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Formulación de alimentos lácteos para el control de peso.

Cambios estructurales durante la digestión *in vitro*.

TESIS DOCTORAL

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HACEN CONSTAR QUE:

El trabajo de investigación "Formulación de alimentos lácteos para el control de peso. Cambios estructurales durante la digestión *in vitro*", que presenta Dña. Jennifer Borreani por la Universitat Politècnica de València, y que ha sido realizado bajo nuestra dirección en el Grupo de Investigación de Microestructura y Química de Alimentos de la Universitat Politècnica de València, reúne las condiciones para optar al grado de Doctor.

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Fdo. Dra. Isabel Hernando Hernando Fdo. Dra. Amparo Quiles Chuliá

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Resumen

La presente tesis doctoral se centra en el desarrollo y obtención de productos lácteos orientados al control del peso, siguiendo dos estrategias diferenciadas: 1) regular la ingesta calórica mediante el diseño de alimentos de alta capacidad saciante y 2) moderar el aporte de grasa al organismo mediante el diseño de alimentos de menor contenido en grasa o de reducida digestibilidad lipídica.

Para modular la **capacidad saciante** se decidió, en primer lugar, añadir diferentes hidrocoloides (goma konjac o alginato) a distintos sistemas modelo con proteína láctea. Dependiendo de las propiedades fisicoquímicas de los hidrocoloides empleados se consiguió influir sobre diferentes mecanismos postingestivos de saciedad que tienen lugar durante la digestión gástrica. La adición de goma konjac (hidrocoloide neutro) permitió incrementar la viscosidad de los sistemas lácteos, incluso a lo largo de la digestión gástrica *in vitro*, lo que podría resultar en un aumento de la distensión gástrica; mientras que la adición de alginato (hidrocoloide cargado negativamente) permitió retrasar la digestibilidad proteica y, en consecuencia, podría prolongar la liberación de hormonas relacionadas con la saciedad.

En segundo lugar, se decidió aumentar, en un postre lácteo como es la panna cotta, la cantidad en proteínas (macronutriente con mayor capacidad saciante) provenientes de diferentes fracciones proteicas de la leche (leche, proteínas del suero lácteo o caseinato). Las proteínas del suero lácteo demostraron aportar mayor firmeza al postre lácteo; resultado que fue potenciado al reducir, además, el contenido en nata. Por otro lado, la digestión gástrica *in vitro* mostró que las proteínas del suero lácteo permanecieron prácticamente intactas después del ataque enzimático (pepsina), lo que está relacionado con una mayor capacidad saciante.

Para **reducir el contenido en grasa** o la **digestibilidad lipídica** se trabajó con panna cottas en las que se decidió sustituir parcial o totalmente la grasa (nata) por emulsiones. Previamente, se estudiaron diferentes emulsiones antes y durante la digestión gastrointestinal *in vitro* para comprender qué factores afectan a su estructura, consistencia y digestibilidad lipídica. En función del tipo de emulsionante o estabilizante empleado (proteínas, lecitina, polisorbato, derivados de celulosa, goma xantana) se obtuvieron emulsiones de diferentes comportamientos reológicos, consistencias y grados de digestibilidad lipídica; las cuales, además, dependiendo de su composición sufrieron una serie de cambios estructurales tras su paso por el tracto gastrointestinal *in vitro*. Por lo tanto, gracias a los diversos estudios realizados, se determinó que para diseñar adecuadamente emulsiones de reducida digestibilidad es fundamental tener en cuenta durante la digestión gastrointestinal *in vitro*: el tamaño de los glóbulos de grasa, la consistencia de la emulsión y/o del sistema digerido, el tipo y localización del estabilizante empleado, así como las interacciones que pueden tener lugar entre algunos de los ingredientes de las emulsiones y otros componentes presentes en los jugos digestivos.

En la reformulación de las panna cottas, tanto el tipo de hidrocoloide empleado (derivados de celulosa o goma xantana) para estabilizar las emulsiones como la cantidad de nata reemplazada por éstas modificaron las propiedades físicas (estructura y firmeza) y sensoriales (principalmente el sabor y la textura) de las panna cottas. Sin embargo, las panna cottas elaboradas con emulsiones estabilizadas con hidroxipropilmetilcelulosa (HPMC) obtuvieron un buen nivel de aceptación sensorial. Además, mediante el uso de estas emulsiones se consiguió reducir, por un lado, el contenido en grasa de las panna cottas hasta un 37,5% y, por otro lado, su digestibilidad lipídica hasta un 20%.

Resum

La present tesi doctoral se centra en el desenvolupament i obtenció de productes làctics orientats al control del pes, seguint dues estratègies diferenciades: 1) regular la ingesta calòrica mitjançant el disseny d'aliments d'alta capacitat saciant i 2) moderar l'aportació de greix a l'organisme mitjançant el disseny d'aliments de menor contingut en greix o de reduïda digestibilitat lipídica.

Per a modular la **capacitat saciant** es va decidir, en primer lloc, afegir diferents hidrocol·loides (goma konjac o alginat) a diferents sistemes model amb proteïna làctica. Depenent de les propietats fisicoquímiques dels hidrocol·loides emprats es va aconseguir influir sobre diferents mecanismes postingestius de sacietat que tenen lloc durant la digestió gàstrica. L'addició de goma konjac (hidrocol·loide neutre) va permetre incrementar la viscositat dels sistemes làctics, fins i tot al llarg de la digestió gàstrica *in vitro*, la qual cosa podria resultar en un augment de la distensió gàstrica; mentre que l'addició d'alginat (hidrocol·loide carregat negativament) va permetre retardar la digestibilitat proteica i, en conseqüència, podria prolongar l'alliberament d'hormones relacionades amb la sacietat.

En segon lloc, es va decidir augmentar, en unes postres làctiques com és la panna cotta, la quantitat en proteïnes (macronutrient amb major capacitat saciant) provinents de diferents fraccions proteiques de la llet (llet, proteïnes del sèrum làctic o caseïnat). Les proteïnes del sèrum làctic van demostrar aportar major fermesa a les postres làctiques; resultat que va ser potenciat en reduir, a més, el contingut en nata. D'altra banda, la digestió gàstrica *in vitro* va mostrar que les proteïnes del sèrum làctic van romandre pràcticament intactes després de l'atac enzimàtic (pepsina), la qual cosa està relacionat amb una major capacitat saciant.

Per a **reduir el contingut en greix** o la **digestibilitat lipídica** es va treballar amb panna cottas en les quals es va decidir substituir parcial o totalment el greix (nata) per emulsions. Prèviament, es van estudiar diferents emulsions abans i durant la digestió gastrointestinal *in vitro* per a comprendre què factors afecten la seua estructura, consistència i digestibilitat lipídica. En funció del tipus d'emulsionant o estabilitzant emprat (proteïnes, lecitina de soja, polisorbat, derivats de cel·lulosa, goma xantana) es van obtindre emulsions de diferents comportaments reològics, consistències i graus de digestibilitat lipídica; les quals, a més, depenent de la seua composició van patir una sèrie de canvis estructurals després del seu pas pel tracte gastrointestinal *in vitro*. Per tant, gràcies als diversos estudis realitzats, es va determinar que per a dissenyar adequadament emulsions de reduïda digestibilitat és fonamental tindre en compte durant la digestió gastrointestinal *in vitro*: la grandària dels glòbuls de greix, la consistència de l'emulsió i/o del sistema digerit, el tipus i localització del estabilitzant emprat, així com les interaccions que poden tindre lloc entre alguns dels ingredients de les emulsions i uns altres components presents en els sucs digestius.

En la reformulació de les panna cottas, tant el tipus de hidrocol·loide emprat (derivats de cel·lulosa o goma xantana) per a estabilitzar les emulsions com la quantitat de nata reemplaçada per aquestes van modificar les propietats físiques (estructura i fermesa) i sensorials (principalment el sabor i la textura) de les panna cottas. No obstant això, les panna cottas elaborades amb emulsions estabilitzades amb hidroxipropilmetilcel·lulosa (HPMC) van obtindre un bon nivell d'acceptació sensorial. A més, mitjançant l'ús d'aquestes emulsions es va aconseguir reduir, d'una banda, el contingut en greix de les panna cottas fins a un 37,5% i, d'altra banda, la seua digestibilitat lipídica fins a un 20%.

Abstract

The research of this doctoral thesis focuses on the development of dairy products aimed at weight management. Two differentiated strategies were followed: 1) to regulate the caloric intake through the design of high satiating capacity foods and 2) to moderate the contribution of fat to the body by designing foods with less fat content or reduced lipid digestibility.

In order to modulate the **satiating capacity**, on the one hand, different hydrocolloids (konjac gum or alginate) were added to dairy protein model systems. Depending on the physicochemical properties of the hydrocolloids used, it was possible to influence different post-ingestive satiety mechanisms that take place during gastric digestion. The addition of konjac gum (neutral hydrocolloid) allowed the viscosity of dairy systems to increase, even during *in vitro* gastric digestion, which could result in an increase in gastric distension; whereas the addition of alginate (negatively charged hydrocolloid) allowed protein digestibility to be delayed and, consequently, could prolong the release of satiety related hormones.

On the other hand, the level of proteins (the most satiating macronutrient) from different dairy fractions (milk, whey proteins or caseinate) was increased in a dairy dessert, such as panna cotta. Whey proteins provided the greatest firmness to the dairy dessert, which was also enhanced by reducing its cream content. Moreover, whey proteins remained practically intact after the enzymatic (pepsin) attack during *in vitro* gastric digestion, which is related to a higher satiating capacity.

In order to **reduce the fat content** or the **lipid digestibility** of panna cottas, the fat (cream) content was partially or totally replaced by emulsions. Previously, different emulsions were studied before and during *in vitro* gastrointestinal digestion in order to understand which factors affect their structure, consistency and lipid digestibility. Depending on the type of emulsifier or stabiliser used (proteins,

lecithin, polysorbate, cellulose derivatives, xanthan gum), emulsions with different rheological behaviours, consistencies and degrees of lipid digestibility were obtained. Furthermore, depending on their composition the emulsions underwent a series of structural changes after their passage through the *in vitro* gastrointestinal tract. Therefore, thanks to the various studies carried out it has been determined that to properly design emulsions with reduced digestibility, it is essential to take into consideration the following factors during *in vitro* gastrointestinal digestion: the size of the fat globules, the consistency of the emulsion and/or of the digested system, the type and localisation of the stabilisers used, as well as, the interactions that take place between some of the ingredients of the emulsions and other components present in the digestive juices.

In the reformulation of the panna cottas, both the type of hydrocolloid used (cellulose derivatives or xanthan gum) to stabilise the emulsions and the amount of cream replaced by these emulsions modified the physical (structure and firmness) and sensory (mainly the taste and texture) properties of the panna cottas. However, the panna cottas elaborated with the emulsions stabilised with hydroxypropyl metilcelullose (HPMC) obtained a good level of sensory acceptability. Moreover, through the use of these emulsions it was possible to reduce the fat content of the panna cottas up to 37,5%, as well as their lipid digestibility up to 20%.

Introducción

1. Control de peso

El sobrepeso y la obesidad representan un problema de salud pública a nivel mundial que aumenta seriamente el riesgo de desarrollar trastornos graves, como la diabetes mellitus tipo II, enfermedades cardiovasculares y algunos tipos de cáncer (Munsters & Saris, 2014). Según la Organización Mundial de la Salud, en 2016 el 39% de adultos tenían sobrepeso, y el 13% eran obesos. Aunque los factores genéticos desempeñan un papel importante en la regulación del peso corporal, los principales factores que contribuyen al incremento de la obesidad son el aumento de la ingesta de alimentos de alto contenido calórico que son ricos en grasa junto con bajos niveles de actividad física (Munsters & Saris, 2014).

En este contexto, la investigación en tecnología de alimentos ha realizado numerosos esfuerzos para desarrollar alimentos con **menor contenido en grasa**, haciendo frente a la gran dificultad que supone mimetizar la estructura y las propiedades sensoriales aportadas por la grasa. En los últimos años, se está empleando también como estrategia, el aumento o la mejora de la **capacidad saciante** de los alimentos, con el objetivo de incidir sobre la regulación de la ingesta calórica (Norton, Espinosa, Watson, Spyropoulos, & Norton, 2015). Otra posible estrategia efectiva, que se está investigando recientemente, es el uso de emulsiones de **reducida digestibilidad lipídica** para el diseño y la reformulación de alimentos, con el objetivo de reducir o posponer la digestión de las grasas, manteniendo al mismo tiempo el atractivo sensorial y la palatabilidad de los alimentos (Norton, Fryer, & Norton, 2013).

No obstante, si bien existe una gran cantidad de estudios en los que se diseñan sistemas modelos capaces de influir en la capacidad saciante o en la digestibilidad lipídica, surge la cuestión de si estas matrices simples mantienen la misma función

cuando forman parte de una matriz más compleja, como son los alimentos. En consecuencia, los alimentos necesitan ser diseñados considerando, no solamente su contenido nutricional (cantidad y tipo de macronutriente), sino también las interacciones entre las diferentes macromoléculas y los demás ingredientes del alimento (Norton et al., 2015).

1.1. Concepto de saciedad

La saciedad es la sensación de plenitud que se produce después de comer y por la cual se suprime la necesidad de volver a comer por un periodo de tiempo determinado. En este sentido, aumentar la capacidad saciante de los alimentos puede ser un buen método para controlar, el deseo de comer en exceso, la ingesta diaria de alimentos y, por tanto, el peso corporal (Hoad et al., 2004).

El concepto de saciedad engloba realmente dos términos: en inglés "satiation" y "satiety". El primero, "**satiation**", se refiere a los procesos que se generan durante la comida y que conducen a su finalización, limitando, de este modo, la duración y la proporción de ésta; mientras que el segundo, "**satiety**", hace referencia a los sucesos postprandiales que evitan que el hambre retorne, afectando por tanto al intervalo entre comidas y a su frecuencia (Cummings & Overduin, 2007). Ambos términos, por tanto, forman parte del complejo sistema del control del apetito del cuerpo involucrado en la regulación de la ingesta de alimentos (Benelam, 2009; Tan, Wei, Zhao, Xu, Zhou, & Peng, 2016).

1.1.1. La cascada de la saciedad

La ingesta de alimentos y bebidas desencadena una serie de señales de diversa naturaleza (sensorial, cognitiva, hormonal y metabólica) que conducen a la

inhibición de la necesidad de comer (Blundell & Bellisle, 2013). Esta serie de complejas, sucesivas y superpuestas influencias se han englobado en un marco conceptual conocido como la "cascada de la saciedad" que fue desarrollada por primera vez por Blundell, Rogers y Hill (1987). Sin embargo, la investigación en las últimas décadas ha revelado la contribución y la interacción de muchos otros factores, por lo que, las modificaciones más recientes han incluido influencias pre y postprandiales (Blundell & Bellisle, 2013) (Figura 1).

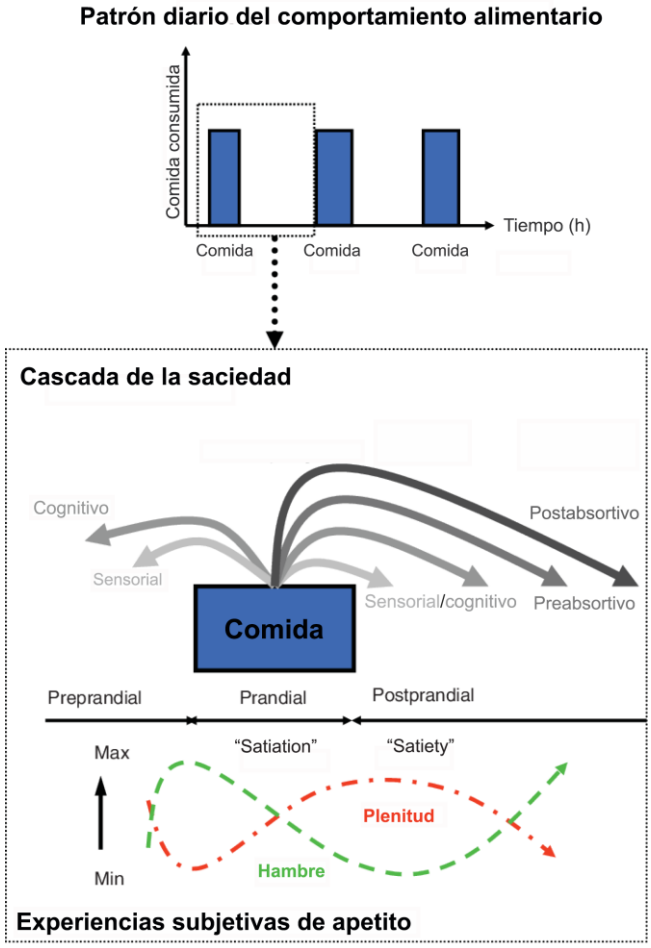

Figura 1. Representación de la cascada de la saciedad (Halford & Harrold, 2012)

Según se observa en la Figura 1, la saciedad, en términos generales ("satiation" y "satiety"), está controlada por una cascada de factores (conductuales, fisiológicos y psicológicos) que surgen, en primer lugar, antes de que se ingiera el alimento (fase preprandial), en segundo lugar, a medida que se consume y entra por el tracto gastrointestinal (fase prandial) y, en tercer lugar, cuando es digerido y absorbido (fase postprandial).

La **palatabilidad** y las **percepciones sensoriales** (apariencia, olor, sabor, textura) y **cognitivas** (expectativas sobre lo que va a ser consumido, cualquier asociación que surge de experiencias previas) del alimento antes y durante su consumo afectan a la cantidad de alimentos que ingerimos en una comida. En respuesta a esas percepciones, así como a la **distensión gástrica** que se produce cuando el alimento alcanza el estómago, tiene lugar la liberación de señales que están involucradas en la regulación de la ingesta energética, las cuales estimulan la "satiation". Una vez los nutrientes alcanzan el intestino y son absorbidos, un número de **señales hormonales** son nuevamente liberadas, las cuales influyen en el vaciado gástrico y tránsito y, promueven tanto la "satiation" como la "satiety". Finalmente, a largo plazo, la saciedad también puede verse afectada por la fluctuación de hormonas tales como la leptina e insulina, que transmiten información sobre el nivel de almacenamiento de nutrientes en el cuerpo (Benelam, 2009). Sin embargo, hay que tener en cuenta que otros factores tales como las condiciones sociales (presencia de otras personas) o ambientales (luz, temperatura), las características intrínsecas de los consumidores (peso corporal, rasgos afectivos o cognitivos, predisposición genética), la falta de sueño, la actividad física o el tamaño de las porciones, entre otros, también afectan de manera significativa al comportamiento a la hora de comer.

1.1.2. Ingredientes con capacidad saciante

Las **proteínas** y **fibras** son ampliamente reconocidas como los nutrientes con mayor potencial para el desarrollo de alimentos con capacidad saciante (Fiszman & Varela, 2013). Se ha demostrado que los alimentos enriquecidos con cantidades sustanciales de proteínas o tipos específicos de fibra, administrados como precargas* o suplementos, disminuyen el hambre y/o refuerzan los procesos de "satiation" en las comidas y "satiety" después de las comidas (Halford & Harrold, 2012; Norton et al., 2013).

Proteínas

La proteína se considera el macronutriente con mayor capacidad saciante, en comparación con cantidades equivalentes de energía provenientes de carbohidratos o grasas. Los mecanismos por los que las proteínas inducen la saciedad son el aumento en 1) la concentración de hormonas que regulan la saciedad, 2) el gasto energético (mediante la termogénesis), 3) la concentración de aminoácidos y, 4) el proceso de glucogénesis (Morell & Fiszman, 2017; Veldhorst et al., 2008).

