 mg pancreatin and incubated at 37 ˚C for 30 min with moderate stirring. The supernatant was taken after centrifuging and determined by the glucose assay kit. The starch solution without NC or MCC was used as the control group. The inhibition ability of pancreatic amylase was calculated using the following equation:

$$\text{Pancreatic amylase inhibition}(\%) = \frac{\text{Produced glucose content in the control group} - \text{Produced glucose content in the sample group}}{\text{Produced glucose content in the control group}} \times 100$$

2.10.2. Effect of cellulose on the activity of pancreatic lipase

13.5 mL PBS (0.1 M, pH 7.2) was incubated with 0.5 mL short-chain fatty acid glycerides (tributyrin as substrate) in a water-jacketed beaker at 37 ˚C. 1 mL of pancreatic lipase filtrate before and after cellulose adsorbing was added in the mixture. The lipase filtrate was obtained after complete adsorption with NC and MCC. The concentration of lipase, NC and MCC used were kept same. The adsorbed lipase in residue was removed and only the free pancreatic lipase in the filtrate were collected. The released FFA was determined by pH-stat titration method. The unabsorbed lipase solution was used as the control. The inhibition ability of pancreatic lipase was calculated using the following equation (Minekus et al., 2014):

$$\text{Pancreatic lipase inhibition}(\%) = \frac{A - B}{A} \times 100$$

where A was the FFA release rate of the control group, and B was the FFA release rate of the sample group.

2.11. Fluorescent spectra

Based on the results of enzyme activity experiments, NC showed inhibitory activity only for α-amylase. Thus, the interaction between NC and α-amylase was further studied via fluorescent spectra. 0.5 mg/mL α-amylase solution was prepared in PBS (0.05 M, pH 6.5) and placed in a four-sided quartz cuvette with optical path of 10 mm. Fluorescence spectrum with a wavelength range between 300 to 400 nm of α-amylase solution containing 1-5 mg/mL NC or without NC were recorded via a M5 fluorescence spectrometer. The excitation wavelength was set at 280 or 295 nm.

2.12. Viscosity determination

A Discovery DHR-2 rheometer was used to determine the rheological properties of the digestion system. The fixture was a 40 mm 2˚ cone plate, the test temperature was 37˚C, and the apparent viscosity of the tested emulsion was measured within the shear rate range of 0.01~500 s^{-1}. The viscosity of different emulsions was compared at a shear rate of 50 s^{-1}.

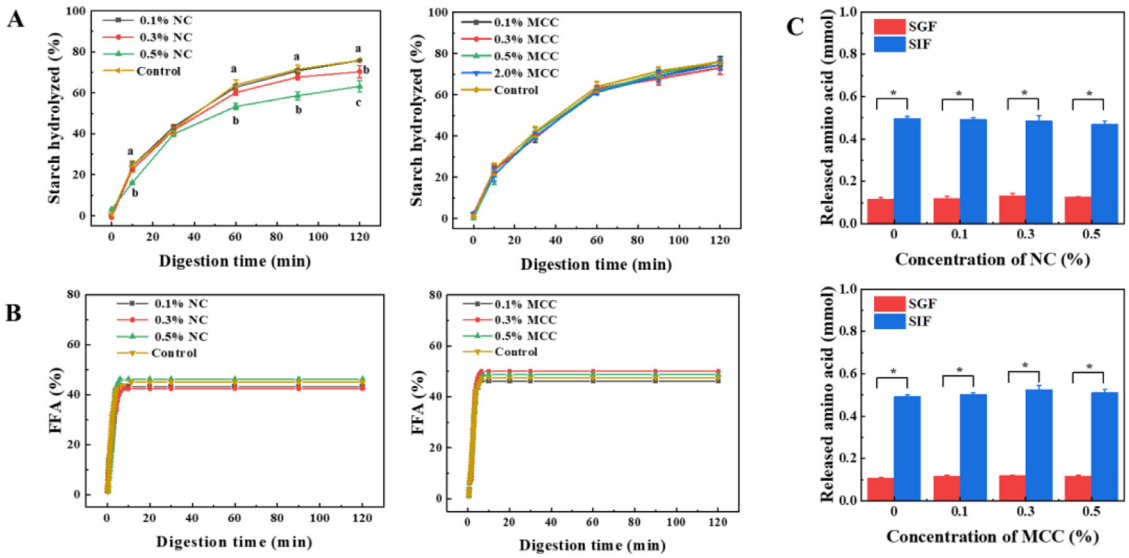

Fig. 1. The effect of NC and MCC with different concentrations on (A) starch hydrolysis; (B) FFA release; (C) free amino acids release (Food mixture without NC and MCC was used as control). Lines with different letters are significantly different ($p < 0.05$).

2.13. Statistical analysis

All measurements were carried out in at least triplicate. Quantitative variables are presented as mean ± standard deviation (SD). The results were subjected to statistical analysis by one-way ANOVA followed by Tukey's test with SPSS software (SPSS Inc., Chicago, IL, USA). The equality of variance was verified with Levene's method. Differences were considered to be significant when p<0.05.

3. Results and discussion

3.1. Effect of NC on the digestion of three major nutrients in food matrix in vitro

The effect of the addition of NC on the digestion of the three macronutrients, that were carbohydrates (waxy corn starch), lipids (peanut oil) and proteins (sodium caseins) were investigated and is shown in Fig. 1, MCC was used as comparison samples to investigate whether the reduced particle size of hydrolyzed cellulose (NC) would slow macronutrients digestion more effectively. The hydrolyzation of starch during intestinal digestion was evaluated by determining the content of released glucose in the digestive system. It can be seen from Fig. 1A that the addition of MCC at any concentration (even 2%) did not cause any significant differences on the amount of glucose released, while 0.1-0.5 wt.% NC addition significantly impaired the glucose diffusion (from 75.9% digestibility of the control sample to 59.2% at 0.5 wt.% presence of NC), indicating the introduction of NC delayed the hydrolysis of starch and reduced its digestibility. The above phenomenon demonstrated that NC can effectively inhibit starch digestion in an *in vitro* simulated digestion system, while MCC would not. Similar results have been also reported by Ji et al. that they found addition of CNC below 0.16% reduced the starch digestibility from 67-78% to around 55% (Ji et al., 2018). This may be attributed to the higher viscosity of the digesta induced by NC, which provided the steric hindrance and delayed the digestion of starch. It was investigated in our previous study that NC could modulate digesta viscosity to inhibit starch digestion depends on particle aspect ratio and charge density (Nsor-Atindana et al., 2018). Another possible reason could be NC inhibited the contact and interaction between enzymes and substrates, which would be discussed in the following part.

According to Figs. 1B and S3, the FFA release rate during simulated intestinal digestion with and without NC or MCC showed similar patterns. Generally, the released FFA increased rapidly at the beginning, and reached saturation at about 46% within 5 min. It is shown that the addition of NC or MCC at any concertation below 0.5% would not influence the digestion pattern of lipids. This phenomenon was different from some other researches that they found NC could delay lipid digestion (Deloid et al., 2018; Liu & Kong, 2019a, 2019b). There were two mechanisms proposed by the authors: (1) NC fibers induce the coalescence of fat droplets that reduce the available surface area for lipase binding; and (2) NC fibers sequester bile salts from food matrix, suppressing the interfacial displacement of bioactive molecules at the lipid droplet surface and thus decreasing the lipolysis. However, these two mechanisms seem did not work in our system, which could probably be explained by the discovery of Liu et al. (2019) They found that low concentrations (below 2%) of NC did not influence initial lipolysis rates and the final lipolysis extent of lipids in the food matrix, while high concentrations would cause significant reductions in the amount of the released FFA. It was reported that even NC with a low concentration could form a dense hydrogel during gastrointestinal digestion, the porous network still could allow the access of lipase with lipids, while at high NC concentrations the viscosity would substantially increase, resulting in the decrease in lipolysis.

As presented in Fig. 1C, free amino acids released during the intestinal digestion were much higher than those in the gastric fluid, that is due to large polypeptides or small oligopeptides were generated under the peptic hydrolysis in SGF, while the major products of protein in the intestinal phase were free amino acids and oligopeptides, thus only small amounts of free amino acids were released during SGF incubation, but more released in the SIF (Liu & Kong, 2019c). However, this does not mean that sodium caseinate was more susceptible to trypsin. As hydrolysis began in the stomach, hydrolyzed peptides were produced under the action of pepsin, while the determined amino acids were further digested and released in the small intestine. The effects of NC and MCC on the free amino acid release during SGF and SIF incubation were negligible. Similar results with us were achieved by Liu et al, who conducted the experiments at a lower NC concentration (0.1%) (Liu & Kong, 2019c). They found 4% CNC could significantly reduce free amino nitrogen release which hardly could be observed at 0.1% CNC, indicating NC concentrations would play a role in protein digestion and nitrogen release. The mechanism for reduced protein digestibility found in