Aunque existen evidencias de que la fuente de proteína empleada puede influir en los diferentes mecanismos por los que se induce la capacidad saciante, en general, no hay un claro consenso que establezca qué tipos de proteínas son más saciantes que otras. Sin embargo, en lo que a las **proteínas lácteas** se refiere, sí parece que, a corto plazo, las proteínas del suero tienen una mayor capacidad saciante que las caseínas (Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013).

* alimento, tentempié o incluso parte de una comida que ha sido diseñada para evaluar a corto plazo su efecto sobre la ingesta de alimentos o energética de la próxima comida (generalmente servida entre 30 a 90 minutos después).

Boirie et al. (1997) introdujo por primera vez el concepto de proteínas "rápidas" y "lentas" para describir las diferencias en la digestión y absorción de las proteínas del suero y de las caseínas respectivamente. Las **caseínas**, a diferencia de las proteínas del suero, precipitan en el medio ácido del estómago, lo que retrasa su vaciado gástrico e induce un lento aumento postprandial de aminoácidos en plasma. **Las proteínas del suero**, por el contrario, inducen un rápido, alto y transitorio aumento de aminoácidos en plasma (Bendtsen et al., 2013; Boirie et al., 1997), el cual está asociado a una mayor capacidad saciante (Hall, Millward, Long, & Morgan, 2003).

Fibras

El efecto que tiene el tipo de fibra sobre el apetito y la ingesta energética depende de sus propiedades físicas cuando es consumida (solubilidad, viscosidad o gelificación, y fermentación) y de sus efectos fisiológicos en el intestino (Norton et al., 2013). Concretamente, algunas **fibras solubles** tienen la capacidad de retener grandes cantidades de agua e hincharse causando un aumento de volumen y viscosidad, incluso a bajas concentraciones (Benelam, 2009), como ocurre en el caso del **glucomanano konjac**; mientras que otras tienen la capacidad de formar un gel más o menos fuerte en el estómago mediante la gelificación iónica o por las condiciones ácidas (pH < 3,5), como ocurre con el **alginato**. Por lo tanto, estos efectos contribuyen, en primer lugar, a que se produzca un aumento de la distensión gástrica y retraso del vaciado gástrico, que a su vez puede aumentar o prolongar la liberación de señales promotoras de la saciedad desde el estómago (Fiszman & Varela, 2013). En segundo lugar, a que se prolongue el tiempo de tránsito hacia el intestino delgado, la absorción de nutrientes y, por tanto, la generación de señales post-absortivas promotoras de la saciedad (Blundell & Bellisle, 2013; Norton et al.,

2013). Además, los ácidos grasos de cadena corta producidos por los microorganismos del intestino grueso durante la fermentación de las fibras podrían influenciar la respuesta de determinadas hormonas relacionadas con la saciedad (Fiszman & Varela, 2013).

1.2. Emulsiones de reducida digestibilidad lipídica

En la mayoría de los alimentos, los lípidos se encuentran formando parte de emulsiones, como por ejemplo en los productos lácteos, chocolates, salsas, mayonesas, sopas, untables, y aliños (Gallier & Singh, 2012). Para poder estabilizar las emulsiones alimentarias, se emplean agentes **emulsionantes**, conocidos como sustancias con actividad superficial que son capaces de adsorberse rápidamente en la interfase aceite/agua durante la homogeneización y de prevenir la futura agregación de las gotas de aceite; y/o agentes **estabilizantes**, los cuales aportan estabilidad a la emulsión mediante el aumento de la viscosidad de la fase continua (Li & Nie, 2016; Traynor, Burke, Frías, Gaston, & Barry-Ryan, 2013). En una emulsión alimentaria, las proteínas y las pequeñas moléculas tensioactivas tienen un papel principal como emulsionantes, mientras que los polisacáridos son empleados como estabilizantes. Sin embargo, existen algunos polisacáridos que actúan también como agentes emulsionantes, como son los derivados de la celulosa, hidroxipropilmetilcelulosa y metilcelulosa (Dickinson, 2009).

El interés por comprender los cambios estructurales que sufren los alimentos, y en especial las emulsiones, debido a los procesos moleculares, fisicoquímicos y fisiológicos que ocurren durante la ingestión, digestión y absorción de los lípidos es cada vez mayor. Este conocimiento permitiría diseñar y desarrollar alimentos, capaces de mejorar la salud y el bienestar (McClements, Decker, Park, & Weiss, 2008;

Singh & Sarkar, 2011), mediante la óptima regulación de la ingesta calórica, la mejora y el aumento de la respuesta saciante y el control de la bioaccesibilidad y/o biodisponibilidad de los lípidos (Golding & Wooster, 2010). No obstante, el desarrollo de dichos alimentos resulta ser una tarea compleja, ya que los procesos que ocurren a lo largo del tracto gastrointestinal dependen de la composición, de las propiedades y de la estructura inicial de los alimentos, así como de las características de los individuos (edad, sexo, genética, salud) y de otros factores (tiempo de consumo, temperatura).

Debido a que, generalmente, el cuerpo humano es suficientemente capaz de digerir los lípidos, el grado de digestión lipídica suele estar controlado por la habilidad de las lipasas en alcanzar la interfase aceite/agua y en adsorberse en ésta, que es donde tiene lugar la hidrólisis de los triglicéridos en mono y diglicéridos, así como en ácidos grasos. Esto, a su vez, está controlado por las **características de los glóbulos**, como son su tamaño (a mayor tamaño, menor área superficial para la adsorción de lipasas) y su composición (longitud de la cadena de los triglicéridos, grado de saturación, estado físico), por las **características de la interfase** (tipo de emulsionante, carga superficial, espesor, actividad superficial), por las **características de la fase acuosa** (a mayor viscosidad, menor movilidad de los compuestos intestinales como sales biliares y lipasa, y por tanto menor acceso a la interfase lipídica), y por la **solubilidad de los lípidos digeridos** (capacidad de incorporar los productos de la digestión en micelas mixtas y vesículas y la formación, solubilidad y transporte de éstas) (Golding & Wooster, 2010; Guo, Ye, Bellissimo, Singh, & Rousseau, 2017). Cabe destacar que las emulsiones o alimentos contienen una amplia variedad de otros componentes capaces de interferir con varios de los procesos mencionados anteriormente, lo que puede afectar al grado de digestibilidad lipídica (McClements et al., 2008; Norton et al., 2013). No obstante, la

obtención de emulsiones de reducida digestibilidad podría conseguirse mediante la manipulación de las características moleculares de los lípidos, del tamaño de las gotas de los lípidos, de las propiedades interfaciales y de la estructura y composición de la matriz de las emulsiones.

2. El aparato digestivo y su funcionamiento

La composición, estructura y las propiedades de los alimentos pueden variar considerablemente durante su paso a través del tracto digestivo humano (Figura 2) debido a cambios específicos en la composición de los jugos digestivos (pH, fuerza iónica, biopolímeros, sustancias tensioactivas), actividad enzimática (amilasas, proteasas, lipasas), y fuerzas o perfiles de flujo (ruptura, mezclado, transporte) asociados con la boca, estómago e intestino delgado (McClements et al., 2008).

2.1. Fase oral

Al ingerir un alimento, éste se mezcla con la saliva, se enfría o calienta a la temperatura corporal (37 °C), sufre un cambio moderado en el pH y fuerza iónica, y puede ser físicamente dividido en trozos más pequeños debido al efecto de cizalla entre la mucosa oral y los dientes (McClements et al., 2008; Singh, Ye, & Horne, 2009). Además, el alimento interactúa con varias enzimas salivares -principalmente α-amilasa-, biopolímeros como la mucina, y diferentes electrolitos (Singh & Sarkar, 2011). Después de permanecer de unos segundos a unos minutos en la boca, tiene lugar la deglución del bolo formado y pasa al estómago (Singh et al., 2009).

2.2. Fase gástrica

Una vez el bolo entra en el estómago, este puede sufrir grandes cambios al estar sometido a un pH altamente ácido (pH entre 1 y 3) y a agitación mecánica debido a los movimientos peristálticos del estómago. Además, el bolo se mezcla con los jugos gástricos que contienen electrolitos, varios compuestos tensioactivos y las enzimas pepsina y lipasa gástrica (McClements et al., 2008), las cuales empiezan la hidrólisis de los macronutrientes para que puedan ser absorbidos una vez alcancen el intestino delgado (Bornhorst & Singh, 2014).

Concretamente, en el estómago tiene lugar la mayor parte de la digestión de las proteínas, siendo la **pepsina** la principal enzima proteasa responsable de hidrolizar las proteínas en oligopéptidos más pequeños y aminoácidos. Sin embargo, la hidrólisis lipídica tiene lugar, generalmente, entre un 10 y 30% en el estómago (McClements et al., 2008) debido a que el pH del estómago no es el óptimo para la acción de la lipasa gástrica.

2.3. Fase intestinal

Al pasar el quimo o alimento parcialmente digerido del estómago al intestino delgado, éste se mezcla con los jugos intestinales a pH entre 5 y 7 que contienen bicarbonato, sales biliares, fosfolípidos, enzimas pancreáticas (tripsina, quimotripsina, carboxipeptidasa, lipasa) y electrolitos, segregados por el hígado, páncreas y vesícula biliar (McClements et al., 2008), lo que asegura que el quimo se descomponga en moléculas suficientemente pequeñas para ser posteriormente absorbidas (Bornhorst & Singh, 2014). Finalmente, las partículas del alimento que no han sido completamente digeridas pasan al intestino grueso donde serán fermentadas.

En el intestino delgado es donde tiene lugar la mayor parte de la digestión de los lípidos (70-90%). En resumen, la lipólisis es un proceso interfacial en el cual la lipasa pancreática debe adsorberse en la interfase aceite/agua preexistente en el alimento o formada tras el paso del alimento por el tracto gastrointestinal. La actividad de la lipasa pancreática depende de la presencia de sales biliares, colipasa y calcio. Las **sales biliares** son moléculas tensioactivas que se adsorben en la superficie de los glóbulos de grasa y que desplazan cualquier molécula existente, preparando de esta manera la interfase y facilitando el acceso a la lipasa. La **colipasa** debe unirse al área interfacial rica en sales biliares, y la **lipasa pancreática** es activada formando un complejo con la colipasa (Gallier & Singh, 2012). Los triglicéridos son consecuentemente hidrolizados en di-, monoglicéridos y ácidos grasos. El **calcio** reacciona con los ácidos grasos liberados, eliminándolos de este modo de la superficie de los glóbulos de grasa e impidiendo que éstos inhiban la acción de la lipasa. Finalmente, las sales biliares junto con los fosfolípidos forman **micelas mixtas y vesículas** en la fase acuosa, las cuales son capaces de incorporar los productos de la digestión lipídica y de eliminarlos de la superficie de los glóbulos de grasa, facilitando que se prolongue la digestión (Hur, Lim, Decker, & McClements, 2011), además de transportarlos hasta la mucosa intestinal donde serán absorbidos (McClements et al., 2008).

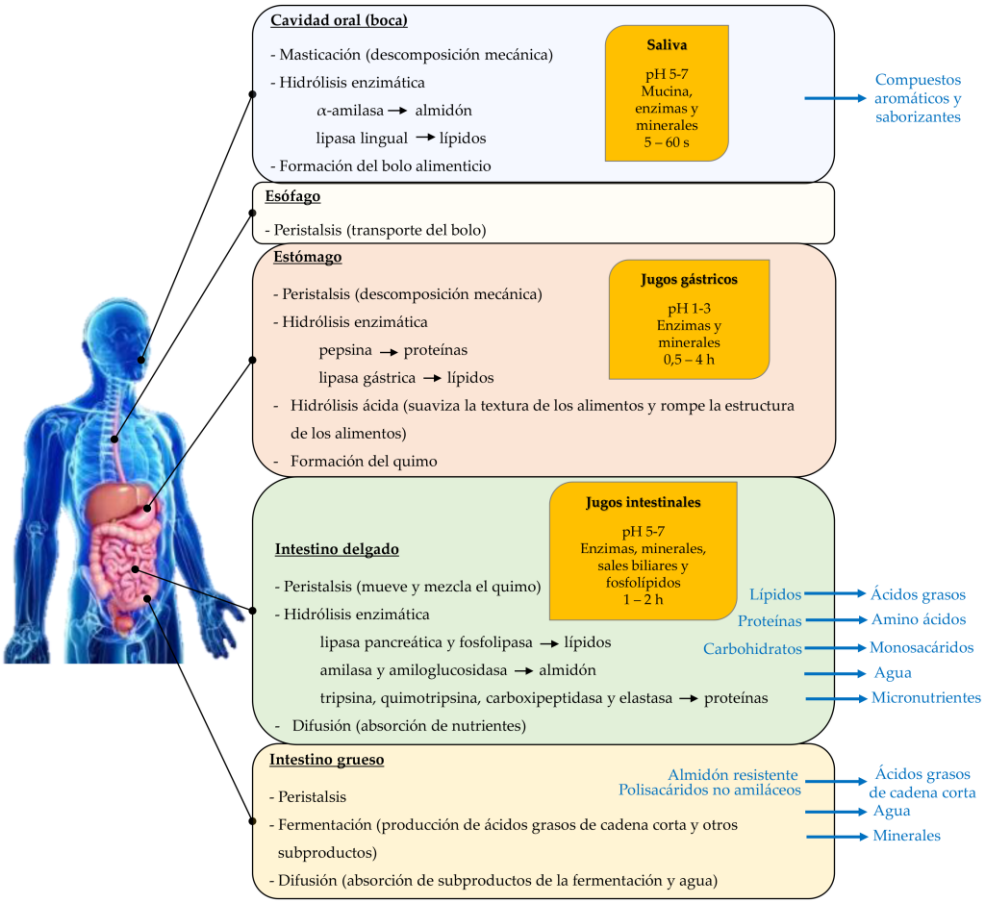

Figura 2. Principales procesos fisicoquímicos que ocurren en el tracto digestivo humano

3. Selección y características de un alimento modelo

La **panna cotta**, en italiano "nata cocida", es un postre lácteo típico de la región italiana del Piamonte, cuyo consumo hoy en día se ha extendido por otros países. Se compone básicamente de nata, azúcar y agentes gelificantes, siendo frecuentemente acompañado con algún tipo de mermelada, sirope, o saborizante.

Los crecientes problemas de salud y la necesidad de hedonismo de los consumidores pueden encontrar una solución conjunta en la producción de alimentos que sean saludables, pero a la vez **indulgentes**, como por ejemplo ocurre con los chocolates con bajo contenido en grasa (Norton et al., 2013) o sin azúcares. En este contexto, la selección de la panna cotta como base para la reformulación de alimentos lácteos para el control de peso se fundamenta esencialmente en su alto contenido en grasa y proteínas, y a su vez, en su carácter **hedónico** o indulgente. Su composición permite modificar la proporción de proteínas y grasas, así como añadir nuevos ingredientes o realizar diversos tipos de sustituciones, con el fin de obtener panna cottas que puedan proporcionar alta capacidad saciante y/o menor contenido en grasa al organismo. En cualquier caso, se busca que las modificaciones realizadas afecten en la menor medida posible a su palatabilidad y aceptación sensorial.

A pesar de que es conocido el vínculo que existe entre el consumo de grasas y la salud, los productos reducidos en grasa no siempre forman parte de la dieta de los consumidores. Generalmente, esto se debe a que los consumidores sienten que las dietas bajas en grasa son difíciles de mantener; en muchas ocasiones, carecen de atractivo sensorial o tienen una calidad sensorial inferior (Norton et al., 2013). Sin embargo, se han producido cambios en algunos sectores del mercado, en particular en el de los productos lácteos (por ejemplo, ha crecido el consumo de leche semidesnatada y desnatada en detrimento de la leche entera, hay un elevado consumo de margarina como sustituto de la mantequilla, y de productos para untar bajos en grasa), que sí han sido perfectamente asimilados por los consumidores. Además, en la actualidad, los consumidores pueden encontrar una gran variedad de productos con un contenido reducido en grasa, tales como los que contienen las declaraciones nutricionales “light”, “bajo en grasa”, “0%”, entre otros.

Desde hace relativamente pocos años, también existen en el mercado productos lácteos, como por ejemplo leche, yogures, helados y quesos, con mayor contenido en proteínas que sus homólogos tradicionales. Estos productos presentan una serie de declaraciones nutricionales como "rico en proteínas", "50% más de proteína", "alto en proteínas", "más proteínas" y su consumo está enfocado tanto a deportistas que quieran mejorar su tono muscular, como a cualquier consumidor que quiera incidir sobre su saciedad a la hora de comer. Además, también existen productos a base de glucomanano konjac, como por ejemplo algunos sobres o cápsulas para tomar unas horas antes de las comidas, y algunas infusiones. En ese tipo de productos, las declaraciones nutricionales empleadas son: "saciante", "ayuda natural al control de peso", "ayuda a adelgazar".

En cuanto a la reducción de la digestibilidad lipídica, hoy en día, solamente se encuentran productos farmacéuticos. Hacen falta más estudios, tanto desde el punto de vista de la tecnología de los alimentos como clínico, y aprobar nuevas declaraciones nutricionales para que algunos productos de reducida digestibilidad lipídica puedan salir al mercado.

4. Bibliografía

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Objetivos

OBJETIVO GENERAL

El objetivo general de la presente tesis doctoral es formular y desarrollar alimentos lácteos capaces de incidir sobre el control del peso. Para ello, se plantean dos estrategias diferenciadas. En primer lugar, que estos alimentos aporten alta capacidad saciante, mediante la adición de hidrocoloides y/o proteínas. En segundo lugar, que aporten al organismo un menor contenido en grasa mediante la sustitución de ésta por emulsiones de menor contenido en grasa y/o de reducida digestibilidad lipídica.

OBJETIVOS PARCIALES

- Analizar el efecto de la adición, eliminación o sustitución de ingredientes sobre las propiedades fisicoquímicas y estructurales de una matriz simple (sistemas proteicos lácteos modelo) y otra compleja (postre lácteo tipo panna cotta).

- Estudiar la relación entre los cambios fisicoquímicos y estructurales que se producen durante la digestión gástrica *in vitro* y la capacidad saciante de los productos lácteos (matriz simple y compleja).

- Analizar el efecto que tiene la presencia de diferentes emulsionantes o estabilizantes en la formulación de emulsiones sobre las propiedades reológicas, texturales, microestructurales, tamaño de partícula y digestibilidad lipídica durante la digestión gastrointestinal *in vitro*.

- Emplear diferentes emulsiones como sustitutos de grasa en la formulación de un postre lácteo (panna cotta) y estudiar cómo influye la presencia de cada emulsión y sus interacciones con los otros componentes del alimento en las propiedades

fisicoquímicas y estructurales del postre lácteo antes y durante la digestión intestinal *in vitro*, y su relación con la lipólisis.

- Determinar y evaluar los atributos sensoriales característicos ("Check All That Apply", CATA) de los nuevos postres lácteos y evaluar su aceptación global utilizando un panel de consumidores.

Estructura de la tesis

El trabajo de investigación realizado ha dado lugar a seis publicaciones científicas, las cuales se han estructurado en dos grandes capítulos.

El primer capítulo se enmarca dentro del proyecto del Ministerio de Economía y Competitividad titulado "Formulación de alimentos con hidrocoloides de efecto saciante. Reología, estructura y percepción sensorial y del consumidor. Estudio de trayectoria oral y digestión *in vitro*" (AGL2012-36753-C02-01), y engloba los dos primeros trabajos que hacen referencia al estudio de la adición de hidrocoloides y/o proteínas lácteas para modular la capacidad saciante de productos lácteos.

En el primer trabajo se estudió el efecto que tiene la adición de hidrocoloides (glucomanano konjac o alginato) sobre las propiedades micro y macroestructurales, de viscosidad, de tamaño de partícula, y electroforéticas de sistemas modelo lácteos antes y durante la digestión gástrica *in vitro*, y su relación con la capacidad saciante.