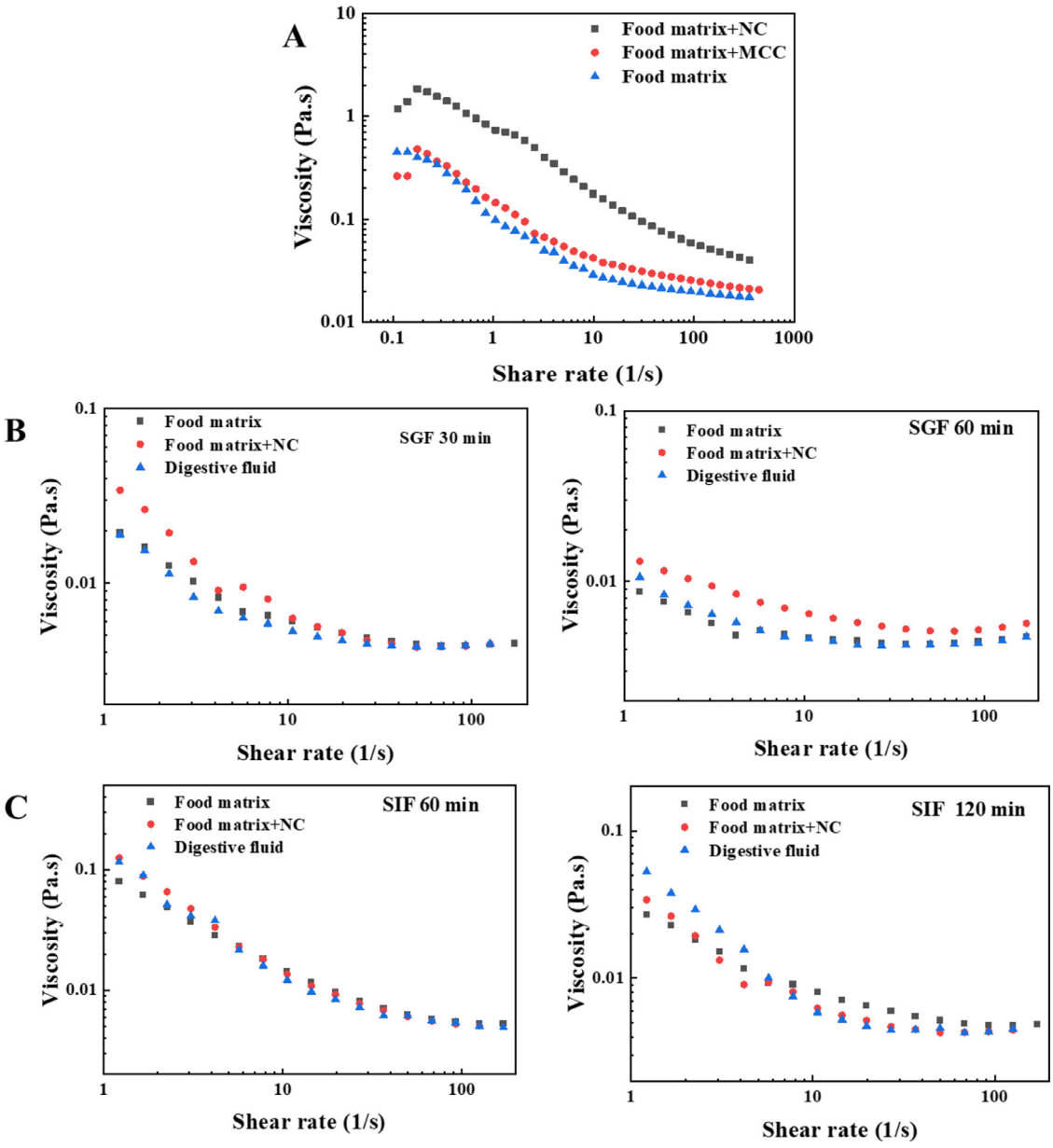

Fig. 2. (A) The effect of NC and MCC on the viscosity of food matrix; (B) the viscosity of the digestion system after 30 and 60 min of simulated gastric hydrolysis and (C) after 60 and 120 min of simulated small intestinal hydrolysis.

Liu's study was attributed to the high viscosity caused by high concentration of CNC, which inhibits the interaction between gastrointestinal enzymes and protein substrates (Liu & Kong, 2019c).

Above research indicated that compared with MCC, NC was found to be able to delay starch digestion, while had almost no effect on lipid and protein digestibility at low concentration (below 0.5 wt.%). However, the potential mechanism how NC could influence the digestion of starch still need to be studied, and we will discuss this in the following part.

3.2. *Effect of NC on the viscosity of food matrix*

As we mentioned before, the effect of NC on the viscosity of the digesta might be the reason why NC can delay and inhibit starch hydrolysis in the simulated *in vitro* digestion experiment. Hence the viscosity of food matrix with or without NC/MCC was evaluated in Fig. 2A. The addition concentration of NC and MCC was 0.5 wt.%, and all the sample exhibited shear thinning behavior. Compared with the food matrix without adding cellulose, the presence of NC has significantly increased the viscosity of the system, while the addition of MCC has no significant effect on the systematic viscosity. It has been reported that the high viscosity contributed to the decreased starch digestion and glucose diffusion (Liu & Kong, 2019a). If NC can remain the high viscosity of the system during the digestion process, the contribution of NC to the systematic viscosity would likely be the reason of the inhibition of starch digestion. Hence, the viscosity of food matrix with/without NC during gastrointestinal digestion was subsequently evaluated in Fig. 2B & C. It can be seen from the figure that after entering the GI tract, the contribution of NC to the systematic viscosity disappeared. The viscosity of food matrix including NC was similar with that of food matrix only, and even with the blank digestive juice (using the same volume of water instead of the food paste). This phenomenon was different from that using other hydrocolloids like xanthan gum which could delay starch digestion by increasing the viscosity of the system, reported by Goff et al. (Fabek et al., 2015; Fabek et al., 2014) The possible reason for the difference of these two cases might be the solubility of NC and xan-