En el segundo trabajo se decidió estudiar cómo afecta la adición de una cantidad extra de diferentes proteínas lácteas (leche, proteínas del suero o caseinato) y, a su vez, la reducción del contenido en grasa sobre las propiedades texturales iniciales, así como sobre los cambios microestructurales y electroforéticos producidos durante la digestión oral y gástrica *in vitro* en una matriz compleja y sometida a tratamiento térmico, como es el postre lácteo panna cotta. Los resultados obtenidos se relacionaron con la capacidad saciante.

El segundo capítulo se enmarca dentro del proyecto del Ministerio de Economía, Industria y Competitividad titulado "Funcionalidad de los hidrocoloides en la reducción de la digestibilidad lipídica *in vitro* de emulsiones alimentarias: reología, estructura y percepción sensorial" (AGL2015-68923-C2-2-R), el cual engloba los cuatro siguientes trabajos, y se centra en el desarrollo y estudio de

emulsiones basadas en hidrocoloides, que sean aptas como sustitutos de grasa y resistentes a la digestión lipídica, para formular alimentos que aporten menor grasa al organismo y que posean óptima calidad fisicoquímica y sensorial.

En el tercer trabajo se estudió la estabilidad física y oxidativa, textura, microestructura, y tamaño de partícula antes y durante la digestión gastrointestinal *in vitro* de emulsiones estabilizadas con diferentes hidrocoloides derivados de celulosa, estableciéndose una relación con la acción de la lipasa mediante la medida de ácidos grasos liberados durante dicha digestión.

En el cuarto trabajo se decidió ahondar más en el estudio de cómo influye el uso de diferentes emulsionantes o estabilizantes (polisorbato, lecitina, goma xantana, metilcelulosa) sobre las propiedades reológicas, microestructurales y de tamaño de partícula de las diferentes emulsiones, relacionando los cambios producidos durante la digestión intestinal *in vitro* con la digestibilidad lipídica de éstas (liberación de ácidos grasos).

En el penúltimo trabajo se estudió de qué manera afecta la sustitución, total o parcial de la grasa, por emulsiones de menor contenido en grasa formuladas con hidrocoloides, metilcelulosa o hidroxipropilmetilcelulosa, en las propiedades texturales, microestructurales y sensoriales de la panna cotta.

Finalmente, en el último trabajo se estudió cómo influye la sustitución de la grasa por emulsiones de reducida digestibilidad lipídica sobre la textura, micro y macroestructura de las panna cottas, así como sobre la digestibilidad lipídica de éstas. Además, se realizó un estudio sensorial para conocer la aceptación de las nuevas panna cottas por parte de los consumidores.

Las referencias de las publicaciones científicas derivadas de esta tesis se presentan a lo largo de los capítulos en el siguiente orden:

Capítulo 1: Diseño de productos lácteos con capacidad saciante

Adding neutral or anionic hydrocolloids to dairy proteins under *in vitro* gastric digestion conditions. Borreani, J., Llorca, E., Larrea, V., and Hernando, I. (2016). *Food Hydrocolloids*, 57, 169-177 (DOI: 10.1016/j.foodhyd.2016.01.030).

Designing dairy desserts for weight management: structure, physical properties and *in vitro* gastric digestion. Borreani, J., Llorca, E., Quiles, A., and Hernando, I. (2017). *Food Chemistry*, 220, 137-144 (DOI: 10.1016/j.foodchem.2016.09.202).

Capítulo 2: Uso de emulsiones para reducir el aporte en grasa al organismo

Oil-in-water emulsions stabilised by cellulose ethers: stability, structure and *in vitro* digestion. Borreani, J., Espert, M., Salvador, A., Sanz, T., Quiles, A., and Hernando, I. (2017). *Food & Function*, 8, 1547-1557 (DOI: 10.1039/c7fo00159b).

How do different types of emulsifiers/stabilisers affect the *in vitro* intestinal digestion of o/w emulsions? Borreani, J., Leonardi, C., Moraga, G., Quiles, A., and Hernando, I. *Food Biophysics*. En revisión.

New hydrocolloid-based emulsions for replacing fat in panna cottas: a structural and sensory study. Borreani, J., Hernando, I., Salvador, A., and Quiles, A. (2017). *Journal of the Science of Food and Agriculture*, 97(14), 4961-4968 (DOI: 10.1002/jsfa.8373).

Cream replacement by hydrocolloid-stabilised emulsions to reduce fat digestion in panna cottas. Borreani, J., Hernando, I., and Quiles, A. *Journal of Food Science*. En revisión.

Resultados y discusión

Capítulo 1

Diseño de productos lácteos con capacidad saciante

Adding neutral or anionic hydrocolloids to dairy proteins under *in vitro* gastric digestion conditions

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Food Hydrocolloids (2016), 57, 169-177

Abstract

The effect of adding uncharged polysaccharides such as konjac glucomannan (KGM) or negatively charged polysaccharides such as alginate to dairy protein ingredients – milk, whey proteins and calcium caseinate – was investigated through simulated *in vitro* gastric digestion. The apparent viscosity, microstructure (light microscopy), particle size distribution and degradation (SDS-PAGE) of the proteins were monitored after different *in vitro* gastric digestion times (0, 30, 60 and 120 min). The addition of KGM increased the viscosity values of the samples during gastric digestion, which probably would increase gastric distention affecting satiety. The microstructure and particle size distribution results showed that the aggregates formed in the dairy protein-konjac glucomannan mixtures at the start of gastric digestion were broken down into smaller ones over time. However, the aggregates formed with the addition of alginate were larger and remained almost unchanged throughout gastric digestion, due to the strong interaction between the opposite charges of the protein and alginate. The SDS-PAGE results showed that whey proteins were more resistant to pepsin digestion than caseins and that the alginate slowed down protein degradation. These findings suggest that a combination of whey proteins and alginate could be used to delay gastric emptying and promote satiety.

Keywords: dairy proteins, konjac glucomannan, alginate, *in vitro* gastric digestion, SDS-PAGE, microstructure

1. Introduction

Over recent decades, the problems of overweight and obesity have increased and, therefore, the interest in formulating satiating foods has grown. The concept of appetite control comprises two components: satiation (the processes that induce meal termination) and satiety (which determines the intervals between meals) (Geraedts, Troost, & Saris, 2011; Solah et al., 2010) . Ingested food evokes satiety in the gastrointestinal (GI) tract by mechanical and humoral stimulation. Post-gastric factors seem to play a key role in satiety through secretion of various peptides by the walls of the small and large intestine in response to ingested food (Geraedts et al., 2011). Satiety signals differ as the meal moves through the gut but include oral (taste and texture), gastric (distension and emptying), and intestinal (distension and nutrient absorption) factors (Hoad et al., 2004). Fibres (carbohydrates resistant to digestion) and various proteins have commonly been used as ingredients in foods and beverages to enhance satiety (Halford & Harrold, 2012).

Proteins suppress food intake, make a stronger contribution to satiety and delay the return of hunger more than fats and carbohydrates (Anderson & Moore, 2004; Geraedts et al., 2011; Solah et al., 2010). The mechanisms by which the peptide products of protein digestion exert their effect on food intake via the gut include slowing stomach emptying and direct or indirect stimulation of gut hormone receptors (Anderson & Moore, 2004). As dairy products contain high levels of protein, they are good for designing satiating food products. Casein is the most abundant milk protein, accounting for 80% of total protein, with whey proteins constituting the remaining 20% (Chen, Chen, & Hsieh, 2016). Hall, Millward, Long, and Morgan (2003) and Veldhorst et al. (2009) found that whey proved more satiating than casein. The digestion and absorption of whey and casein differ in that

casein, unlike whey, coagulates in the stomach due to its precipitation by gastric acid. Furthermore, casein is considered a "slow" protein, whereas whey protein is a relatively "fast" protein (Boirie et al., 1997; Veldhorst et al., 2009), so whey consumption leads to higher plasma concentrations of factors known to contribute to satiety, such as amino acids, glucose-dependent insulinotropic polypeptide, glucagon-like peptide-1 and cholecystokinin (Anderson & Moore, 2004; Hall et al., 2003).

A number of studies shows that fibre-rich foods can increase the feeling of satiety and decrease short-term food intake. Certain fibre types bind water and swell, causing increased viscosity, which is associated with delayed gastric emptying and increased satiety (Halford & Harrold, 2012; Hoad et al., 2004; Peters et al., 2011).

Konjac glucomannan (*Amorphophallus konjac* K. Koch) and alginate are often used to formulate satiating food. Their mechanisms to induce satiation are different due to differences in their charge and structure: konjac glucomannan (KGM) is a neutral polysaccharide while alginate is a negatively-charged polysaccharide.

KGM is a soluble (Fang & Wu, 2004), fermentable and highly viscous dietary fibre (Keithley & Swanson, 2005), due to its high water-absorption capacity (Chua, Baldwin, Hocking, & Chan, 2010). It has a mechanical function in slowing food intake and reducing appetite (Chen, Cheng, Liu, Liu, & Wu, 2006). KGM promotes satiety through the induction of cephalic and gastric-phase signals, delayed gastric emptying and slowed bowel transit time due to the increased viscosity of the gastrointestinal content, and a reduced rate of food absorption in the small intestine leading to attenuated postprandial glucose and insulin surges (Chua et al., 2010).

Alginate gel formation can be triggered by low pH or the presence of divalent cations such as Ca^{2+}. Once the alginate comes into contact with acids in the stomach it can become a gel, leading to prolonged gastric emptying and a considerably slower rate of intestinal absorption (Brownlee et al., 2005). Torsdottir, Alpsten, Holm, Sandberg, and Tolli (1991) found that a small dose of alginate induced delayed gastric emptying. Peters et al. (2011) showed that a specific alginate that gelled strongly in the presence of Ca^{2+} increased satiety more than an alginate that formed a weak gel. Hoad et al. (2004) suggested that a sense of fullness can be obtained by using a palatable, relatively low-viscosity meal (low-G alginate) which forms solids in the stomach, due to distension of the gastric antrum and/or transport of nutrients to the small intestine in the lumps.

Because alginate, KGM and milk proteins are negatively, neutrally and positively charged, respectively, it may be expected that the interaction between the milk proteins and the two hydrocolloids will be different, and therefore, that the protein digestion will also differ. Many interactions can occur between proteins and polysaccharides, depending on the pH and ionic strength of the environment, the ionisation and charge density and the structure and concentration of the different biopolymers. Protein-polysaccharide complexes form due to strong interactions such as covalent bonding, or to several weak interactions (electrostatic, van der Waals', hydrogen or hydrophobic bonding) (Dickinson, 1998; Mouécoucou, Villaume, Sanchez, & Méjean, 2004).

Several authors (El Kossori et al., 2000; Mouécoucou et al., 2004; Polovic et al., 2007; Shah, Atallah, Mahoney, & Pellett, 1982) have reported that the protein digestion rate depends on the chemical composition of the fibres and their physical properties, such as viscosity and protein-polysaccharide complex formation, which

provide a physical obstacle at the protein to pepsin enzymatic cleavage site and reduce its activity (Larsen, Wilson, & Moughan, 1994). Therefore, both KGM, due to its viscosity when mixed with water, and alginate, due to its electrostatic interactions with proteins, may be expected to decrease pepsin activity. The rate of proteolysis will be higher or lower depending on the accessibility of peptide bonds to pepsin, so more or fewer peptides will be broken down into smaller ones or even into amino acids, influencing satiety.

The present study aimed to evaluate the effect of adding neutral or charged hydrocolloids on the *in vitro* gastric digestibility of different milk proteins when formulating satiating dairy products. The digested samples were characterised by apparent viscosity, light microscopy (LM), particle size distribution and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. Materials and methods

2.1. Ingredients

The ingredients used to prepare the samples were skimmed milk powder (Central Lechera Asturiana, Siero, Spain), whey protein concentrate (AVONLAC 482, Glanbia Nutritionals Ltd., Kilkenny, Ireland), calcium caseinate (Fonterra Co-operative Group Ltd, Reference 385, Palmerston North, New Zealand), konjac glucomannan (GLUCOMANNAN 86 TDF, 120 MESH M202, Trades S.A., Barcelona, Spain), sodium alginate (MANUCOL DMF, FMC Biopolymer, Philadelphia, USA) and distilled water.

2.2. Sample preparation

Three protein solutions were prepared by dissolving skimmed milk powder, whey powder or casein powder, respectively, in distilled water. While slowly adding the powder, the water was stirred and heated (50 °C) for 1 h. Two polysaccharide solutions, konjac glucomannan and alginate, were prepared in the same way. Each protein solution was mixed with each polysaccharide solution to obtain six different samples: MK (10% w/w of skimmed milk powder + 0.5% w/w of konjac glucomannan), MA (10% w/w of skimmed milk powder + 0.55% w/w of alginate), WK (10% w/w of whey protein concentrate + 0.5% w/w of konjac glucomannan), WA (10% w/w of whey protein concentrate + 0.55% w/w of alginate), CK (10% w/w of calcium caseinate + 0.5% w/w of konjac glucomannan) and CA (10% w/w of calcium caseinate + 0.55% w/w of alginate). The percentage of KGM was calculated on the basis of the manufacturer's recommendations and the percentage of alginate was selected through a preliminary study to obtain a similar apparent viscosity to that of KGM systems at low shear rates at 37 °C. Three control samples (M, W and C, all without any polysaccharide) were also analysed.

2.3. *In vitro* gastric digestion

The simulation of gastric digestion was performed in a jacketed glass reactor (1 L capacity) maintained at 37 °C in a temperature-controlled circulating water bath with continuous magnetic stirring throughout the test.

The simulated gastric fluid (SGF) consisted of 0.034 M NaCl, with the pH adjusted to 1.2 using HCl 10 N. The SGF (200 mL) was pre-incubated for 5 min with continuous stirring (Zhang & Vardhanabhuti, 2014b) at 300 rpm.

Each sample (200 g) was mixed with simulated gastric fluid. The pH value was reduced to 1.9 (Abdel-Aal, 2008) with HCl 10 N. Pepsin (P7125, pepsin from porcine gastric mucosa, ≥ 400 units/mg protein, Sigma-Aldrich) was added at a pepsin to protein ratio of 1:250 on a weight basis, in accordance with Zhang and Vardhanabhuti (2014b). The mix was maintained at 37 °C with continuous stirring (650 rpm) for a recommended time of 120 min, which corresponds to a half-gastric emptying (Minekus et al., 2014). Aliquots (28 mL) were withdrawn into a glass beaker containing 22 mL NaOH (0.1 N) to inactivate the enzyme after 0, 30, 60 and 120 min of incubation. The samples were centrifuged at 5000 g for 15 min at 4 °C. The hydrolysed protein content in the supernatant was measured at 280 nm using a UV-visible spectrophotometer (Cecil Instruments Limited, Cambridge, UK).

2.4. Viscosity measurement

The apparent viscosity was determined using a viscometer (Haake ViscoTester VT6R Plus, Thermo Scientific, Walthman, Mass., USA) equipped with spindle 1, at 10-200 rpm, at 37 ºC. Measurements were performed in duplicate.

2.5. Light microscopy (LM)

A Nikon ECLIPSE 80i (Nikon Co., Ltd., Tokyo, Japan) light microscope (LM) was used. A 20 μL aliquot of each formulation was placed on a glass slide and observed at 10x magnification (objective lens 10x/0.45 DIC N1 ∞/0.17 WD 4.0, Nikon, Tokyo, Japan). A camera (ExWaveHAD, model no. DXC-190, Sony Electronics Inc, Park Ridge, New Jersey, USA) was attached to the microscope and connected to the video entry port of a computer. The images were captured and stored at 1280 x 1024

pixels using the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan). The software interfaced directly with the microscope, enabling image recording control. Toluidine blue (1%) was used to stain the proteins.

2.6. Particle size measurements

The particle size distributions of the samples were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments Limited, Malvern, UK) with two laser sources. The instrument was set up as follows: refractive index of proteins: 1.52, refractive index of the dispersion medium (water): 1.33, assumed absorbance of proteins: 0.1. At least two replications were conducted for each sample.

2.7. Extraction of proteins

The preparation of the samples for electrophoretic study was carried out according to the method of Laemmli (1970) with modifications. Each sample was mixed with Laemmli buffer (4.8% (w/v) sodium dodecyl sulphate, 0.1 M dithiothreitol, 0.001 M ethylenediaminetetraacetic acid, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 0.125 M Tris-HCl; pH 6.8) and heated at 100 °C for 5 min, adjusting the protein concentration of the samples to 2 mg mL^{-1}.

2.8. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) on a Multiphor II Electrophoresis

System (Pharmacia Biotech, Piscataway, USA), using 15% polyacrylamide gels (ExcelGel SDS Homogeneous) at 600 V, 18 mA, 18 W and 15 °C. Of each sample, 8 μL were loaded into the gel.

The protein bands were stained with Coomassie Brilliant Blue tablets (Phastgel Blue R., Pharmacia). De-staining was performed in an aqueous solution of 25% ethanol and 8% acetic acid. The samples were preserved in a solution of 10% glycerol and 7.2% acetic acid.

The high molecular weight calibration kit used as the standard (Amersham GE Healthcare, UK) consisted of phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Gels were scanned with an ImageScanner III LabScan 6.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and analysed with the ImageQuant TL Image Analysis Software v7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

3. Results and discussion

3.1. Viscosity measurement

The apparent viscosities of the samples were measured during *in vitro* gastric digestion for 0, 30, 60 and 120 min. In sample M, prepared only with milk, the viscosity values practically remained unchanged during gastric digestion (they varied from 20.5 mPa s at 0 min to 18 mPa s at 120 min). The addition of KGM (sample MK) led to higher viscosity values (101.5 mPa s at 0 min), which decreased along digestion to 80 mPa s at 120 min. In contrast, the addition of alginate barely

affected the viscosity at gastric level, being 21.0 mPa s at 0 min and 19.0 mPa s at 120 min.

Samples formulated with whey proteins (W, WK and WA) exhibited slightly lower viscosity values than the correspondent milk samples (M, MK and MA). The values of viscosity kept the same tendency than in milk samples: the addition of KGM in sample WK led to higher viscosity values, which decreased along digestion from 88 mPa s at 0 min to 55 mPa s at 120 min, and the addition of alginate did not affect the viscosity.

In the case of samples C and CA, the viscosity values were even lower than the correspondent samples elaborated with milk -M and MA- and with whey protein -W and WA-. In sample CK, the addition of KGM also led to high viscosity values (710 mPa s at 0 min), which decreased at min 30 (516 mPa s) and remained practically unchanged along *in vitro* digestion.

Regardless the protein used, the samples prepared with KGM had the highest apparent viscosity values and the samples prepared with alginate or without polysaccharides presented similar viscosity values. Several authors reported that some soluble fibres bind water and swell, causing increased viscosity and thereby increasing gastric distention, which is associated with delayed gastric emptying and increased satiety (Halford & Harrold, 2012; Logan, Wright, & Goff, 2015; Schroeder, Gallaher, Arndt, & Marquart, 2009). KGM has been found to have volumetric effects, creating a sense of fullness while slowing down physiological processes associated with food digestion (Marcano, Hernando, & Fiszman, 2015). Therefore, the addition of KGM to the milk proteins studied in our work would delay gastric emptying giving place to an increased satiety.

3.2. Light microscopy (LM)

The microstructure of the nine samples was monitored by LM at different moments of the simulated *in vitro* digestion (Figs. 1-3). The LM results were correlated with the macroscopic appearance of the samples (Fig. 4).