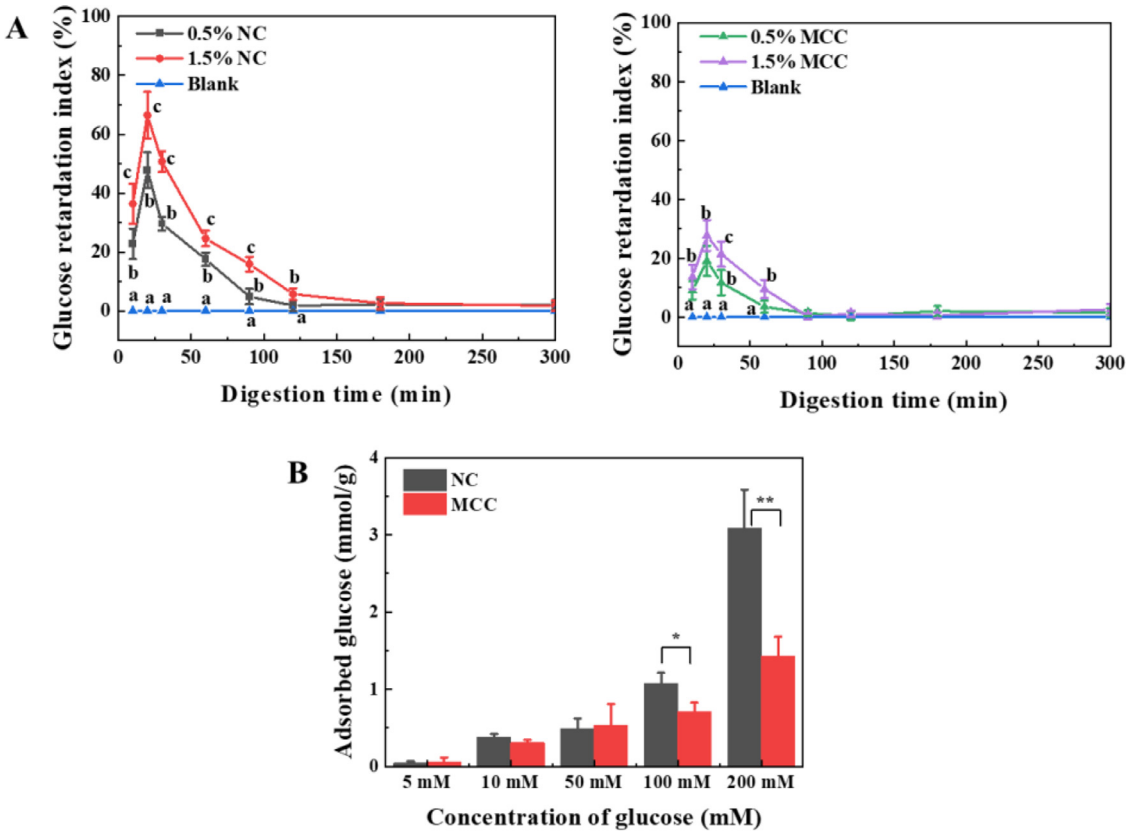

Fig. 3. (A) The effect of NC and MCC on the diffusion of glucose; (B) glucose-adsorption capacity of NC and MCC. Lines with different letters are significantly different ($p < 0.05$). * $p < 0.05$; ** $p < 0.01$.

than gum was different, NC is an insoluble fiber, while xanthan gum is a water-soluble colloid. The increased viscosity induced by NC cannot tolerated 8 times of dilution with digestive juice. However, the opposite phenomenon has been reported by several literatures that they found an increased viscosity of NC-containing system after entering the gastric and intestinal phase (Liu et al., 2019; Liu & Kong, 2019a, 2019b, 2019c). The difference of the nanocellulose used could account for this. In our previous study, we found the effect of gastrointestinal digestion on the viscosity of NC-containing system was highly depends on the type of NC used (Nsor-Atindana et al., 2018). This conclusion was further confirmed by Liu et al. that they discovered an increased cellulose nanocrystals (CNC) viscosity after gastric digestion due to the high ionic strength of SGF, while a decrease in viscosity for cellulose nano-fibrils (CNF) because of the occurrence of aggregates formation and phase separation (Liu et al., 2019). The nanocellulose we used in this study probably belongs to the later one. Therefore, in our study, the modulation of the systemic viscosity with NC was not the cause of the inhibition of starch digestion.

3.3. Effect of NC on the glucose diffusion and adsorption

The glucose dialysis retardation index (GDRI) was used to evaluate the influence of cellulose on the glucose diffusion in the small intestine, which was a useful *in vitro* index to predict the delay in glucose adsorption property of cellulose (Chau et al., 2003; Ou et al., 2001). Fig. 3A presents the effects of NC and MCC at low (0.5 wt.%) or high (1.5 wt.%) concentration on the glucose diffusion. Both NC and MCC have a strong ability to inhibit the diffusion of glucose, even at low concentration. It could be observed that GDRI maximal values were reached after 20 min during simulated intestinal digestion, which are 47.8% for 0.5 wt.% NC, 66.4% for 1.5 wt.% NC, 19.1% for 0.5 wt.% MCC and 27.8% for 1.5 wt.% MCC, respectively. While comparing with MCC containing system, it was revealed that NC at all concentrations have a significantly stronger (about 2.5 times of MCC) ability to inhibit the diffusion of glucose ($p<0.05$). Among all the samples containing cellulose, the GDRI generally diminished over time. According to the comprehensive report of previous researchers (Chau et al., 2003; López et al., 1996; Ou et al., 2001), the greater glucose diffusion inhibition ability of NC was probably attributed to the higher viscosity of NC suspension compared with MCC. As the concentration of NC in the dialysis bag increased, the viscosity of the solution elevated, delaying the diffusion of glucose. However, in last section it has been confirmed that the viscosity of digesta containing cellulose would be extreme low after dilution, which means the viscosity of NC containing system was not the major reason. On the other hand, it was found that the particle size of NC (65 nm, Fig. S2) was much smaller than MCC (7 μm, Fig. S2), indicating a greater specific surface area of NC that would contribute to a higher glucose adsorption capacity and then reduce the diffusion rate of glucose. In addition to the glucose adsorption, the physical obstacle and the entrapment of glucose within the network provided by insoluble cellulose might be another reason for the retardation in glucose diffusion. Greater GDRI of insoluble fibers compared with soluble ones has been demonstrated by López et al. (1996).

As the glucose diffusion reached equilibrium (normally 6 h), the difference of glucose in the dialysate between control and treatment can be regarded as the amount of glucose adsorbed (Ou et al., 2001). Fig. 3B presents the isothermal adsorption of glucose by NC and MCC with a series of different concentrations. This experiment was used to prove the above speculation. It was shown that all of the cellulose can bind glucose, but they showed only slightly adsorption ability for glucose when the glucose concentration was below 10 mM. When the glucose concentration is higher than 100 mM, NC has a significant stronger ability ($p<0.05$) to adsorb glucose compared with MCC. Up to 200 mM, the glucose adsorption capacity of NC (3.08 mmol/g) is 2.2 times that of MCC

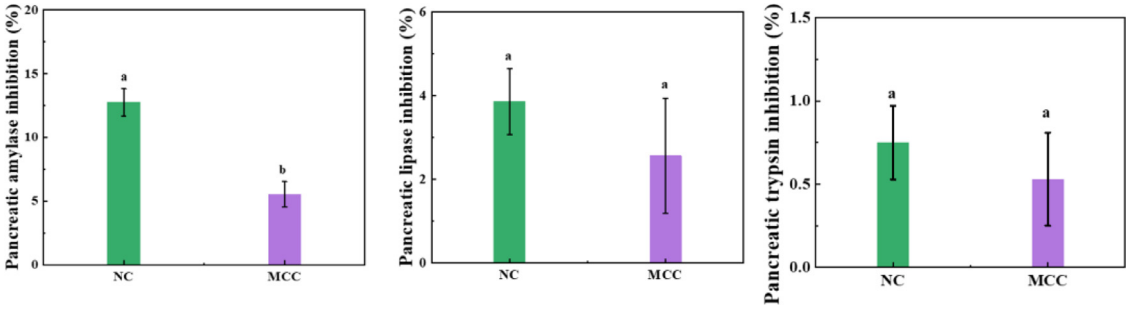

Fig. 4. The inhibition ability of NC and MCC on the pancreatic α-amylase, lipase and trypsin. Different letters indicated significantly different between NC and MCC ($p < 0.05$).

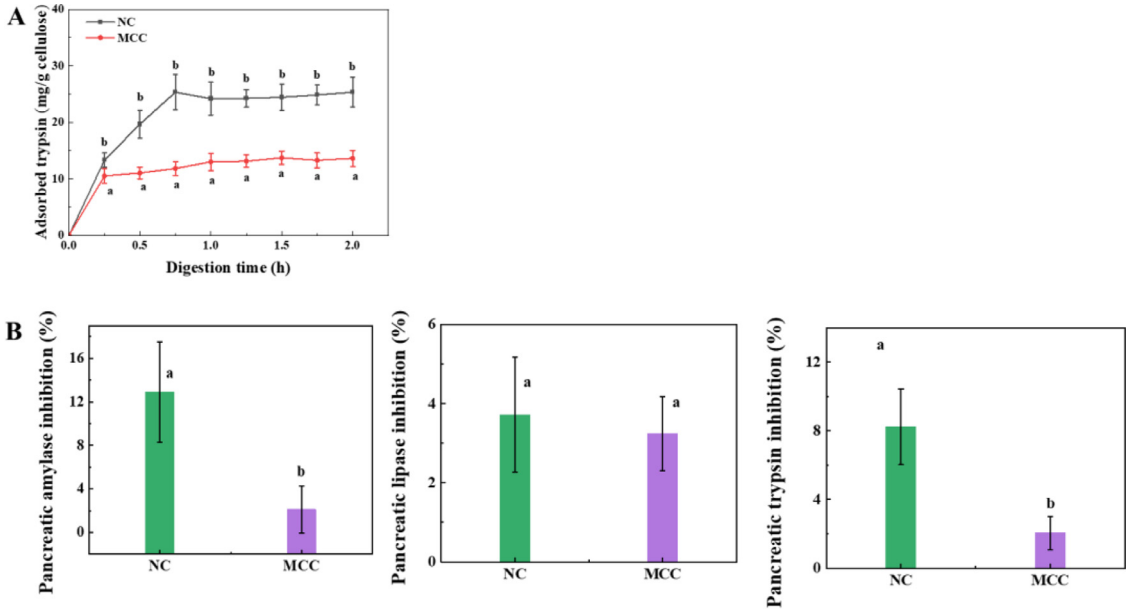

Fig. 5. (A) The pancreatin adsorption ability of NC and MCC; (B) the activity inhibition ratio of pancreatic α-amylase, lipase, and trypsin after adsorption by NC and MCC. Different letters indicated significantly different between NC and MCC ($p < 0.05$).

(1.42 mmol/g). In our simulated *in vitro* digestion system, the glucose concentration of food matrix reached about 50 mM after 2 h intestinal incubation. According to Fig. 3B, the adsorption capacity of NC and MCC on glucose at this level was very low, with no significant difference. From these results, the effect of NC on glucose is not the cause of delaying starch digestion, but it was still conceivable that these insoluble NC could effectively adsorb glucose, delay the glucose diffusion, and then postpone the glucose absorption in the real human gastrointestinal tract.

3.4. Effect of NC on the enzyme activity

Above experiments demonstrated that the delay of starch digestion or the inhibition of glucose diffusion with the addition of NC was not caused by the change of systemic viscosity or the adsorption of released glucose. Hence, the last hypothesis that the interactions between NC and digestive enzymes might be the potential mechanism. As shown in Fig. 4, the inhibition effect of NC and MCC on the enzyme activity of pancreatic α-amylase, lipase and trypsin was explored. NC presented a significant inhibitory effect on α-amylase activity with an inhibition ratio of 14.6%, but had almost no effect on lipase and trypsin. Compared with NC, the inhibitory effect of MCC was negligible. Undoubtedly, the effect of NC on α-amylase activity is the reason why NC could delay starch digestion, but this effect was based on the interaction with the substrate or the enzyme itself still need to be further investigated.