Figure 1 shows the microstructure of the samples prepared with milk (samples M, MK and MA). The protein (sample M) or protein-polysaccharide polymers (samples MK and MA) are stained purple. In sample M, the aggregates formed at 0 min became slightly larger at 30 min and then showed a gradual degradation of the protein network as the digestion time progressed. Nevertheless, no visual changes were observed at a macroscopic level (Fig. 4). Similar results were obtained during *in vitro* gastric digestion of dairy proteins by Nguyen, Bhandari, Cichero, and Prakash (2015). They observed that at the start of the gastric digestion, the dairy protein (caseins and whey proteins) was clustered into large aggregates, but after 1 h of proteolysis in the stomach these large aggregates of milk protein had become smaller compared to those at 0 min. In sample MK, the aggregates formed at 0 min broke down at a constant rate up until 120 min. Swollen konjac glucomannan particles were observed within the protein aggregates at all the digestion times, showing that no important interactions took place between the protein and the polysaccharide. This was reflected by lower turbidity and a separation of phases (Fig. 4). In contrast, sample MA showed aggregation of the network from 0 to 30 min and no structural changes were observed from 30 min onwards. This can be seen in Fig. 4 as the emergence at 30 min of gelled particles which remained unchanged up to 120 min, and is in accordance with a study by Hemar, Tamehana, Munro, and Singh (2001), who observed that the addition of

xanthan gum led to protein aggregation and subsequent phase separation in skimmed milk powder using confocal scanning laser microscopy.

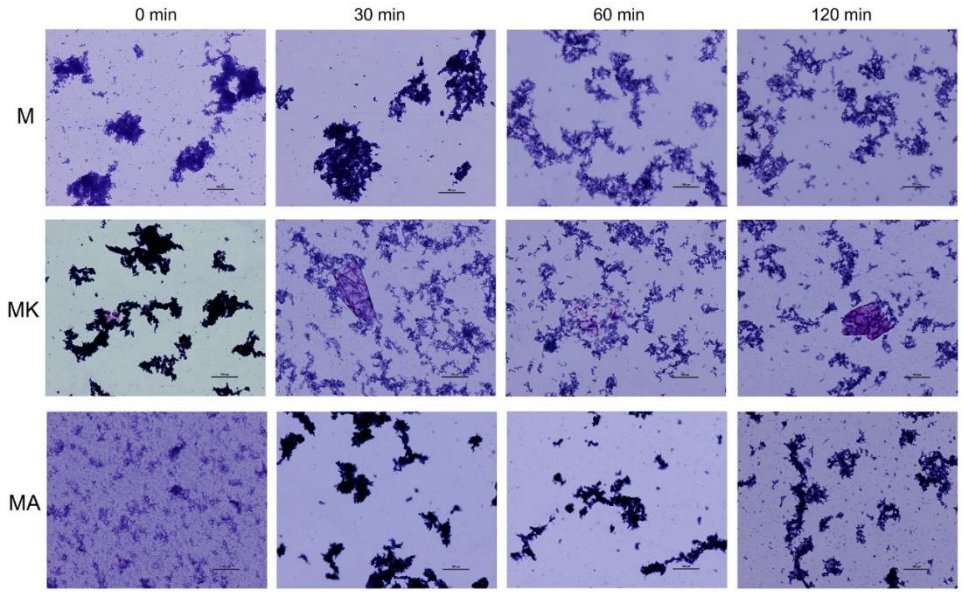

Fig. 1. Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of milk protein microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. M: milk; MK: milk with konjac glucomannan; MA: milk with alginate.

The samples prepared with whey proteins (samples W, WK and WA) are shown in Fig. 2. The protein network was observed to be finer in the samples with whey proteins than in the milk samples. Samples W and WK showed a similar trend in the degradation of the protein network: slight degradation from 0 to 30 min and no visible changes afterwards.

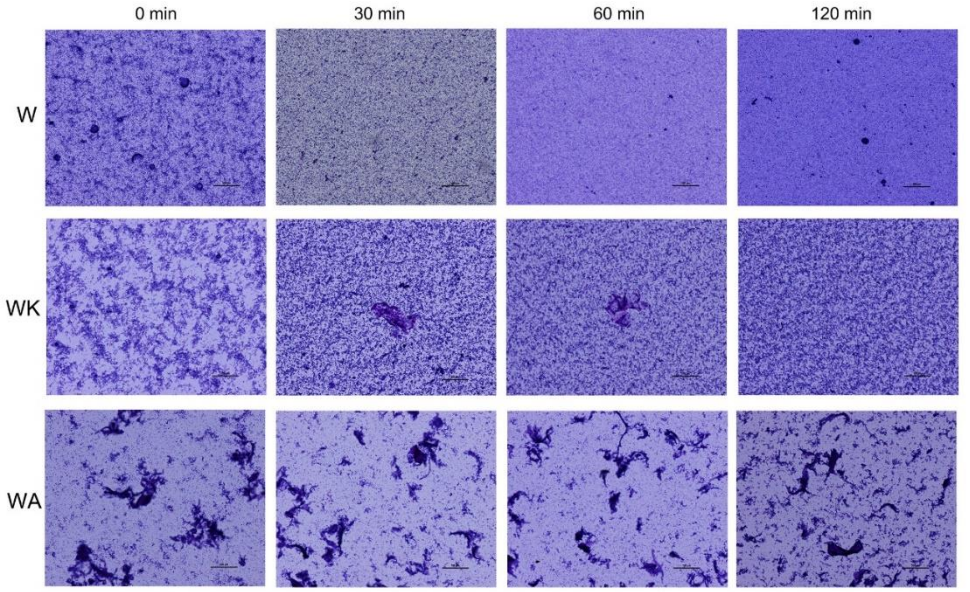

Fig. 2. Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of whey protein microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate.

As in sample MK, isolated particles of Konjac were detected in sample WK. The microstructure of samples W and WK corresponded to their macroscopic appearance, where a very slight decrease in turbidity was appreciated (Fig. 4). However, sample WA presented large aggregates due to the strong bond formed between the alginate and the whey proteins, which remained almost unchanged as the digestion time progressed. In accordance with the LM results, no visual changes were observed in sample WA at macroscopic level (Fig. 4). This was also observed by Hemar et al. (2001) in whey protein isolate-xanthan mixtures: the protein aggregates were larger when xanthan was added to whey protein isolate (WPI) than in WPI without xanthan, and they did not observe phase separation in WPI-xanthan mixtures. Agreeing with these results, Zhang, Zhang, and Vardhanabhuti (2014)

observed that the protein aggregates in WPI-carrageenan gels formed large clusters, which was likely to be because of the strong attraction between protein aggregates and carrageenan.

Figure 3 presents the microstructure of samples prepared with calcium caseinate (samples C, CK and CA). Unlike samples M and W, which tended to degrade during digestion, it was observed that sample C tended to aggregate. This was also noticeable in the macroscopic appearance of sample C, where a slight increase in turbidity was observed (Fig. 4).

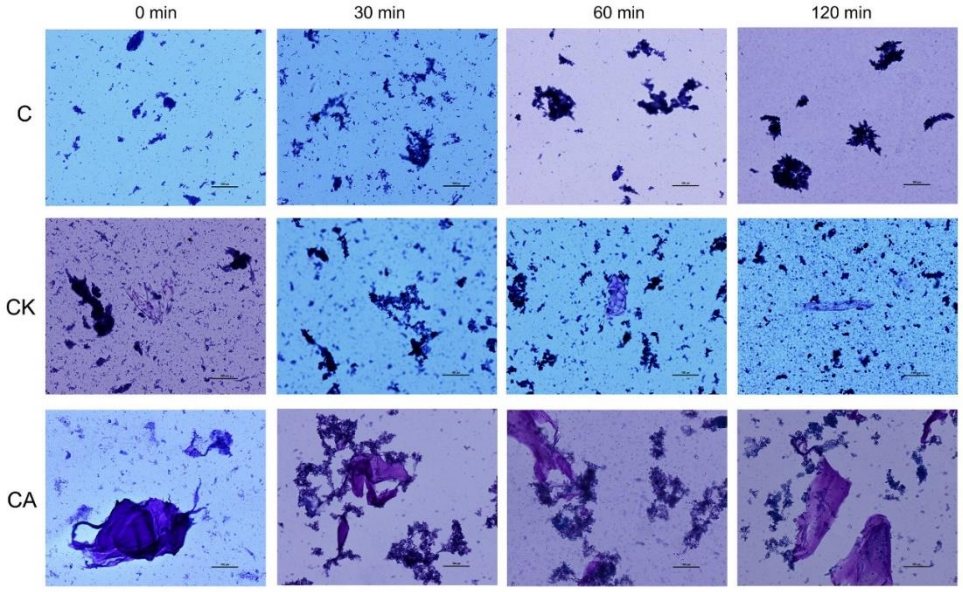

Fig. 3. Light microscopy. Blue toluidine staining. Magnification 10x. Bar 100 μm. Evolution of calcium caseinate microstructure without and with hydrocolloids during *in vitro* gastric digestion at 0, 30, 60 and 120 min. C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

In contrast, in sample CK, the aggregates formed at 0 min showed gradual degradation up to 120 min. The presence of konjac particles seems to limit polymer interactions, decreasing protein coagulation. Hence, the microstructure of CK showed a similar trend to sample MK and also displayed a similar appearance at macroscopic level (Fig. 4). The phase separation in the MK and CK mixtures is likely to be due to flocculation of casein micelles by depletion mechanisms, as observed by Syrbe, Bauer, and Klostermeyer (1998). Sample CA, like samples MA and WA, remained practically unchanged. Large pieces of alginate linked to caseinate could be observed, which are seen in Fig. 4 as gelled particles. Similarly, Hemar et al. (2001) observed "thread-like" dark objects composed of individual strands in confocal micrographs of sodium caseinate-xanthan mixtures.

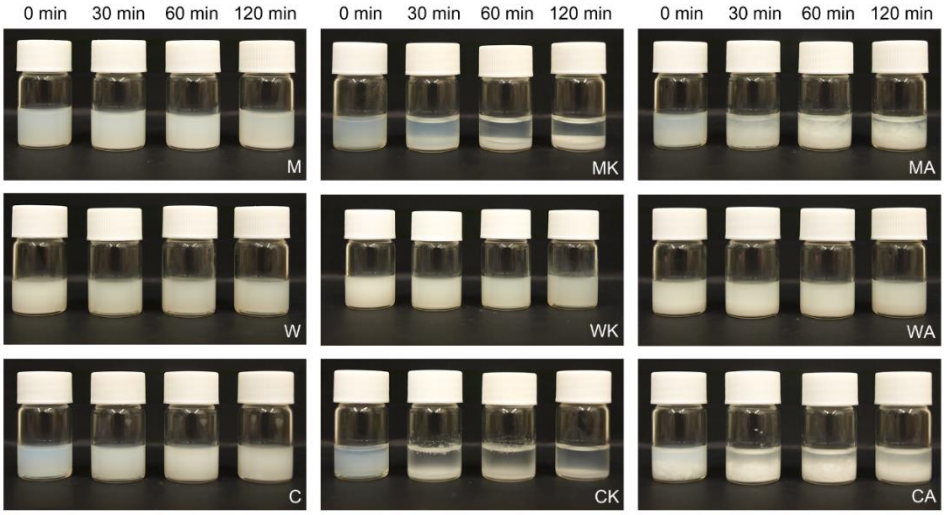

Fig. 4. Macroscopic appearance after 24 h of samples digested for 0, 30, 60 and 120 min. M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

3.3. Particle size distribution

The particle size measurement was used to quantify the degradation rate of the aggregates formed during digestion (Fig. 5).

When the samples prepared with milk (samples M, MK and MA) were analysed, sample M showed aggregates with a particle size between ~35 and 500 μm with a peak at ~170 μm at 0 min, and between ~50 and 260 μm with a peak at ~130 μm at 30 min. From 30 to 60 min, the particle size decreased considerably, to ~30 – 40 μm, and from 60 to 120 min it decreased slightly, to ~25 – 30 μm. This decreasing particle size was also observed by Nguyen et al. (2015) in infant dairy formulations during *in vitro* digestion. In the case of sample MK, the aggregates found at the different gastric digestion times had similar particle sizes to sample M, except at 30 min, where the peak value was lower (~25 μm). For sample MA, the aggregates formed at 0 min were larger (between ~40 and 2000 μm) than those of M and MK. The particle size in sample MA increased from 0 to 30 min and remained practically unchanged afterwards, with a value of ~700 μm. The formation of large aggregates is due to the strong interaction between the protein and the alginate. These results are in accordance with the LM observations, where aggregation of the protein network was observed in the first 30 min in the case of sample MA, whereas samples M and MK showed protein degradation.

When the samples were prepared with whey proteins (samples W, WK and WA), the particle size of the aggregates was smaller than that of the milk samples. The aggregates were also seen as a finer network when observed by LM. Like the samples prepared with milk, samples W and WK had similar particle sizes but those of sample WA were bigger due to the strong protein-alginate interactions.

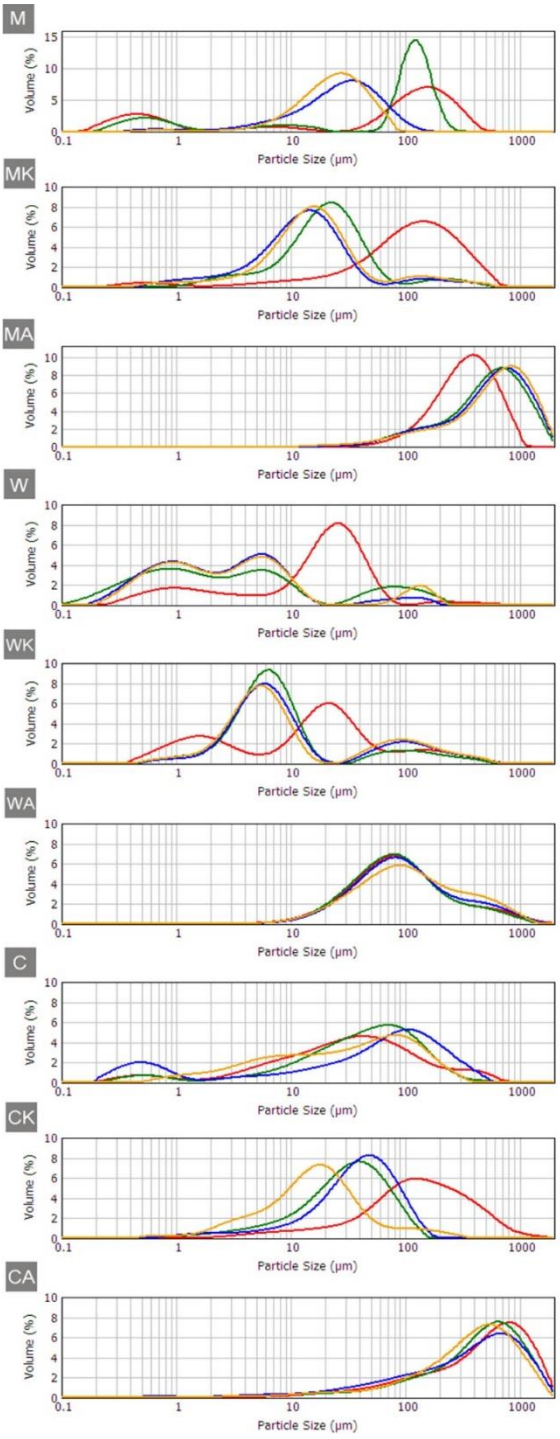

Fig. 5. Particle size distribution of the samples digested for: 0 min (red), 30 min (green), 60 min (blue) and 120 min (yellow). M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

In sample W, the particle size of the aggregates at 0 min was between ~0.25 – 90 μm, with a peak at ~25 μm. From 30 to 120 min, the particle size distribution became bimodal (one peak at ~0.8 μm and another at ~5.5 μm). In the case of sample WK, the particle size was practically the same as in sample W at 0 min and from 30 to 120 min the particle size distribution shifted from bimodal to monomodal (only one peak, at ~5.5 μm). The particle size of the aggregates in sample WA over the digestion time was between ~10 and 1500 μm, with a peak at ~80 μm. The presence of big aggregates of similar size that remained unaltered over the digestion time was also observed by Zhang and Vardhanabhuti (2014b) when studying the interaction between whey protein isolate and another charged polysaccharide, pectin.

The particle size of samples prepared with caseinate (samples C, CK and CA) was more similar to that of the milk samples than the whey ones, because casein is the most abundant milk protein. Nevertheless, the particle size of sample C increased as the digestion time advanced, rising from ~40 μm to 100 μm (maximum peaks) between 0 and 60 min, although the particle size decreased slightly, to ~90 μm, at 120 min. In sample CK, the particle size decreased from ~120 μm to 17 μm over the digestion time, following a similar trend to sample MK. As in samples MA and WA, sample CA showed large aggregates, but in sample CA the size decreased slightly over the 120 min digestion time, from ~800 μm to 500 μm.

The particle size results matched the LM results in all the samples. Overall, the addition of KGM induced practically no modification of aggregate particle size compared to the respective control samples (samples M, W and C), and a decrease in the particle size was observed over the digestion time. However, the presence of alginate increased the particle size of the aggregates and no important changes were observed during digestion.

3.4. SDS-PAGE

The *in vitro* gastric digestion patterns of the nine samples prepared with different dairy proteins and hydrocolloids were examined using the SDS-PAGE technique under reducing conditions. Fig. 6 presents the electropherogram, showing the protein profiles before *in vitro* pepsin digestion (0 min) and after different times of simulated *in vitro* digestion (30, 60 and 120 min).

The samples showed similar proteolysis patterns during simulated gastric digestion, with peptide bands appearing below the α-lactalbumin (α-La) band (14.4 kDa) at 30 min. In the electropherogram, the hydrolysis products can be seen to be more intense with prolonged digestion times.

In the milk sample without hydrocolloids (sample M), peptides were observed immediately after 30 min and their intensity increased from 10.93% at min 30 to 22.83% at min 120, while the α_S-casein (including α_{S1}-CN and α_{S2}-CN) and β-casein (β-CN) bands lost intensity, especially at 120 min. The band of α_S-CN lost more intensity (from 28.98% at 0 min to 0.71% at 120 min) than the band of β-CN (25.04% and 7.7% at 0 and 120 min, respectively). In agreement with these results, Dupont et al. (2010) and Nguyen et al. (2015) found that bands corresponding to caseins disappeared during digestion of skimmed milk powder and whey protein isolate-casein mixtures, respectively. The bovine serum albumin (BSA) band also lost intensity during gastric digestion, being 7.42% at 0 min, 3.7% at 30 min, 1.89% at 60 min and 0% at 120 min. However, the band at the κ-casein (κ-CN) molecular weight became more intense at 30 min due to the degradation of caseins by pepsin action. The proteolysis pattern of sample MK was very similar to sample M, agreeing with the particle size and LM results, the only difference being the greater degradation of the α_S-CN and β-CN bands at 30 and 60 min. For example, the

intensity of αs-CN band in sample M was 28.98% at 0 min, 13.42% at 30 min and 11.65% at 60 min; however in sample MK it decreased from 26.96% to 6.24% and to 1.13%, at respectively times. For sample MA, there was less degradation of the αs-CN and β-CN bands until the 120 min profile compared to samples M and MK. In the case of αs-CN band, in samples M and MK, the intensity percentages were 28.98% and 0.7% (0 and 120 min, respectively), but in sample MA, the intensity varied from 31.68% to 9%. Furthermore, the peptide bands were much less intense than in samples M and MK (at min 120, the intensity of peptides bands was 28.83%, 21.93% and 13.71%, for sample M, MK and MA, respectively). This could mean that the presence of alginate decreases the digestibility of protein. Chater, Wilcox, Brownlee, and Pearson (2015) found that alginate is able to interact with and disrupt the catalytic mechanism of pepsin, which leads to a reduced level of protein digestion.