To clarify this question, the adsorption ability of NC on pancreatin was studied. Fig. 5A shows the adsorption curve of NC and MCC towards pancreatin as time went by. The maximum adsorption of NC and MCC was reached after 45 and 15 min respectively, with adsorbed amount of 25 mg/g and 13 mg/g. The adsorption effect of NC on pancreatin is significantly stronger than that of MCC, which may be attributed to the smaller particles size and larger specific surface area of NC that resulted in more pancreatin being adsorbed and bound. Hereafter, NC and MCC were incubated with the pancreatin solution and the filtrate was collected after complete adsorption. The enzyme solution directly subjected to the same filtration operation was used as the control. The inhibition ratio of the enzyme activity based on the filtrate and control was then calculated, shown in Fig. 5B. Similar with the results in Fig. 4, only NC presented a notable inhibitory effect on α-amylase with an inhibition ratio of 13.1%, without any significant effect on other enzymes. In addition, the enzyme activity of the filter residue after NC adsorption was also examined, and it could not be measured, indicating that the enzyme adsorbed by NC will lose its activity. Thus, it can be confirmed that the inhibition of pancreatic α-amylase activity by NC was caused by the adsorption of NC to enzyme.

The interaction of NC with α-amylase was further investigated using fluorescence spectroscopy presented in Fig. 6. In usual, the tyrosine and tryptophan residues of the pancreatic α-amylase will produce fluorescence (Hua et al., 2018). When NC was interacted with α-amylase, fluorescence quenching would occur, then the tested fluorescence intensity

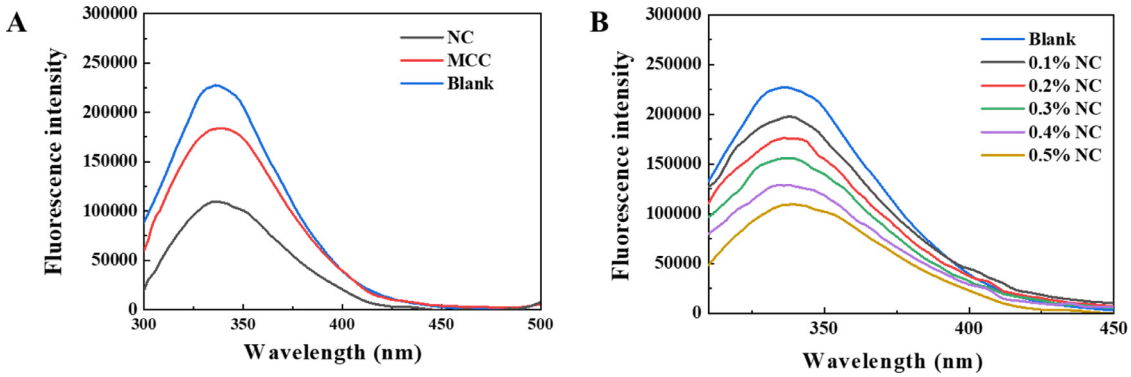

Fig. 6. Effect of NC and MCC on fluorescent spectrum of pancreatic α-amylase (A) at 280 nm and (B) 295 nm.

would decrease. As shown in Fig. 6A, the addition of 0.5 wt.% NC exhibited a significant fluorescence quenching effect on α-amylase, while the effect of MCC at the same concentration was weaker. This suggests the interaction between NC and α-amylase, such as electrostatic or hydrogen bond interaction, changed the hydrophobic microenvironment of the fluorescent chromophore (Zhang et al., 2014). In addition, at the emission wavelength of 280 nm, the addition of NC induced a slight red-shifting of fluorescence peak of α-amylase at 336 nm, indicating the influence of NC on the conformation of pancreatic amylase.

Fig. 6B shows a gradually reduced fluorescence intensity of α-amylase as the NC concentration increased. To confirm quenching process of NC to the fluorescence of α-amylase and illustrated the mechanism in it, the fluorescence spectra were analyzed using the Stern-Volmer equation (Kandagal et al., 2006; Zhang et al., 2014).

$$\frac{F_0}{F} = 1+k_q\times\tau_0\times[Q] = 1+K_{SV}\times[Q]$$

where F_0 and F are the relative fluorescence intensities with or without the quencher, [Q] is the concentration of quencher, K_{SV} is the Stern-Volmer constant, K_q is the quenching constant of bimolecular, which is 2.0×10^{10} L•moL^{-1}•s^{-1} here, τ_0 is the lifetime of the endogenous fluorophore without quencher with a value 10^{-8} s^{-1}. K_{SV} calculated from this equation was about 1.25×10^{14}, which is far greater than 10^{10}, which means the interaction between NC and α-amylase is static quenching possibly caused by Van der Waals forces or other forces.

The number of binding sites (n) between NC and α-amylase can be calculated from the following equation (Zhang et al., 2014).

$$\lg\left(\frac{F_0-F}{F}\right) = \lg K_{SV}+n\lg[Q]$$

n was 1.5618 calculated by this equation, suggesting the number of binding sites was 1-2. Therefore, the delay of starch digestion is due to the inhibitory effect of NC on the α-amylase activity, which is caused by the adsorption of NC towards pancreatic amylase resulting in the static quenching of enzyme.