In the case of the W samples (samples W, WK and WA) the bands corresponding to β-lactoglobulin (β-Lg), β-Lg dimer and α-La remained practically unchanged during digestion. These results have also been observed by other researchers. Mouécoucou, Frémont, Villaume, Sanchez, and Méjean (2007) found that the β-Lg dimer, α-La and β-Lg were poorly digested by pepsin in samples prepared with β-Lg/polysaccharide (gum arabic, low methylated pectin or xylan) mixtures. In different infant dairy formulations, Nguyen et al. (2015) observed that β-Lg and α-La completely resisted proteolysis by pepsin during the duration of digestion in the stomach. Other authors have also stated that β-Lg is resistant to pepsin digestion (Mouécoucou et al., 2004; Zhang & Vardhanabhuti, 2014a, b; Zhang et al., 2014). It has been suggested that the acid stability of β-Lg could result in increased internal hydrogen bonding between either two titrated carboxyl groups or one amide and one carboxyl group, reducing the accessibility of its cleavage

bonds to the enzyme (Mouécoucou et al., 2004). Faint peptide bands were observed in the electropherograms. This could be related to poor digestion of the β-Lg dimer, β-Lg and α-La by pepsin. Moreover, in samples W and WK, the BSA band disappeared completely within 30 min, whereas in sample WA, the BSA band was still observed as digestion progressed (20.04%, 7.43%, 5.22% and 2.41% at 0, 30, 60 and 120 min, respectively). In sample W, the peptide bands appeared discretely at 30 min (10.31%) and their intensity was higher at 120 min (27.30%). In sample WK, the peptide bands were more intense at 30 min (18.45%) and gained further intensity at 60 and 120 min (28.37%). As in the case of sample MA, the intensity of the peptide bands of sample WA was much weaker than those of W and WK: the intensity of peptides bands at min 120 was 27.3% for sample W, 28.37% for sample WK and 17.13% for sample WA. Zhang et al. (2014) reported that the decrease in the intensity of the peptide bands indicates a decrease in digestion rate with time. Once again, this could suggest that the presence of alginate decreased the digestibility of protein.

In the caseinate samples (with and without hydrocolloids), high molecular weight bands (bands above 45 kDa) were present initially but gradually broke down during digestion. In sample C, the αs-CN and β-CN bands lost intensity from 0 to 30 min (from 13.54% and 17.9% at min 0 to 11.1% and 9.96% at min 30, respectively) and remained almost unchanged afterwards. Moreover, the bands that appeared below 30 kDa (including the κ-CN band) gained intensity at 30 - 120 min (53.36% and 64.89% at 30 and 120 min) compared to 0 min (14.05%), due to the degradation of caseins into molecules of lower molecular weight. In sample CK, similar results were observed: degradation of the αs-CN and β-CN bands and the appearance of bands below 30 kDa. In sample CA, the αs-CN and β-CN bands lost intensity slightly during digestion. For example, in the case of β-CN, the intensity of the band was 14.02%, 4.43%, 1.25% and 0.61% at 0, 30, 60 and 120 min, respectively. As in samples

C and CK, the bands below 30 kDa became more intense from 30 (60.7%) to 120 min (80.11%). No peptide bands were observed on the electropherogram of the caseinate samples (C, CK and CA). When urea was used for casein extraction, peptide bands could be seen at the same molecular weight as in the milk samples (M, MK and MA) (results not shown).

The whey bands (sample W) were less degraded than the caseinate bands (sample C), which is likely to be due to the fact that whey proteins (WP) remain soluble in the stomach as an intact protein. As Lundin, Golding, and Wooster (2008) stated, the folded structure of β-Lg inhibits enzyme access to potential cleavage sites and, consequently, the protein is considerably more resistant to hydrolysis by pepsin. As a result, WP empties rapidly and is further hydrolysed by pancreatic proteases, being more distally absorbed than casein (Mahé et al., 1996). In contrast, clotted casein is more exposed to gastric peptic hydrolysis – the open structure of proteins in caseinate allows enzymes greater access to target residues, resulting in rapid proteolysis (Lundin et al., 2008) – and empties slowly from the stomach in the form of degraded products, which are subsequently hydrolysed by pancreatic proteases and absorbed in the upper part of the intestine (Mahé et al., 1996).

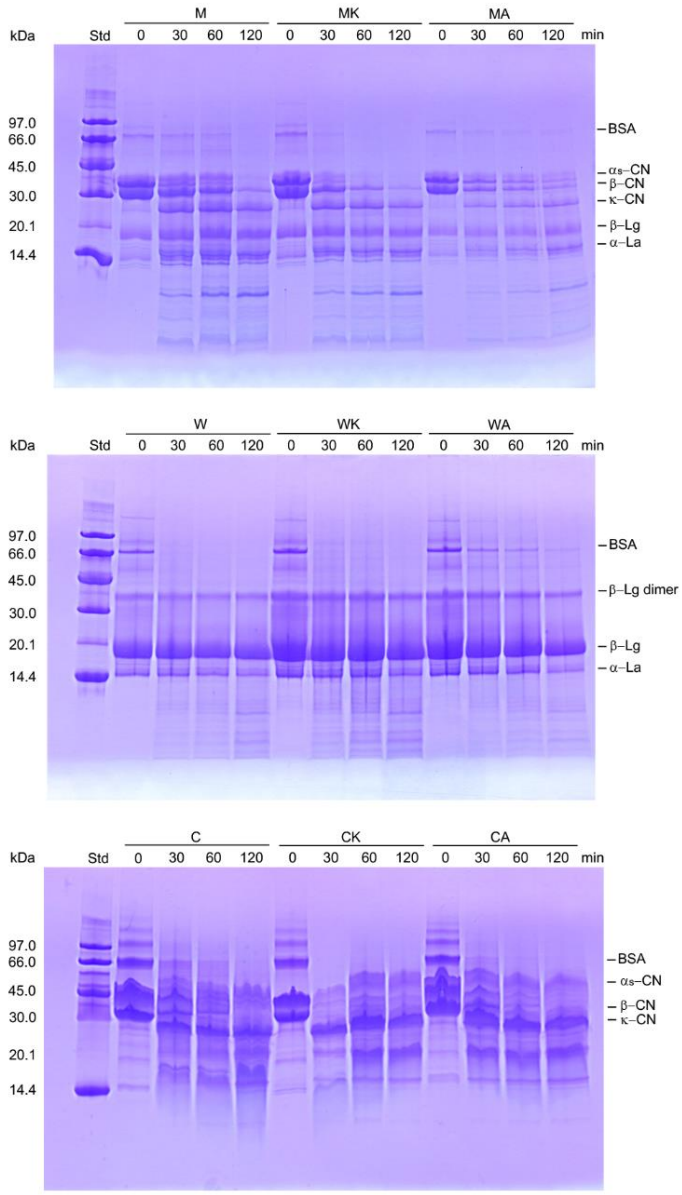

Fig. 6. SDS-PAGE analysis of the samples digested for 0, 30, 60 and 120 min. Std: Standard marker; M: milk; MK: milk with konjac glucomannan; MA: milk with alginate; W: whey proteins; WK: whey proteins with konjac glucomannan; WA: whey proteins with alginate; C: calcium caseinate; CK: calcium caseinate with konjac glucomannan; CA: calcium caseinate with alginate.

4. Conclusions

The effect of adding neutral or charged hydrocolloids on the *in vitro* gastric digestibility of different milk proteins was investigated. According to the results, whey proteins remained practically undigested over the digestion time, which is known to be related to rapid gastric emptying. In contrast, caseins formed clusters in the acidic gastric environment and were gradually degraded, therefore a slow emptying of the stomach may be expected.

When formulating satiety products fortified with dairy proteins, polysaccharide-protein interactions have to be taken into account. Regardless of the protein used, its digestion is affected less by the presence of the neutral polysaccharide (KGM) than by the presence of the charged one (alginate). The addition of KGM led to high values of apparent viscosity during gastric digestion, which probably will increase gastric distention and therefore, will affect satiety. However, the strong interaction between alginate and dairy proteins at the gastric pH gave place to large aggregates, which led to limited pepsin access to the protein during gastric digestion. Few hydrolysis products were observed, indicating a significant slowing down of the protein digestion rate, mainly in the case of the whey protein-alginate mixture. These results suggest that this combination could be used to delay gastric emptying and promote satiety.

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Designing dairy desserts for weight management: structure, physical properties and *in vitro* gastric digestion

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Abstract

The first aim of this study was to observe the effect of adding dairy proteins and reducing the cream content in order to obtain healthier dairy desserts for use in weight management. The extra-whey protein low-cream sample had the densest, firmest matrix, which is related to increased satiety. The second aim was to investigate the *in vitro* gastric digestion behaviour of whey and casein proteins in a heat-treated semisolid real food. The extra-casein protein sample matrix broke down more slowly than the others because the caseins clotted at the gastric pH. Despite being heated, the whey proteins in the panna cottas were more resistant to pepsin digestion than caseins; this is related with a higher satiety capacity. These findings suggest that the combination of reducing fat content (to obtain a reduced energy density product) and adding whey protein (to increase satiety capacity) allows obtaining dairy desserts for weight management.

Keywords: dairy dessert, dairy proteins, *in vitro* digestion, microstructure, SDS-PAGE, texture

1. Introduction

Excess weight and obesity represent an increasing health problem worldwide that seriously raises the risk of developing severe metabolic disorders and cardiovascular diseases (Munsters & Saris, 2014). The contribution of energy-dense high-fat sugary foods to weight gain is well recognized (Halford & Harrold, 2012). Energy-dense and high-fat foods are associated with high palatability, and vice versa. However, a significant inverse correlation between palatability ratings and satiety index scores has been found, so the more palatable foods are generally less satiating (Holt, Brand Miller, Petocz, & Farmakalidis, 1995). Moreover, although all types of fat contain almost the same amount of energy, increases in animal fat—rich in saturated fatty acids (SFA)—and in *trans*-fat have a stronger association with weight gain than increases in vegetable oils—rich in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (Field, Willett, Lissner, & Colditz, 2007). Consequently, designing healthy foods with low energy density, by reducing its animal fat content, and high satiating capacity, through adding extra protein, which is the most effective food macronutrient in providing a satiating effect (Geraedts, Troost, & Saris, 2011; Lundin, Golding, & Wooster, 2008; Morell, Hernando, Llorca, & Fiszman, 2015; Solah et al., 2010), could be an important way to fight excess weight and obesity.

Ingested food evokes satiation through two primary effects on the gastrointestinal (GI) tract: gastric distention (mechanical stimulation) and the release of gut peptides, which are hormones from the intestine (chemical stimulation) (Cummings & Overduin, 2007; Geraedts et al., 2011). Both effects slow down gastric emptying. Skimmed milk powder (SMP) is widely used to fortify dairy desserts, but the new milk and whey fractionation technologies produce a large diversity of dairy

ingredients, such as caseinates and whey protein concentrates (WPC). These ingredients have different properties and can be used separately or blended to replace SMP in dairy desserts (Remeuf, Mohammed, Sodini, & Tissier, 2003). Some authors have found differences between whey and casein in satiating terms. Casein, unlike whey, coagulates in the stomach, due to precipitation by gastric acid. Therefore, casein has a longer gastric emptying time than whey, which is not subject to acid precipitation and empties rapidly into the duodenum (Lundin et al., 2008). The concept of 'fast' and 'slow' proteins has been introduced to describe these differences in the digestion and absorption of proteins. According to this concept, a fast protein such as whey is more satiating than a slow protein such as casein (Mahé et al., 1996). Although the behaviour of these proteins (whey and casein) has been extensively studied in simplified model systems or liquid preloads (Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013; Hoad et al., 2004; Lacroix et al., 2006; Solah et al., 2010; Zhang & Vardhanabhuti, 2014a, 2014b; Zhang, Zhang, & Vardhanabhuti, 2014), few satiety-related studies have incorporated both proteins into semi-solid or solid real food (Kopf-Bolanz et al., 2014; Morell, Hernando et al., 2015; Morell, Piqueras-Fiszman, Hernando, & Fiszman, 2015).

The structure of food and its physical properties, such as texture or volume, are also important when designing reduced-fat and satiating foods. On the one hand, the structure and textural characteristics are altered when formulating reduced-fat or low-fat foods, affecting product acceptance by the consumer (Lobato-Calleros et al., 2007). Therefore, most authors have investigated the effect of using dairy proteins (whey proteins principally) as fat replacers in order to improve the texture of low-fat dairy foods such as yogurt and cheese and maintain their sensory characteristics. According to Damin, Alcântra, Nunes, and Oliveira (2009), the nature and proportions of the different proteins (skimmed milk, whey protein

concentrates, caseinates) in the formulation significantly affect the texture of yogurts. Equally, the incorporation of a high level of dairy proteins plays a key role in satiety (Zhang & Vardhanabhuti, 2014b). The reason is, firstly, that protein is recognized as the macronutrient with the highest satiating ability, and secondly, as mentioned in the previous paragraph, that adding proteins can enhance texture, and several authors have reported that a solid meal has a greater effect on satiety than a liquid meal of equivalent size and energy content (Chambers, McCrickerd, & Yeomans, 2015; Hoad et al., 2004; Solah et al., 2010).

The microstructure and physicochemical properties of foods also have significant effects on digestibility (Zhang & Vardhanabhuti, 2014a). Studies have demonstrated that food disintegration and gastric emptying are a complex process involving numerous variables, including particle size, meal volume, calories and composition of the meal, viscosity, and physical properties such as density, texture and microstructure (Kong & Singh, 2008). Therefore, a better understanding of how protein structures relate to degradation properties under gastric conditions might help to provide complementary information on gastric emptying, and thus also on their influence on satiety in the GI tract, and can assist food manufacturers in developing the next generation of structured food for health (Zhang & Vardhanabhuti, 2014a).

The primary objective of this study was to reformulate a dairy dessert, panna cotta, in order to obtain a healthier product which could be used in weight management. Two different approaches were used: reducing the fat to diminish the calorie content and adding extra milk protein from different sources to increase satiety. The second aim of this study was to investigate whether the digestion behaviour of whey and casein proteins incorporated into a heat-treated semi-solid

real food was similar to their behaviour in the liquid preloads or model systems studied until now. Accordingly, their microstructural (confocal laser scanning microscopy), textural (puncture test) and electrophoretic (SDS-PAGE) properties were assayed before and after *in vitro* oral plus gastric digestion.

2. Materials and methods

2.1. Panna cotta formulations

A control panna cotta (sample P) was prepared with skimmed milk powder (Central Lechera Asturiana, Siero, Spain) reconstituted in distilled water, liquid cream (Hacendado, Esnelat S.L., Urnieta, Spain) with a 35% fat content, and κ-carrageenan (Satiagel™ ME5, Cargill France SAS, Saint-Germain-en-Laye, France) (Table 1). Nine panna cotta samples were formulated with reduced amounts of cream (medium, low or zero) and the addition of different dairy proteins in order to obtain panna cottas with higher protein contents than the control. The proteins added to the formulation were skimmed milk powder (M); whey protein concentrate (W) (Avonlac™ 482 IP, Glanbia Nutritionals Ltd., Kilkenny, Ireland), or calcium caseinate (C) (Fonterra Co-operative Group Ltd, Reference 385, Palmerston North, New Zealand) (Table 1).

2.2. Sample preparation

To avoid phase separation in samples C_M, C_L and C_0, the calcium caseinate powder was pre-dissolved in 100 mL of distilled water and heated at 80 °C for 10 min before adding it to the mixtures.

The different ingredients were placed in a cooking device (Thermomix™ 31, Wuppertal, Germany) where they were heated to 90 °C with continuous stirring (700 rpm). After reaching this temperature, the mixtures were maintained under the same conditions for 6 min, then placed in silicone moulds and cooled to ambient temperature. The samples were stored at 4–5 °C in a refrigerator until they were analysed.

Table 1. Formulation of panna cotta samples with decreasing amounts of cream and the addition of different dairy proteins.

Ingredients	Samples									
	P	**M_M**	**M_L**	**M_0**	**W_M**	**W_L**	**W_0**	**C_M**	**C_L**	**C_0**
Skimmed milk (g)	50	100	100	100	50	50	50	50	50	50
Whey (g)	-	-	-	-	50	50	50	-	-	-
Caseinate (g)	-	-	-	-	-	-	-	50	50	50
Cream (mL)	200	50	25	0	50	25	0	50	25	0
κ-Carrageenan (g)	3	3	3	3	3	3	3	3	3	3
Water (mL)	500	500	500	500	500	500	500	500	500	500

P: control; M_M, M_L, M_0: skimmed milk powder added and medium, low or zero amount of cream, respectively; W_M, W_L, W_0: whey protein concentrate added and medium, low or zero amount of cream, respectively; C_M, C_L, C_0: calcium caseinate added and medium, low or zero amount of cream, respectively.

2.3. Texture analysis

The firmness of the panna cottas was determined with a puncture test, using a TA.XT-Plus Texture analyser (Stable Microsystems, Godalming, UK) equipped with a 30 kg load cell and a 12 mm diameter flat-ended cylindrical plastic probe. The crosshead speed was set at 10 mm s^{-1} and the penetration distance at 10 mm. The

firmness of the panna cotta was defined as the maximum force (N) attained during sample penetration (Salvador & Fiszman, 2004). Six replications were performed for each sample.

2.4. *In vitro* oral plus gastric digestion

To simulate oral digestion, samples P, M_0, W_0 and C_0 were mixed with artificial saliva in a hand blender (Ufesa, model BP4566, Barcelona, Spain) for 15 s. The ratio of saliva to sample was 1:4 on a weight basis. The artificial saliva was prepared according to the method described by Morell, Fiszman, Varela, and Hernando (2014). To simulate gastric digestion, an adaptation of the *in vitro* digestion model proposed by Abdel-Aal (2008) was used. It consisted of a jacketed glass reactor (1 L capacity) with continuous magnetic stirring, maintained at 37 °C in a temperature-controlled circulating water bath throughout the test. Each sample was mixed with simulated gastric fluid (SGF, a solution containing 0.034 M NaCl) in a proportion of 50 mL SGF/100 g panna cotta, and digested. The pH value was reduced to 1.9 with HCl 10 N, and pepsin (P7125, pepsin from porcine gastric mucosa, $\geqslant$ 400 units/mg protein, Sigma-Aldrich) was added at a pepsin to protein ratio of 1:4 on a weight basis. The mix was maintained at 37 °C with continuous stirring for 120 min. Digestion was stopped by raising the pH to 7 with NaOH 1 N. Sampling was carried out at 0, 30, 60 and 120 min.

2.5. Confocal laser scanning microscopy (CLSM)

A Nikon C1 confocal microscope unit fitted on a Nikon Eclipse E800 V-PS100E microscope (Nikon, Tokyo, Japan) was used. An argon laser line (488 nm) was

employed as the light source to excite Rhodamine B and Nile Red fluorescent dyes. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) was solubilised in distilled water at 0.2%. This dye was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) was solubilised in PEG 200 at 0.01%. This dye was used to stain fat. The detection wavelengths were 515 nm and 570 nm for Nile Red and Rhodamine B, respectively. A 40×/1.0/Oil/Plan Apo Nikon objective lens was used. Twenty microliters of the sample were placed in the central microscope slide. The Rhodamine B and Nile Red solutions were added, and the cover slide was carefully positioned to exclude air pockets. The observations were made 10 min after diffusion of the dyes into the sample. The images were observed and stored with a 1024 × 1024 pixel resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

2.6. Protein electrophoresis

2.6.1. Extraction of proteins

Samples P, M_0, W_0, C_0 were freeze-dried (TELSTAR Lioalfa-6, Terrassa, Spain) for 48 h at 1 kPA and −45 °C. Sample P was previously defatted using the Soxhlet method. Each sample was mixed with Laemmli buffer (4.8% (w/v) sodium dodecyl sulfate, 0.1 M dithiothreitol, 0.001 M ethylenediaminetetraacetic acid, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue and 0.125 M Tris-HCl; pH 6.8) (Borreani, Llorca, Larrea, & Hernando, 2016), adjusting the protein concentration of the samples to 8 mg mL^{-1}.

2.6.2. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Multiphor II Electrophoresis System (Pharmacia Biotech, Piscataway, USA), using ExcelGel SDS Homogeneous 15% polyacrylamide gels at 600 V, 20 mA, 20 W and 15 °C (Borreani et al., 2016). The samples were loaded at a protein concentration of 64 μg of protein/well.

The protein bands were stained with Coomassie Brilliant Blue tablets (Phastgel Blue R., Pharmacia). De-staining was performed in an aqueous solution of 25% ethanol and 8% acetic acid. The samples were kept in a solution of 10% glycerol and 7.2% acetic acid.

A high molecular weight calibration kit consisting of: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) (Amersham GE Healthcare, UK) was used as standard.

The gels were scanned with an ImageScanner III LabScan 6.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and analysed with the ImageQuant TL Image analysis Software v7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed on the data using the Statgraphics Centurion VI.II software package (Statistical Graph Co., Rockville, USA). Fisher's Least Significant Difference (*LSD*) test was used to assess the differences in mean values ($P < 0.05$).

3. Results and discussion

3.1. Texture analysis

The firmness values obtained by the puncture test are shown in Table 2. The control (P) and M_M panna cottas showed the lowest values, without significant differences ($P > 0.05$) between them. The firmness values of samples M_L and M_0 were higher than those obtained in samples P and M_M ($P < 0.05$). Therefore, it seems that as the amount of cream increased, the firmness values decreased. The panna cottas formulated with added whey protein (W_M, W_L, W_0) showed the highest firmness values and significant differences between them ($P < 0.05$). The samples formulated with caseinate (C_M, C_L, C_0) showed significantly higher firmness values than those with added milk (M_M, M_L, M_0) or the control (P), but significantly lower values than the samples with added whey protein (W_M, W_L, W_0). As in the milk samples (M_M, M_L, M_0), there were no significant differences between the samples with a low cream content (C_L) and without cream (C_0), but there were significant differences between these and the sample with a medium cream content (C_M).

In general, the firmness values decreased as the cream content rose. The samples with a medium cream content exhibited significantly lower firmness values ($P < 0.05$) than those with a low or zero cream content, regardless of the type of dairy protein added. As the fat content is reduced, a high degree of cross-linking of protein molecules may occur, resulting in three-dimensional networks that exhibit high resistance to deformation as observed by Lobato-Calleros et al. (2007). Moreover, as observed in CLSM section, the panna cottas prepared with the medium cream content (M_M, W_M, C_M) showed more and larger fat globules than the samples with the low cream content (M_L, W_L, C_L), which could lead to softer, smoother and

creamier textures. Softer cheese pies when cream was added to the formulation was also reported by Marcano, Morales, Vélez-Ruiz, and Fiszman (2015).

Table 2. Firmness values of the control sample and of the panna cottas formulated with extra protein and with different amounts of cream.

Sample	Firmness (N)
P	1.10 ± 0.12^{a}
M_M	1.12 ± 0.06^{a}
M_L	1.29 ± 0.10^{b}
M_0	1.32 ± 0.08^{b}
W_M	3.95 ± 0.11^{c}
W_L	4.59 ± 0.07^{d}
W_0	4.24 ± 0.06^{e}
C_M	2.92 ± 0.05^{f}
C_L	3.30 ± 0.07^{g}
C_0	3.21 ± 0.09^{g}

Values in parentheses are the standard deviations. Different superscript letters in the same row denote values with statistically significant differences ($P < 0.05$) according to the LSD multiple range test.
P: control; M_M, M_L, M_0: skimmed milk powder added and medium, low or zero amount of cream, respectively; W_M, W_L, W_0: whey protein concentrate added and medium, low or zero amount of cream, respectively; C_M, C_L, C_0: calcium caseinate added and medium, low or zero amount of cream, respectively.

On the other hand, although the increase in firmness values could also be due to the different carrageenan concentration among the samples, the firmness values of control (P) and M_M samples, which exhibited differences in carrageenan concentration (0.40 and 0.46%, respectively), were not significantly different ($P > 0.05$). Moreover, the firmness values between the samples with low and without

cream content (0.48 and 0.50% carrageenan, respectively) in M and C samples (M_L, M_0, C_L, C_0), neither were significantly different. Therefore, it seems not to be a direct relationship between the carrageenan concentration and firmness values. Regarding the type of protein added, the firmness values increased significantly ($P < 0.05$) as follows: extra milk protein < caseinate < whey protein. Similarly, Morell, Piqueras-Fiszman, et al. (2015) found that the dairy ingredient source significantly affected the yogurt's firmness, as the samples with extra caseinate and the samples with extra milk protein showed significantly lower firmness values than the samples with extra whey protein concentrate, but higher values than the control sample. Therefore, the increase in firmness values seems to be more related to the addition of the different dairy proteins and the reduction of cream content than to the increase in carrageenan concentration.

3.2. Confocal laser scanning microscopy (CLSM)

3.2.1. CLSM before oral plus gastric digestion

The microstructure of the panna cottas stained with Nile Red and Rhodamine B is shown in Fig. 1. Fat is observed in green-orange, stained with Nile Red, and protein and carrageenan are observed in red, stained with Rhodamine B.

The control panna cotta (P) exhibited an open protein matrix interspersed with fat globules in the intervening spaces. Some large fat globules could be observed, owing to emulsion destabilisation processes involving coalescence. Coalescence occurs when two or more droplets merge to form a bigger droplet (losing their individual interfacial films) and their contents mix (Mao & Miao, 2015). As expected, the panna cottas prepared with the medium cream content (M_M, W_M,

C_M) showed more and larger fat globules and more coalescence than the samples with the low cream content (M_L, W_L, C_L). Nevertheless, regardless of the protein used (M, W or C), no visual differences in the structure of the protein matrix were observed between the samples formulated with a medium, low or zero amount of cream. Therefore, the presence or absence of cream did not appear to influence the protein structure.

In contrast, the M_0 panna cotta, devoid of fat content, showed a continuous homogeneous network. This became more aggregated when part of the milk protein was replaced by whey protein in sample W_0 or by caseinate in sample C_0, leading to a more heterogeneous structure (Fig. 1). In consequence, these samples (W_0 and C_0) exhibited higher resistance to deformation than sample M_0 in the texture analysis assay. Similarly, Lobato-Calleros et al. (2007) found that incorporating whey protein concentrate (WPC) into matrices yielded a higher protein/fat ratio that gave rise to a relatively large area of compact and continuous protein matrix. Moreover, the protein network of sample W_0 seemed to be more heterogeneous, showing a higher degree of aggregation, which resulted in higher firmness values than for sample C_0. This is probably because the cross-linking capacity of denatured whey proteins plays a key role in the structure after heating by contributing to increased bridging between protein particles (Remeuf et al., 2003). In contrast, Damin et al. (2009) reported that casein-based (sodium caseinate) products tended to produce firmer gels than yogurts supplemented with WPC.

In general, the protein network became denser and more aggregated in panna cottas made with extra protein and no cream, as follows: sample W_0 showed the highest aggregation, followed by sample C_0, while sample M_0 exhibited the lowest aggregation.

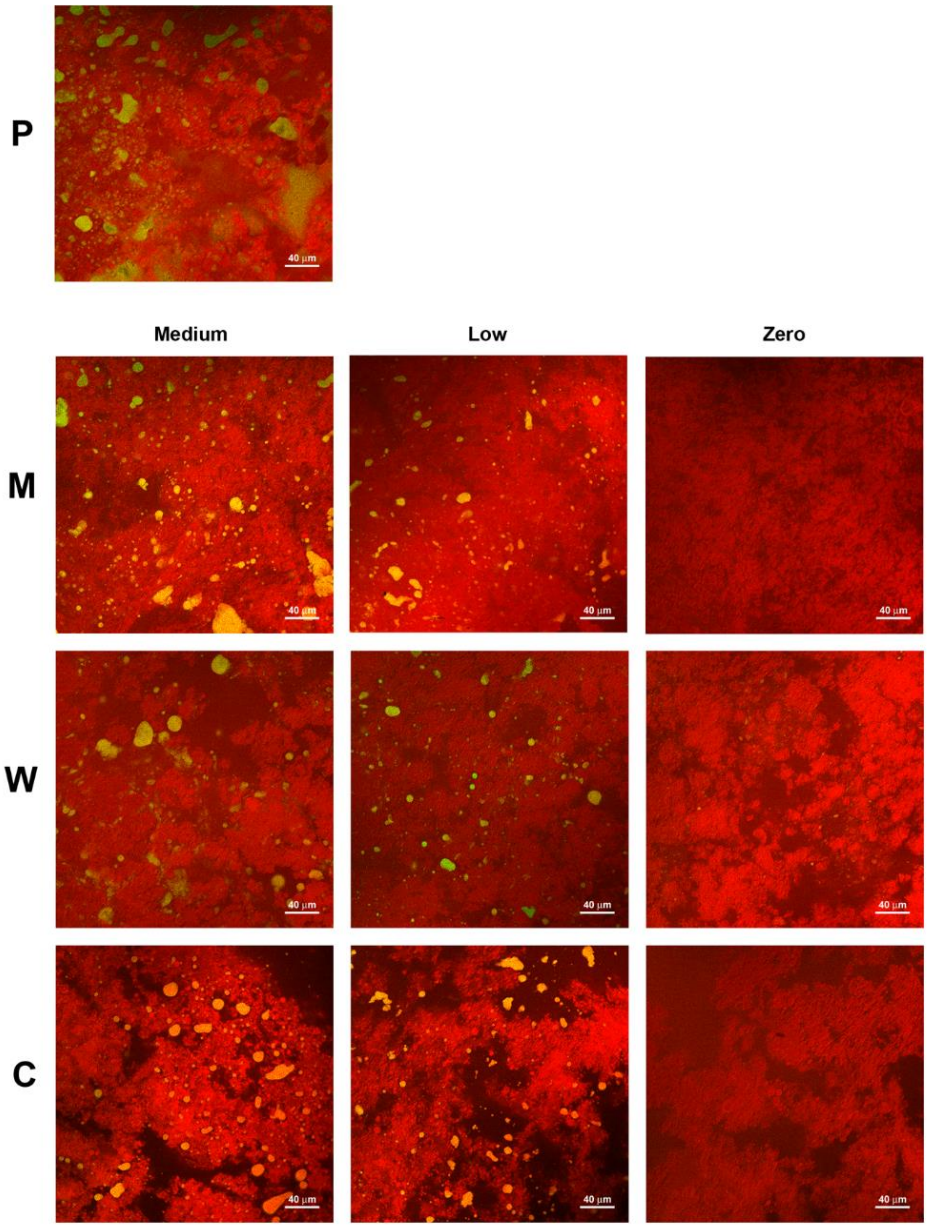

Fig. 1. Microstructure of the panna cottas before *in vitro* oral plus gastric digestion. Nile Red stained fat *green-orange*, Rhodamine B stained protein *red*. Magnification 40x. The scale bars measure 40 µm. P: control; M: skimmed milk powder added; W: whey protein concentrate added; C: calcium caseinate added; Medium, Low, Zero: amount of cream.

3.2.2. CLSM during oral plus gastric digestion

During *in vitro* gastric digestion (Fig. 2), the control panna cotta (P) exhibited protein network degradation and fat globule coalescence. These were more pronounced at min 120. In the same way, Guo, Ye, Lad, Dalgleish, and Singh (2014) observed a continuous protein network with coalescence of oil droplets in the emulsions during *in vitro* gastric digestion, which they attributed to flocculation of the released oil droplets and mechanical shearing as the likely cause. Ye, Cui, and Singh (2011) observed that fat globules in raw milk flocculated during incubation in SGF at 10 min and that this flocculation was enhanced at longer incubation times. The flocculation may have arisen from hydrolysis of charged milk fat globule membrane (MFGM) proteins, resulting in a decrease in electrostatic repulsion (through the fat globules' linking together) or from aggregation of the casein micelles in the serum phase, induced by a combination of low pH and pepsin hydrolysis, trapping the fat globules in the casein aggregates (Ye et al., 2011).

In sample M_0, protein network degradation was appreciable at 60 min and even more at 120 min. This degradation could be observed as the loss of continuity of the protein network, giving place to a discontinuous matrix that forms little "islands". In contrast, sample W_0 had the highest protein matrix degradation compared to samples M_0 and C_0, as the protein network was degraded at 30 min and the degradation continued at 60 and 120 min. Sample C_0 was the least degraded compared with the other samples (P, M_0 and W_0). In particular, its protein network was slightly degraded at 30 min and this degradation was more pronounced at 60 min but remained almost unchanged at 120 min. The lower protein degradation of sample C_0 was probably due to the fact that caseins coagulate at gastric pH, so large aggregates could be seen practically unchanged afterwards.

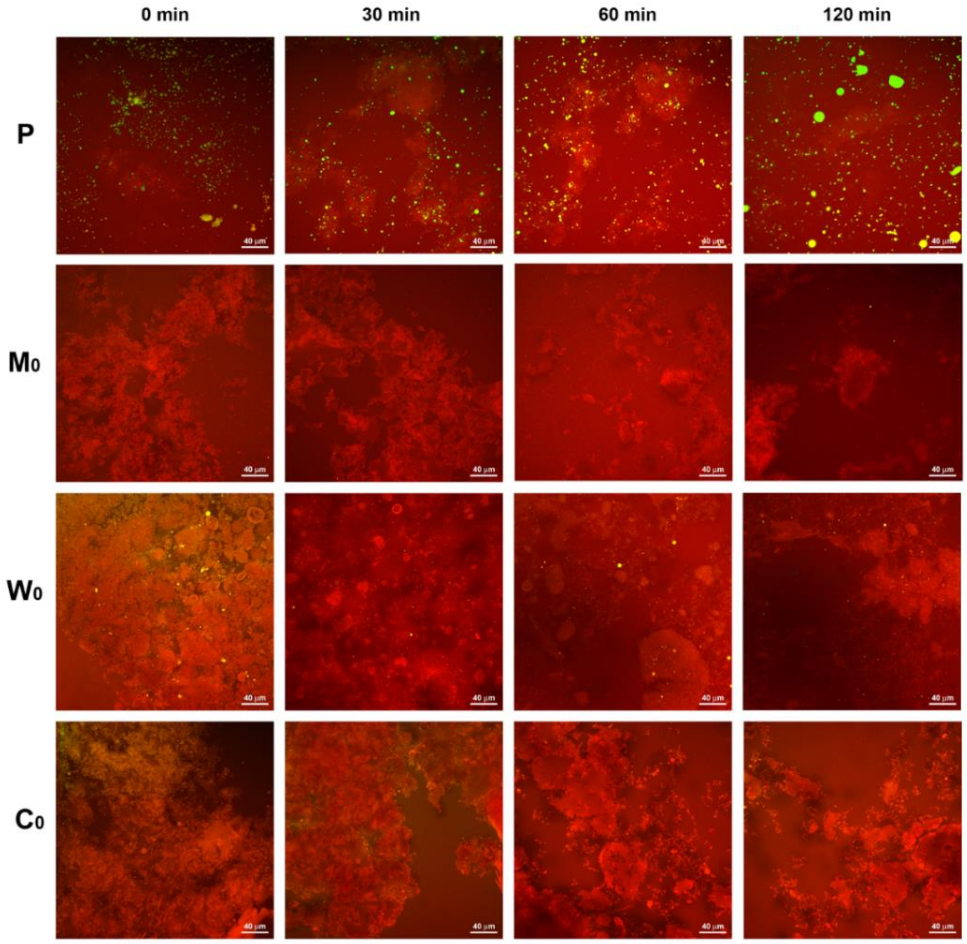

Fig. 2. Microstructure of four panna cottas during *in vitro* gastric digestion (0, 30, 60 and 120 min). Nile Red stained fat *green*, Rhodamine B stained protein *red*. Magnification 40x. The scale bars measure 40 μm. P: control; M_0: skimmed milk powder added, no cream; W_0: whey protein concentrate added, no cream; C_0: calcium caseinate added, no cream.

3.3. Electrophoresis

The *in vitro* oral plus gastric digestion patterns of the control sample and the samples prepared with added protein and without fat (P, M_0, W_0 and C_0) were examined using the SDS-PAGE technique under reducing conditions. Figure 3 presents the electropherogram, showing the protein profiles before (0 min) and after different simulated *in vitro* gastric digestion times (30, 60 and 120 min).

In the control (P) sample before digestion (0 min), the bovine serum albumin (BSA) band was observed at approximately 66.0 kDa; α_S-casein (α_S-CN), β-casein (β-CN) and κ-casein (κ-CN) at approximately 33.6, 27.2 and 21.3 kDa respectively; and β-lactoglobulin (β-lg) and α-lactalbumin (α-la) at approximately 17.5 and 14.4 kDa. After 30 min of gastric digestion, the BSA band disappeared almost completely and the casein band (α_S-CN and β-CN) lost much of its intensity (falling from 32.25% to 7.74% and from 29.39% to 22.06% respectively), whereas the κ-CN, β-lg and α-la bands remained practically unchanged. Moreover, peptide bands appeared below the α-la band (14.4 kDa), due to the degradation of high molecular weight proteins into proteinaceous molecules with a low molecular weight. After 60 and 120 min of pepsin digestion, the casein bands (α_S-CN, β-CN and κ-CN) disappeared, but β-lg and α-la remained almost unchanged and the peptide bands were slightly more intense.

In the M_0 sample, as in the control sample, the BSA, α_S-CN and β-CN bands lost much of their intensity between 0 and 30 min of gastric digestion (from 10.81%, 45.14% and 34.83% to 2.89%, 18.05% and 7.8%; respectively), κ-CN remained practically unchanged and several peptide bands were visible. Kim et al. (2007) found similar results: high molecular weight bands, such as BSA, were completely eliminated from SDS-PAGE electropherograms when studying native and heated

dairy hydrolysates produced by pepsin after a 30 min incubation period. After 60 min, α_s-CN and β-CN lost more intensity (1.1% and 1.39% respectively), and at 120 min they disappeared completely, together with κ-CN. This result was in accordance with Barbé et al. (2013), who observed that caseins are rapidly cleaved by pepsin, so rapid hydrolysis was expected. Indeed, Tunick et al. (2016) and Kopf-Bolanz et al. (2014) found that the degradation of casein bands was very rapid during gastric digestion. Nevertheless, the electrophoretic bands corresponding to β-lg and α-la remained intense throughout the digestion period. Although Kopf-Bolanz et al. (2014) observed β-lg in decreasing amounts depending on the heat treatment of the milk, the β-lg band was still present in differently heated milk. In fact, with regard to β-lg, several studies have shown insignificant differences among milk samples subjected to heat treatments throughout gastric digestion (Inglingstad et al., 2010; Tunick et al., 2016; Wada & Lönnerdal, 2014).

In the W_0 sample, the β-lg dimer (indicated by an arrow in Fig. 3) and β-lg bands lost intensity at 30 min of gastric digestion, whereas the α-la band, which had a poor intensity at min 0 (6.48%) did not seem to change. Similarly, Nguyen, Bhandari, Cichero, and Prakash (2015) and Inglingstad et al. (2010) found that α-la was the most resistant protein to human digestive enzymes and therefore underwent limited digestion. As in the other samples, at min 30 the peptide bands were slightly visible (26.45%). At min 60, the β-lg dimer, β-lg and α-la bands had disappeared.

In the C_0 sample, as in the control (P) and M_0 samples, from 0 to 30 min the BSA band disappeared, the α_s-CN and β-CN bands lost most of their intensity (from 18% and 67.9 to 9.71% and 6.48% respectively) and some slight peptide bands

appeared. From 30 to 120 min of gastric digestion no major changes were found, and only the casein bands (α_S-CN, β-CN and κ-CN) had disappeared at min 120.