4. Conclusion

This study explored the effect of NC on the digestion of the three macronutrients through an *in vitro* digestion model. It was found that 0.5 wt.% of NC has a significant inhibitory effect on starch digestion with the inhibition ratio of 14.3%, but no obvious effect on the digestion of lipids and protein. Low concentration of NC (below 0.5 wt.%) can increase the systemic viscosity before digestion, but the viscosity of food matrix would significantly reduce after entering the gastrointestinal tract. NC presented a high adsorption capacity towards glucose when the glucose concentration was above 100 mM, however in our digestive system, the glucose concentration was only 50 mM, indicating the effect of NC on glucose is not the cause of delaying starch digestion. Therefore, the possible reason for the inhibited starch digestion was focused on the interaction of NC with the digestive enzymes. NC exhibited a great adsorption capacity on α-amylase and showed an enzyme activity inhibition ratio of 14.6%. According to the results of fluorescence spectroscopy, it was found that the interaction between NC and α-amylase is static quenching and the number of binding sites was 1-2. Above experiments demonstrated that NC could delay the starch digestion through adsorption towards the pancreatic amylase and does have the potential to be used as a dietary fiber in functional foods for obesity and other special populations.

However, in this study, the functional properties of NCs were indirectly evaluated by the method of static digestion models, so animal and even human experiments are needed to confirm this result more realistically. In addition, we only examined the effect of NC with a narrow concentration range on a fixed food matrix. A further investigation should be conducted into how NC with higher or lower concentrations affects other kinds of food systems.

Funding

This research was supported by the National Natural Science Foundation of China (No. 32072153) and the International S&T Innovation Cooperation Key Project (2017YFE0135400).The research is also supported by 111 project-B07029, national first-class discipline program of Food Science and Technology (JUFSTR20180204) and program of "Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province", China.

Ethical statement

The research presented does not involve any animal or human study.

Declaration of Competing Interest

The authors confirm that they have no conflict of interest to declare for this publication. Given his/her role as Editor in Chief at the time of submission, Fang Zhong had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another editor, as per the Journal guidelines.

Data Availability

No data was used for the research described in the article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2022.100098.

References

Chassaing, B., Miles-Brown, J., Pellizzon, M., Ulman, E., Ricci, M., Zhang, L., Patterson, A. D., Vijay-Kumar, M., & Gewirtz, A. T. (2015). Lack of soluble fiber drives diet-induced adiposity in mice. *American Journal of Physiology Gastrointestinal and Liver Physiology, 309*(7), G528–G541.

Chau, C. F., Huang, Y. L., & Lee, M. H. (2003). *In vitro* hypoglycemic effects of different insoluble fiber-rich fractions prepared from the peel of Citrus sinensis L. cv. Liucheng. *Journal of Agricultural and Food Chemistry, 51*(22), 6623–6626.

Deloid, G. M., Soha, I. S., Lorente, L. R., Molina, R. M., Pyrgiotakis, G., Stevanovic, A., Zhang, R., McClements, D. J., Geitner, N. K., Bousfield, D. W., Ng, K. W., Loo, S. C. J., Bell, D. C., Brain, J., & Demokritou, P. (2018). Reducing intestinal digestion and absorption of fat using a nature-derived biopolymer: Interference of triglyceride hydrolysis by nanocellulose. *ACS Nano* acsnano.8b03074-.

Eastwood, M., & Kritchevsky, D. (2005). Dietary fiber: How did we get where we are? *Annual Review of Nutrition, 25*(1), 1–8.

Fabek, H., & Goff, H. D. (2015). Simulated intestinal hydrolysis of native tapioca starch: Understanding the effect of soluble fibre. *Bioactive Carbohydrates and Dietary Fibre, 6*(2), 83–98.

Fabek, H., Messerschmidt, S., Brulport, V., & Goff, H. D. (2014). The effect of *in vitro* digestive processes on the viscosity of dietary fibres and their influence on glucose diffusion. *Food Hydrocolloids, 35*, 718–726.

Hsu, P.-K., Chien, P.-J., Chen, C.-H., & Chau, C.-F. (2006). Carrot insoluble fiber-rich fraction lowers lipid and cholesterol absorption in hamsters. *LWT - Food Science and Technology, 39*(4), 338–343.

Hua, F., Zhou, P., Wu, H. Y., Chu, G. X., Xie, Z. W., & Bao, G. H. (2018). Inhibition of α-glucosidase and α-amylase by flavonoid glycosides from Lu'an GuaPian tea: Molecular docking and interaction mechanism. *Food Function, 9*(8), 4173–4183.

Hughes, J. S., Acevedo, E., Bressani, R., & Swanson, B. G. (1996). Effects of dietary fiber and tannins on protein utilization in dry beans (Phaseolus vulgaris). *Food Research International, 29*(3), 331–338.

Jarunglumlert, T., Nakagawa, K., & Adachi, S. (2015). Digestibility and structural parameters of spray-dried casein clusters under simulated gastric conditions. *Food Research International, 75*, 166–173.

Ji, N., Liu, C., Li, M., Sun, Q., & Xiong, L. (2018). Interaction of cellulose nanocrystals and amylase: Its influence on enzyme activity and resistant starch content. *Food Chemistry, 245*, 481–487.

Jiang, F., & Hsieh, Y. L. (2015). Holocellulose nanocrystals: Amphiphilicity, oil/water emulsion, and self-assembly. *Biomacromolecules, 16*(4), 1433–1441.

Kandagal, P. B., Ashoka, S., Seetharamappa, J., Shaikh, S. M., Jadegoud, Y., & Ijare, O. B. (2006). Study of the interaction of an anticancer drug with human and bovine serum albumin: Spectroscopic approach. *Journal of Pharmaceutical and Biomedical Analysis, 41*(2), 393–399.

Kendall, C. W. C., Esfahani, A., & Jenkins, D. J. A. (2010). The link between dietary fibre and human health. *Food Hydrocolloids, 24*(1), 42–48.