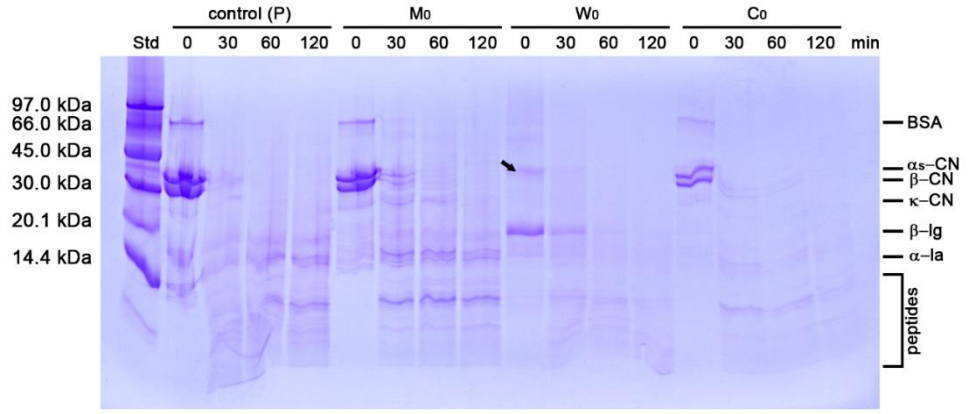

Fig. 3. SDS-PAGE patterns under reducing conditions of panna cottas during *in vitro* gastric digestion (0, 30, 60 and 120 min). Std: Standard marker; P: control; M_0: skimmed milk powder added, no cream; W_0: whey protein concentrate added, no cream; C_0: calcium caseinate added, no cream.

According to the results, although the panna cottas were heated to 90 °C—which can denature whey proteins (Matignon et al., 2014), resulting in increased susceptibility to pepsin proteolysis (Lundin et al., 2008)—it seemed that in the first 30 min of gastric digestion the whey proteins (β-lg and α-la) were more resistant to pepsin attack than the casein proteins (α_S-CN and β-CN), regardless of the panna cotta to which they were added. This finding suggests that despite the whey proteins' losing some resistance to hydrolysis, the behaviour of the dairy proteins was the same when incorporated into real food as when incorporated into simple formulations without heating.

In the present study, the addition of whey proteins and elimination of cream obtained the highest firmness values. This is probably due to a major cross-linking of proteins, which exhibited high resistance to deformation leading to high firmness values, as discussed in Section 3.1. These results are in accordance with the microstructure results, where the samples formulated with whey proteins exhibited a denser or more aggregated protein matrix than the caseinate, milk and control samples. Moreover, Marcano et al. (2015), Morell, Fiszman, et al. (2014), Morell, Hernando, et al. (2015) and Morell, Piqueras-Fiszman, et al. (2015) found that texture had a positive effect on expected satiating capacity, as the expected satiating capacity scores were completely aligned with the TPA firmness and penetration instrumental texture assessment (Marcano et al., 2015). It is worth noting that density, thickness, and compactness/firmness are attributes that consumers generally tend to associate with a product's satiating ability (Morell, Piqueras-Fiszman, et al., 2015). Therefore, the samples with added whey protein and a low fat content, which obtained the highest firmness values and showed the densest protein network, probably would have the highest satiating capacity.

At gastric level, in conjunction with gastric distension (involved in the mechanical effect on satiation), the release of peptides and resulting concentration of amino acids play a key role in satiation (Cummings & Overduin, 2007) and in satiety (Geraedts et al., 2011). The present study focused on the release of peptides by protein-degrading pepsins attacking at gastric level. The SDS-PAGE results showed that in the panna cottas, the whey proteins were more resistant than the casein proteins to gastric digestion by pepsin in the first 30 min. Although caseins coagulate in the stomach and empty more slowly from the stomach into the intestine than whey proteins, they are more exposed to gastric peptic hydrolysis, as the open structure of the caseinate proteins allows enzymes greater access to target residues,

resulting in rapid proteolysis (Lundin et al., 2008). In this regard, some authors (Bowen, Noakes, Trenerry, & Clifton, 2006; Veldhorst et al., 2008) have found that whey proteins exhibit a faster rate of gastric emptying, resulting in a stronger increase in the postprandial plasma amino acid concentration than casein proteins, which would increase satiety. Moreover, gastrointestinal satiety hormones are secreted in response to protein ingestion and they exhibit a greater plasma concentration with the ingestion of whey proteins than with that of casein proteins (Veldhorst et al., 2008). Therefore, whey protein from panna cottas could be emptied from the stomach into the intestine faster and in a more unaltered form than casein, giving rise to a faster and higher release of plasma amino acids and satiety-related hormones.

4. Conclusions

This study demonstrates that the addition of extra dairy proteins and the reduction of cream content in panna cottas leads to a denser and more aggregated matrix than in the control sample, and therefore increases the firmness values of the panna cottas, which can be related with increased satiety. In particular, the samples with whey protein and a low fat content exhibited the highest firmness values and the densest protein network. Therefore, this panna cotta could be expected to have the highest satiating capacity.

The digestion behaviour of dairy proteins in a heat-treated complex food matrix (panna cotta) during *in vitro* gastric digestion was also studied. Although caseins clotted at gastric pH, and thus showed a dense, aggregated matrix which broke down more slowly than the other samples over the digestion time, they were very susceptible to proteases being rapidly cleaved by pepsin. Regardless of the

panna cotta formulation and despite the heating process, the whey proteins were more resistant to pepsin digestion than the caseins. Therefore, although the heat treatment reduced their resistance to pepsin attack, these proteins behaved in the same way as in simple model systems. The resistance of whey proteins to pepsin digestion can also be related to a high satiating capacity.

Considering the results as a whole, panna cottas with whey protein added and a low fat content would exhibit the highest satiation and satiety effects.

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Capítulo 2

Uso de emulsiones para reducir el aporte en grasa al organismo

Oil-in-water emulsions stabilised by cellulose ethers: stability, structure and *in vitro* digestion

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Abstract

The effect of cellulose ethers in oil-in-water emulsions on stability during storage and on texture, microstructure and lipid digestibility during *in vitro* gastrointestinal digestion was investigated. All the cellulose ether emulsions showed good physical and oxidative stability during storage. In particular, the methylcellulose with high methoxyl substituents (HMC) made it possible to obtain emulsions with high consistency which remained almost unchanged during gastric digestion, and thus could enhance fullness and satiety perceptions at gastric level. Moreover, the HMC emulsion slowed down lipid digestion to a greater extent than a conventional protein emulsion or the emulsions stabilised by the other cellulose ethers. Therefore, HMC emulsions could be used in weight management to increase satiating capacity and decrease lipid digestion.

1. Introduction

Fat-rich diets have been associated with high incidences of obesity and a higher risk of coronary heart disease, diabetes, and certain forms of cancer.[1] A potential strategy for combating these chronic diseases is to develop healthier foods by reducing the amount of fat.[1,2] However, the development of fat-reduced products is challenging because fats have a major impact on the physicochemical, sensory, and nutritional properties of foods.[1] Since this makes it difficult to formulate foods whilst maintaining consumer satisfaction, another solution is to develop functional foods by using strategies associated with controlling lipid digestion in the gastrointestinal human tract in order to reduce the bioavailability of the fat.[3,4] This solution requires a good understanding of the relationship between food structure and its behaviour before and during digestion.[5]

A large part of the lipids in processed foods is consumed in the form of oil-in-water (o/w) emulsions, in which the lipids are embedded in the form of droplets in an aqueous medium in order to enhance their stability and organoleptic quality. Emulsions are thermodynamically unstable systems, but they can be physically stabilised by emulsifiers to avoid immediate separation into oil and aqueous phases.[6] Proteins and polysaccharides have been widely used as emulsifiers, stabilisers, thickening or gelling agents in the food industry, to form physically stable emulsions and to control microstructure, texture, flavour and shelf life.[7–9]

Besides their physical instability, o/w emulsions also suffer oxidative deterioration (such as lipid oxidation) during storage.[7] This is directly associated with negative effects on taste, appearance, texture and shelf life and also leads to the formation of off-flavours (rancidity) and toxic compounds.[10] Various factors can influence the rate of lipid oxidation in emulsion-based foods, such as droplet size,

composition of the interfacial layer, colloid structures in the aqueous phase, the presence of antioxidants and pro-oxidants (transition metals), *etc*.[6,10]

Nevertheless, in whatever form they are consumed, lipids are emulsified in the mouth, the stomach, and/or the small intestine due to: (i) the mechanical stresses they experience, (ii) the presence of various endogenous and dietary surface-active and stabilising components[4] and (iii) lipid digestion being an interfacial process[3] in which gastric and pancreatic lipases have to bind to the o/w interface, *via* complexation with co-lipase which adsorbs onto bile salts in the case of pancreatic lipase.[11] Hence, the substrate for dietary fat digestion is usually lipid droplets coated by a complex layer of surface-active material.[4] In general, in a state of lipase abundancy in the duodenum[12] the human body has an excess capacity for fat digestion, so the rate and extent of fat digestion are controlled by the ability of lipase to bind to emulsion interfaces. This ability is controlled in turn by the lipid droplet's characteristics (its surface area and the composition of the lipid itself) and interfacial composition (*e.g.* the presence of bile salts and the nature of the interfacial layer).[13] Thus, in order to obtain healthier foods, the choice of emulsifiers, particle size and fatty acid composition are major factors to be taken into account when processing food with the aim of delaying or limiting lipid digestion and absorption.[3]

Moreover, several studies have shown that adding polysaccharides in the form of fibre can increase viscosity and induce the formation of gels in the stomach, and these properties can slow down gastric emptying and concurrently increase gastric volume/stomach distension,[14] which is positively and linearly correlated with postprandial fullness.[15] Therefore, controlling the emulsion structure and thus its digestion behaviour could make it possible to obtain emulsions with enhanced

satiating capacity and lower lipid digestion which could be used in weight management.

Cellulose ethers are non-ionic dietary fibres that differ principally in molecular weight, viscosity and degree of substitution. In recent studies, the effect of different hydroxypropyl methylcelluloses (HPMCs) on lipid digestion of o/w emulsions has been investigated.[2,16] Torcello-Gómez and Foster[2] found similar results of lipolysis curves regardless the molecular weight, substitution pattern or initial concentration in the bulk of the HPMCs. However, Pizones Ruiz-Henestrosa *et al*.[16] attributed the slight difference in lipolysis extent between two types of HPMC to the molecular events occurring at the interface upon bile salts adsorption, due to their different methyl/hydroxyl ratio. Therefore, there is no a clear trend on the lipid digestion and its relation to the molecular weight or degree and type of substitution of the cellulose ethers used as emulsifiers in o/w emulsions and thus further investigation is needed. In this regard, two types of hydroxypropyl methylcellulose (HPMC) and two types of methylcellulose (MC) are used in this study as emulsifiers in o/w emulsions in order to design new emulsions with satiating capacity and low lipid digestion. These new emulsions are prepared with high fat content, thus they can be used as fat replacers of conventional sources of solid fat in the diet, such as butter or shortening.

The first aim of this study, therefore, was to study the physical and oxidative stability of o/w emulsions stabilised by cellulose ethers and the second aim was to study their microstructure, texture and lipid digestion (free fatty acid release – FFA) during *in vitro* gastrointestinal digestion.

2. Materials and methods

2.1. Emulsion ingredients

Oil-in-water emulsions were prepared with commercial sunflower oil (Koipe Sol, Deoleo SA, Córdoba, Spain), drinking water (Bezoya, Calidad Pascual SAU, Burgos, Spain) and four different cellulose ethers with thermo-gelling ability (METHOCEL™ K4M, F4M, A4M and MX, from now on referred to as HHPMC, HPMC, MC and HMC, respectively), supplied by The Dow Chemical Company. HHPMC and HPMC are hydroxypropyl methylcelluloses. HHPMC (high hydroxypropyl methylcellulose) has a higher percentage of hydroxypropyl (7.7% hydroxypropyl, 22.5% methoxyl) than HPMC (hydroxypropyl methylcellulose) (6.8% hydroxypropyl, 29% methoxyl). MC and HMC are methylcelluloses. MC (methylcellulose) has less methoxyl substitution (30% methoxyl) than HMC (high methylcellulose) (methoxyl >30%). HHPMC, HPMC and MC have approximately the same viscosity (4000 mPa s, measured at 2% aqueous solution at 20 °C by The Dow Chemical Company following reference methods ASTM D1347 and ASTM D2363) while HMC has a higher viscosity (50 000 mPa s, measured in the same way).

2.2. Emulsion preparation

The emulsions were prepared according to Sanz *et al.*[17] with some modifications. Each cellulose ether (2% w/w) was dispersed in the oil (47% w/w) using a Heidolph stirrer (Heidolph RZR 1, Schwabach, Germany) at 283 rpm for 5 min. The mixture was then hydrated by gradually adding water at 1 °C while continuing to stir. A water temperature of 1 °C was selected in accordance with the specific hydration requirement of HMC and was employed for the other emulsifiers

as well. Stirring continued using a homogenizer (Ultraturrax T18, IKA, Germany) at 6500 rpm for 15 s and subsequently at 17 500 rpm for 60 s. Sorbic acid (0.1% w/w) was added as an antimicrobial agent to prevent microbial growth in the emulsions during storage (30 days at 4 °C).

A control emulsion with calcium caseinate (CaCN) (Fonterra Co-operative Group Ltd, Palmerston North, New Zealand) was also prepared for the *in vitro* digestion study. The CaCN powder (4.5% w/w) was slowly dispersed in the oil and then hydrated by gradually adding water, as previously described for the cellulose ether emulsions. In order to form an emulsion with similar oil droplet size to that of the cellulose ether emulsions, the homogenisation conditions were also modified slightly: the first homogenizer speed (6500 rpm) was maintained for 30 s and the second (17 500 rpm) for 120 s.

2.3. Physical stability

Physical stability was examined according to Goyal *et al.*[18] with few modifications. Immediately after preparation, approximately 20 g of sample were transferred into glass tubes (internal diameter 27 mm, height 100 mm), which were sealed with a plastic cap and stored at a low temperature (4 °C) for a period of 30 days. Digital photographs (Olympus E-510, Tokyo, Japan) of the samples were taken every 10 days. This physical stability investigation was performed in duplicate.

2.4. Oxidative stability

Fresh emulsions were placed in glass beakers, covered with aluminium foil and stored at a low temperature (4 °C) for 30 days. Before oxidative stability determination, lipid extraction was carried out according to Timm-Heinrich *et al.*[19] with some modifications. The samples (approximately 20 g) were deep-frozen (−70 °C) for 24 h in a conical centrifuge tube and thawed before centrifugation at 10 765 rpm for 10 min.

The primary lipid oxidation products were measured by the peroxide value method (PV) according to Hornero-Méndez *et al.*[20] In addition, formation of the secondary products was measured by the specific extinction value method (K270) according to ISO 3656.[21] Three replications were performed every 10 days during the storage time.

2.5. *In vitro* digestion model

An *in vitro* gastrointestinal tract model consisting of oral, gastric and intestinal phases was used to simulate the biological fate of ingested samples, following Morell *et al.*,[22] Sanz *et al.*[23] and Qiu *et al.*[24] with some modifications.

To simulate oral digestion, 15 g of fresh emulsion sample were gently mixed for 5 s with 0.33 mL of fresh artificial saliva (pH 6.8, 62 mM $NaHCO_3$, 6 mM $K_2HPO_4 \cdot 3H_2O$, 15 mM NaCl, 6.4 mM KCl, 3 mM $CaCl_2$, mucin type II from porcine stomach (M2378, Sigma-Aldrich), α-amylase type VI-B from porcine pancreas (A3176, Sigma-Aldrich)).

To simulate gastric digestion, the sample obtained after the oral phase (bolus) was mixed with 4.8 mL of pre-incubated (37 °C; 5 min) simulated gastric fluid (pH 2, 53 mM NaCl, 1 mM $CaCl_2$, 14.8 mM KCl, 5.7 mM Na_2CO_3). The pH was adjusted to 2.0 with HCl and 0.7 mg of pepsin (P7125, pepsin from porcine gastric mucosa, ≥ 400 units per mg protein, Sigma-Aldrich) was added. The mix was maintained at 37 °C with continuous stirring for 60 min.

To simulate intestinal digestion, 3.18 mL of bile extract (B8631, Sigma-Aldrich) solution (46.87 mg mL^{-1} phosphate buffer pH 7) and 1.2 mL of electrolyte mixture (pH 7, 21 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$) were added and the pH was increased to pH 7 with NaOH 1 M. After this, 0.5 g of pancreatin from porcine pancreas (P3292, Sigma-Aldrich) and 2.8 g of lipase from porcine pancreas (L3126, type II, 100–500 units per mg protein, Sigma-Aldrich) dissolved in 1.62 mL of phosphate buffer (pH 7) were added and the mix was maintained at 37 °C and pH 7 with continuous stirring for 120 min.

2.6. Texture analysis

Emulsion texture measurement was carried out with a TA.XT.plus Texture Analyser (Stable Microsystems, Godalming, UK) using a 30 kg load cell. A back extrusion test was conducted using an A/BE-D40 back extrusion cell (40 mm diameter). The samples (50 g) were placed into an extrusion cylinder (50 mm internal diameter and 75 mm height) and one cycle was applied (speed: 1 mm s^{-1}; distance: 15 mm). The area under the curve (N s) after reaching the maximum force was recorded from the force-time profiles. The texture analysis was performed in triplicate.

2.7. Free fatty acid release

The extent of lipolysis was measured through the amount of free fatty acids (FFA) released during the intestinal phase. The pH of the mixture was monitored and the volume of NaOH 0.5 M used to neutralise the FFA released through lipid digestion was recorded using a pH-stat (Mettler-Toledo DL 50, Greinfensee, Switzerland). The amount of FFA released was calculated as the percentage of FFA (% FFA) released during the digestion time as described by Li and McClements.[25] The measurement was carried out in duplicate.

2.8. Microstructure analysis

A Nikon ECLIPSE 80i (Nikon Co., Ltd, Tokyo, Japan) light microscope was used as described by Borreani *et al*.[26] An aliquot of each formulation was placed on a glass slide and observed at 20× magnification. A camera (ExWaveHAD, model no. DXC-190, Sony Electronics Inc, Park Ridge, New Jersey, USA) was attached to the microscope and connected to the video entry port of a computer. The images were captured and stored at 1280 × 1024 pixels using the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan). The software interfaced directly with the microscope, enabling image recording control. The images were taken from fresh samples and at the end of each digestion phase. Toluidine blue (0.2%) was used to stain the proteins and celluloses.

A Nikon confocal microscope C1 unit that was fitted on a Nikon Eclipse E800 V-PS100E microscope (Nikon, Tokyo, Japan) was used. An aliquot of each formulation was placed on a glass slide and Nile Red (0.01%) and Rhodamine B (0.2%) solutions were added to stain fat and proteins and/or carbohydrates,

respectively. Observations were performed 10 min after diffusion of the dyes into the sample at 60× magnification. Images were observed and stored with 1024 × 1024 pixels resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

The droplet size of the fresh and intestinal-digested emulsions was determined from CLSM images. The diameter of 180 droplets from each sample was measured with the microscope software (NIS-Elements F, Version 4.0, Nikon, Tokyo, Japan).

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed on the data using XLSTAT statistical software (version 2014.5.02, Addinsoft, New York, NY, USA). Fisher's Least Significant Difference (LSD) test was used to assess the differences in mean values ($P < 0.05$).