Lin, Q., Rong, L., Fang, Z., Ye, A., & Singh, H. (2018). Effect of degree of octenyl succinic anhydride (OSA) substitution on the digestion of emulsions and the bioaccessibility of β-carotene in OSA-modified-starch-stabilized-emulsions. *Food Hydrocolloids*.

Lin, Q., Rong, L., Ye, A., Singh, H., & Fang, Z. (2017). Effects of calcium on lipid digestion in nanoemulsions stabilized by modified starch: Implications for bioaccessibility of β-carotene. *Food Hydrocolloids, 73*, 184–193.

Liu, L., Kerr, W. L., & Kong, F. (2019). Characterization of lipid emulsions during *in vitro* digestion in the presence of three types of nanocellulose. *Journal of Colloid & Interface Science, 545*, 317–329.

Liu, L., & Kong, F. (2019a). *In vitro* investigation of the influence of nano-cellulose on starch and milk digestion and mineral adsorption. *International Journal of Biological Macromolecules, 137*, 1278–1285.

Liu, L., & Kong, F. (2019b). *In vitro* investigation of the influence of nano-fibrillated cellulose on lipid digestion and absorption. *International Journal of Biological Macromolecules, 139*, 361–366.

Liu, L., & Kong, F. (2019c). Influence of nanocellulose on *in vitro* digestion of whey protein isolate. *Carbohydrate Polymers, 210*, 399–411.

López, G., Ros, G., Rincón, F., Periago, M. J., Martínez, M. C., & Ortuño, J. (1996). Relationship between physical and hydration properties of soluble and insoluble fiber of artichoke. *Journal of Agricultural and Food Chemistry, 44*(9), 2773–2778.

Lu, H., Gui, Y., Guo, T., Wang, Q., & Liu, X. (2015). Effect of the particle size of cellulose from sweet potato residues on lipid metabolism and cecal conditions in ovariectomized rats. *Food Function, 6*(4), 1185–1193.

Mackie, A. R., Bajka, B., & Rigby, N. (2016). Dietary fibre: More than a prebiotic. In *Gums and stabilisers for the food industry 18: Hydrocolloid functionality for affordable and sustainable global food solutions* (pp. 227–234). The Royal Society of Chemistry.

Maćkowiak, K., Torlińska-Walkowiak, N., & Torlińska, B. (2016). Dietary fibre as an important constituent of the diet. *Postepy Higieny i Medycyny Doswiadczalnej, 70*, 104–109 (Online).

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carri'ere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Feunteun, S. L., Lesmes, U., Macierzanka, A., Mackie, A., et al., (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food & Function, 5*(6), 1113.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., et al., (2014). A standardised static *in vitro* digestion method suitable for food - an international consensus. *Food Function, 5*(6), 1113–1124.

Ng, Z. X., & Rosman, N. F. (2019). *In vitro* digestion and domestic cooking improved the total antioxidant activity and carbohydrate-digestive enzymes inhibitory potential of selected edible mushrooms. *Journal of Food Science and Technology, 56*(2), 865–877.

Ng, Z. X., & See, A. N. (2019). Effect of *in vitro* digestion on the total polyphenol and flavonoid, antioxidant activity and carbohydrate hydrolyzing enzymes inhibitory potential of selected functional plant-based foods. *Journal of Food Processing and Preservation, 43*(4), e13903.

Nsor-Atindana, J., Douglas Goff, H., Liu, W., Chen, M., & Zhong, F. (2018). The resilience of nanocrystalline cellulose viscosity to simulated digestive processes and its influence on glucose diffusion. *Carbohydrate Polymers, 200*, 436–445.

Ou, S., Kwok, K., Li, Y., & Fu, L. (2001). *In vitro* study of possible role of dietary fiber in lowering postprandial serum glucose. *Journal of Agricultural and Food Chemistry, 49*(2), 1026–1029.

Phillips, J., Muir, J. G., Birkett, A., Lu, Z. X., Jones, G. P., O'Dea, K., & Young, G. P. (1995). Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *American Journal of Clinical Nutrition, 62*(1), 121–130.

Takahashi, T., Karita, S., Ogawa, N., & Goto, M. (2005). Crystalline cellulose reduces plasma glucose concentrations and stimulates water absorption by increasing the digesta viscosity in rats. *Journal of Nutrition, 135*(10), 2405–2410.

Tsujita, T., Sumiyosh, M., Han, L. K., Fujiwara, T., Tsujita, J., & Okuda, H. (2003). Inhibition of lipase activities by citrus pectin. *Journal of Nutritional Science and Vitaminology, 49*(5), 340–345.

Vaĭnshteĭn, S. G., Zhulkevich, I. V., Petropavlovskiĭ, G. A., & Kotel'nikova, N. E. (1987). [Protective properties of microcrystalline cellulose in experimental diabetes mellitus in rats]. *Biulleten Eksperimentalnoi Biologii I Meditsiny, 103*(2), 167–168.

Wooster, T. J., Day, L., Xu, M., Golding, M., Oiseth, S., Keogh, J., & Clifton, P. (2014). Impact of different biopolymer networks on the digestion of gastric structured emulsions. *Food Hydrocolloids, 36*, 102–114.

Zhang, X., Li, L., Xu, Z., Su, J., Li, B., & Huang, J. (2014). Studies on the interaction of naringin palmitate with lysozyme by spectroscopic analysis. *Journal of Functional Foods, 8*, 331–339.

Zhong, Y., Nyman, M., & Fåk, F. (2015). Modulation of gut microbiota in rats fed high–fat diets by processing whole-grain barley to barley malt. *Molecular Nutrition & Food Research, 59*(10), 2066–2076.