3. Results and discussion

3.1. Physical stability

Phase separation was investigated to assess the stability of o/w emulsions during the storage time (Fig. 1). None of the cellulose ether emulsions exhibited phase separation during the 30-day storage period. The excellent physical stability of the emulsions containing cellulose ethers was probably due to the ability of these polysaccharides to increase the viscosity of the continuous phase, which decreased droplet collisions, thus decreasing flocculation and coalescence and therefore

reducing the creaming rate.[8] In the same way, Karlberg *et al.*[27] reported that the viscosity of the continuous phase and the adsorption of the hydrophobically modified cellulose at the o/w interface are the key factors for the stabilisation mechanism of the emulsion.

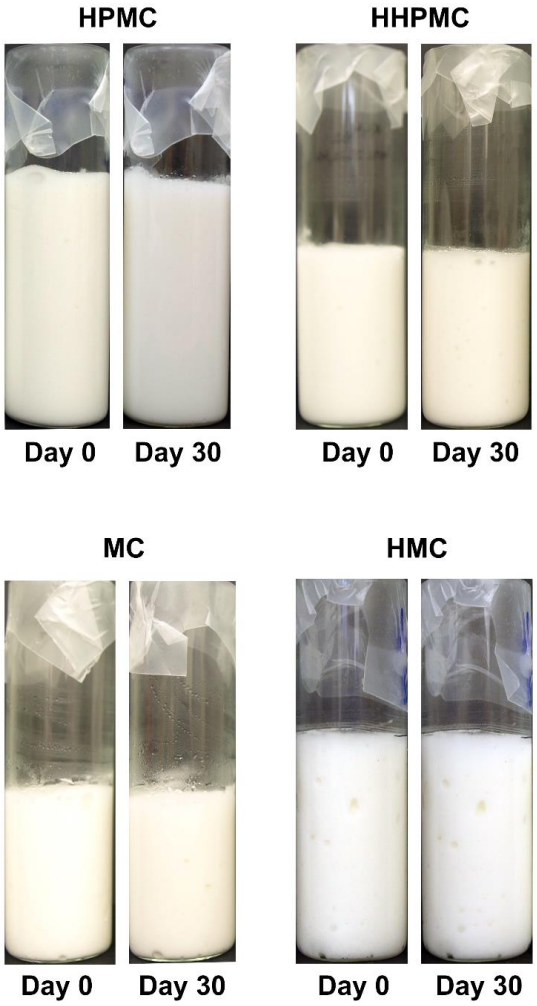

Fig. 1. Effect of emulsifier type and storage time on physical stability. HPMC: hydroxypropyl methylcellulose emulsion; HHPMC: high hydroxypropyl methylcellulose emulsion; MC: methylcellulose emulsion; HMC: high methylcellulose emulsion.

3.2. Oxidative stability

The peroxide value (PV) of the emulsions over time is shown in Fig. 2A. In general, a continuous rising trend in PV was found throughout the storage time. However, the increase in PV seemed to differ according to the cellulose ether used to stabilise the emulsion. Specifically, the PV increased during storage in the following order: HPMC < MC < HHPMC < HMC. Therefore, the HPMC emulsion seemed to be the most oxidative-stable of the emulsions, as the PV increased slightly but significantly ($P < 0.05$) between day 0 (5.4 meq kg^{-1}) and day 10 (6.6 meq kg^{-1}), then remained almost constant ($P > 0.05$). This could mean that a smaller fraction of lipids was susceptible to oxidation due to the good protection afforded by HMPC in this emulsion. In general, the oxidation stability provided by these cellulose ethers could be due to their adsorption ability on the o/w interface, acting as a physical barrier and thus separating the lipid substrates from the pro-oxidants present in the aqueous phase.[28] In addition, the amount of unadsorbed celluloses present in the continuous phase of the emulsions could enhance viscosity, resulting in slow diffusion of pro-oxidants and hence a decreased lipid oxidation rate, as observed by Khouryieh *et al.*[8] in whey protein-stabilised o/w emulsions with xanthan-locust bean gum mixtures. In this regard, as the HMC emulsion exhibited the highest viscosity (visual observations and textural results in section 3.3), it could be expected to be the most stable emulsion. However, the HMC emulsion exhibited a significant ($P < 0.05$) increase in PV during the storage time (from 5.0 meq kg^{-1} at day 0 to 11.5 meq kg^{-1} at day 30), so it was the least oxidative-stable emulsion. This could be because some air bubbles formed inside the gel (Fig. 1) during the preparation of the HMC emulsion. The presence of these bubbles, and thus the presence of oxygen, could have promoted the formation of hydroperoxides. Moreover, some authors have found a positive correlation between oil droplet size and lipid oxidation.[29–31] In this

regard, the higher PV in HMC emulsion than in the other ones could be associated with its larger oil droplet size (see fresh emulsions mean diameters in microstructure section). Nonetheless, other works have shown no effect of droplet size on lipid oxidation[32,33] or an inverse correlation between droplet diameter and lipid oxidation.[34,35] Therefore, no consistent results are found in literature and thus there is no a clear trend on the lipid oxidation and its relation to the particle size measurements.[31] In conclusion, the good protection against oxidation afforded by cellulose ethers in o/w emulsions could be mainly due to their ability to separate the lipid substrate from the pro-oxidants (physical barrier on the interface) and their high capacity to thicken the aqueous phase (high bulk viscosity), which would result in slow diffusion of pro-oxidants.

As a consequence of hydroperoxide degradation, secondary oxidation products such as conjugated triens, aldehydes and ketones are formed and can be measured using the specific extinction coefficient at 270 nm (K270) (Fig. 2B). High initial values could be due to the refined sunflower oil's containing oil refining products that also absorb at 270 nm. In general, all the emulsions showed a slight change in K270 values over the storage time. Therefore, few secondary oxidation products were expected to be formed. The HPMC emulsion exhibited a significant ($P < 0.05$) increase in the K270 coefficient, from 5.46 (day 0) to 6.45 (day 20). This could show that hydroperoxides formed during those 10 days degraded into few secondary oxidative products. Although the HMC emulsion exhibited a sharp increase in PV during storage, no significant ($P > 0.05$) changes were observed in its K270 values.

In conclusion, in general, cellulose ethers provide good oxidative stability for o/w emulsions.

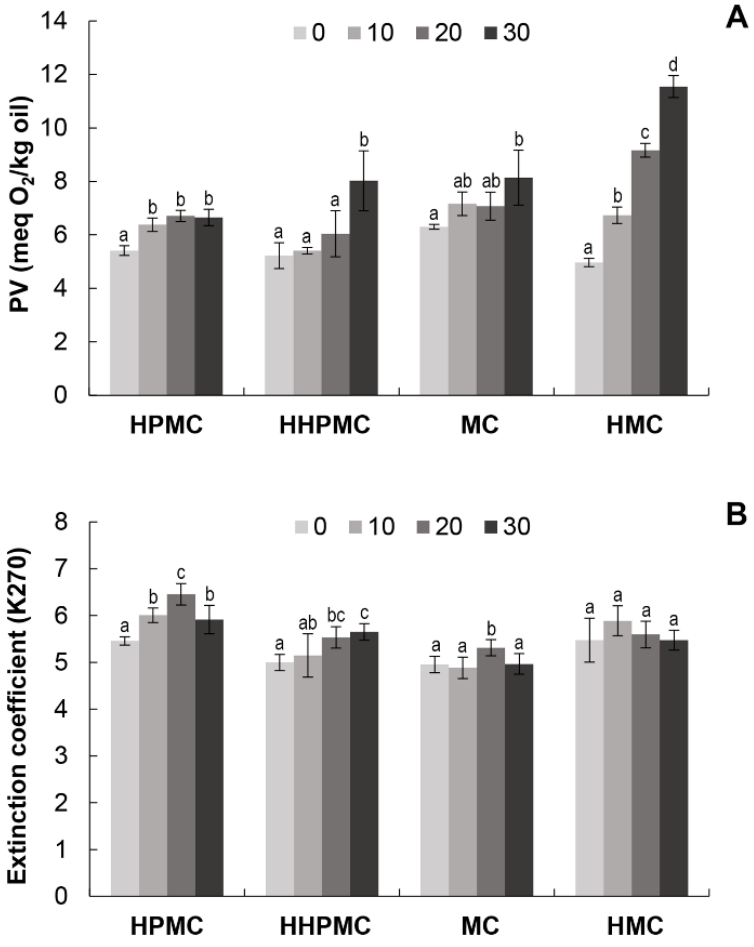

Fig. 2. Lipid oxidation rate of o/w emulsions stabilised with different cellulose ethers. **A)** Peroxide value (PV) and **B)** specific extinction coefficient at 270 nm (K270) during a storage period of 30 days. The error bars represent standard deviations. Different lowercase letters on the bars indicate significant differences ($P < 0.05$) during the storage period within each emulsion. 0; 10; 20 and 30 denote storage time in days. HPMC: hydroxypropyl methylcellulose emulsion; HHPMC: high hydroxypropyl methylcellulose emulsion; MC: methylcellulose emulsion; HMC: high methylcellulose emulsion.

3.3. Texture analysis

The area under the curve (AUC) was taken as representative of the extrusion force profiles in relation to time, indicating the consistency of the samples (Table 1). On the one hand, the results showed that all the samples behaved in the same way, as the AUC values of each cellulose ether emulsion decreased during the digestion phases as follows: fresh emulsion > after oral digestion > after gastric digestion. Specifically, the initial AUC values (fresh emulsions) exhibited a slight decrease after the oral phase but an accentuated decrease after the gastric phase. The reduction in AUC values for all the emulsions during the digestion phases was mainly due to the dilution effect of adding simulated oral and gastric fluids, because approximately the same results were obtained on carrying out the same test with water instead of simulated fluids (data not shown). Espert *et al.*[36] also observed that the decrease in force values in highly concentrated methylcellulose o/w emulsions should be attributed to water dilution rather than stomach conditions (acid pH and pepsin activity). Moreover, Espinal-Ruiz *et al.*[1] noted that the viscosity of all the emulsions they analysed (o/w emulsions stabilised by Tween-80 mixed with methylcellulose, chitosan or pectin) was relatively low under simulated gastric and intestinal conditions. They suggested that this could be attributed to the progressive dilution that occurs after passage through each stage of the gastrointestinal model. The emulsions stabilised with HPMC, HHPMC and MC exhibited similar AUC values in each phase, and therefore possessed a similar consistency. Although HPMC and MC emulsions were significantly different ($P < 0.05$) before digestion (fresh emulsions), they did not exhibit significant differences ($P > 0.05$) after the oral and gastric phases. The HMC emulsion showed significantly higher AUC values ($P < 0.05$) compared to the other emulsions in all the phases. Hence, the HMC emulsion

presented the highest resistance to extrusion, as it was the most consistent in all phases.

Table 1. Area under the curve (AUC) values (N s) of the cellulose ether emulsions before (fresh emulsion) and after oral and gastric *in vitro* digestion.

Sample	Fresh emulsion	Oral phase	Gastric phase
HPMC	24.69 ± 1.20 ^{a}	21.53 ± 0.99 ^{a}	5.03 ± 0.47 ^{ab}
HHPMC	28.01 ± 0.42 ^{ab}	17.44 ± 1.25 ^{b}	4.46 ± 0.83 ^{b}
MC	31.52 ± 2.68 ^{b}	23.82 ± 1.73 ^{a}	6.27 ± 0.29 ^{a}
HMC	69.79 ± 4.94 ^{c}	53.89 ± 1.27 ^{c}	21.31 ± 1.50 ^{c}

Values in parentheses are standard deviations. Different superscript letters in the same column denote values with statistically significant differences ($P < 0.05$) according to the LSD multiple range test.
HPMC: hydroxypropyl methylcellulose emulsion; HHPMC: high hydroxypropyl methylcellulose emulsion; MC: methylcellulose emulsion; HMC: high methylcellulose emulsion.

These results could offer an initial approach to weight management, because simply increasing the viscosity of foods and beverages increases subsequent satiety responses.[37] The intake of food or fluid distends the stomach and triggers mechanoreceptors and vagal afferents, which regulate satiation and satiety,[15] as the postprandial gastric volumes are linearly associated with perceptions of fullness and satiety.[38] As a consequence of larger gastric volumes, gastric emptying is delayed.[39] However, it must be taken into account that the intestine also plays a dominant role in satiation and satiety. The digestion and absorption of the nutrients influence gastrointestinal processes related with satiation and satiety. Therefore, the HMC emulsion, which exhibited the highest consistency at gastric level, may be expected to slow down gastric emptying and concurrently increase gastric distension to a

higher extent than the other cellulose emulsions and thus to increase fullness and satiety perceptions. Hence, this could be a good way to combat excess weight and obesity.

3.4. FFA release during *in vitro* digestion

The free fatty acids (FFA) released during digestion of the cellulose ether emulsions were compared with a CaCN emulsion in order to check the effectiveness of cellulose ethers in decreasing fat digestibility. Figure 3 shows the profiles of the FFA released from the different emulsions over the digestion time. As a general trend, there was a relatively rapid release of FFA during the first 10 min, after which the rate of lipid digestion decreased, reaching an almost constant value at the longer times. The slower rate of FFA release could be associated with an accumulation of lipolysis products at the droplet surface, which could compete with the lipase molecules for adsorption at the interface, reducing the lipase activity.[4,11] The CaCN emulsion had the highest digestion rate and extent of FFA release (approximately 50%). Conversely, the cellulose ether emulsions seemed to stabilise at 40 min and therefore showed an appreciable decrease in the extent of lipid digestion compared to the CaCN emulsion. The relatively slower initial digestion rate of the cellulose ether emulsions might have been due to the higher time taken for the surface-active components in the bile extract or lipase to adsorb to the droplet surfaces and displace the initial emulsifier layer.[24] Among the emulsions stabilised with different types of cellulose ethers, the HHPMC, HPMC and MC emulsions behaved in the same way.

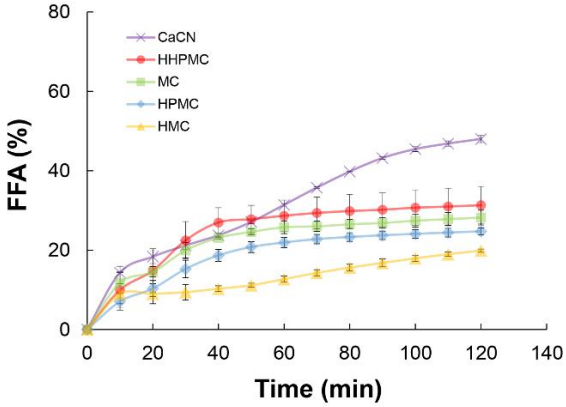

Fig. 3. Free fatty acid (FFA) released under simulated *in vitro* intestinal conditions from emulsions stabilised by protein (CaCN ×) and by different cellulose ethers (HHPMC •, MC ■, HPMC ♦ and HMC ▲). The error bars represent standard deviations.

They exhibited the same FFA release profile and small differences in the extent of lipid digestion (25–30% FFA released). This agrees with Pizones Ruiz-Henestrosa *et al.*,[16] who found that the amount of FFA released during the digestion of two emulsions stabilised by two different HPMCs (different molecular weight and hydrophobicity) was similar (45–50%). Moreover, in another study, all the emulsions stabilised with different types of HPMC had very similar digestion profiles regardless of their molecular weight or methoxyl content.[2] In contrast, in the present study the HMC emulsion was the least-digested one, exhibiting a very slow increase in FFA release and reaching approximately 20% of FFA released. The results obtained suggest that the lipase was able to access the emulsified lipid more readily in the CaCN-coated droplets than in the cellulose ether-coated droplets, with the HMC emulsion being the least accessible. Similarly, the lipid hydrolysis experiments of Mun *et al.*[4] suggested that the initial caseinate layer surrounding the droplets did not prevent the formation of free fatty acids in the emulsions. In addition, some

authors have found non-ionic surfactants (such as different celluloses ethers and polysorbate 20 (Tween20) emulsions) to be more resistant to lipid digestion than protein or other polysaccharide-stabilised emulsions.[1,4,11] Moreover, different types of HPMC-stabilised emulsions have been reported as being more resistant to lipid digestion than a Tween20 emulsion.[2]

The low release of FFA from cellulose ether emulsions in the present study might have been due to a number of possible reasons. Firstly, cellulose ethers may have been able to form interfaces that were more resistant to displacement by bile salts, which may make it difficult for lipase to access the interface required for lipid digestion.[2,24] Secondly, greater interactions between the cellulose and bile salts might take place, hindering the access of bile salts to the o/w interface.[2] Thirdly, the high consistency of cellulose emulsions at the end of the gastric phase (especially that of the HMC emulsion) may have been able to alter mass transport, inhibiting the ability of lipase to reach the lipid droplet surfaces.[1] Therefore, these results, together with those of several other researchers, demonstrate that lipase activity, and hence lipid digestion, could depend on the nature of the emulsifier, among other physicochemical factors.

The present results could offer a second approach to weight management: reducing lipid digestion and thus, possibly, lipid absorption. Several gastrointestinal processes affect satiation and satiety. They include gastric distension and gastric emptying, as previously mentioned, but also digestion and absorption, which are influenced by the physicochemical properties of the nutrients present in a meal.[40] When fat is emptied from the stomach into the small bowel, the presence of fatty acids is sensed by the small intestinal mucosa, which leads to secretion of gut peptides such as cholecystokinin (CCK) and peptide YY (PYY), the two important

satiety hormones.[12] In turn, these hormones lead to a delay in gastric emptying,[41] influencing hunger and food intake.[42] Therefore, although reducing lipid digestion in order to reduce lipid absorption could be a good strategy for combating chronic diseases associated with overweight and obesity, it is important to digest a relatively small part of the lipids in order to influence satiety as well and avoid possible digestive problems. In this regard, the possibility of controlling the structure and lipid digestion of novel emulsions in order to control the appetite (increasing the feeling of satiation and satiety, which might lead to lower total calorie consumption) and nutrient delivery is of considerable interest.[1,16]

3.5. Microstructure analysis

LM and CLSM were used to observe the initial microstructure of the cellulose and caseinate emulsions and follow the microstructural changes that took place during gastrointestinal digestion (Fig. 4 and 5A). The fresh emulsions had a heterogeneous distribution of oil droplets size (Fig. 5B). The mean diameter of the oil droplets was 9.4 ± 4.1 μm for CaCN emulsion, 10.3 ± 3.1 μm for HPMC emulsion, 9.2 ± 2.9 μm for HHPMC emulsion, and 10.5 ± 4.6 μm for MC emulsion. Therefore, these emulsions exhibited a similar mean droplet diameter. On the contrary, the HMC emulsion showed several large oval oil droplets (Fig. 4 and 5A) with a mean diameter of 16.4 ± 5.6 μm and a droplet size distribution with higher values than those of the other emulsions (Fig. 5B), as well as some air spaces among the oil droplets. These results were in accordance with the visual aspect observed in Fig. 1 (shown in section 3.1), where some holes could be observed in the HMC emulsion but none were visible in the other emulsions.

Although the consistency values decreased after the oral phase (see results section 3.3), no dilution effect in the cellulose ether emulsions was appreciable in the micrographs. This effect could be due to water holding capacity of the cellulose ethers. However, this effect was noticeable in the CaCN emulsion, where the oil droplets seemed to be more dispersed and the protein network formed by the CaCN could be clearly seen in purple.

After the gastric phase (Fig. 4), the purple-stained protein network of the CaCN emulsion disappeared, due to pepsin digestion, and several oil droplets therefore appeared flocculated, forming a large floc (around 267 μm). Mun *et al.*[4] also observed many clustered droplets rather than large individual droplets in a caseinate emulsion, indicating that it appeared to be more prone to droplet flocculation than coalescence. One the one hand, this fact could be due to the drop of the pH from the oral phase (pH 6.8) to the gastric phase (pH 2) that could destabilise CaCN, because CaCN reaches its isoelectric point (around pH 4.6) and could aggregate and precipitate. On the other hand, the proteolysis of the interfacial layer promotes the formation of oil droplet aggregates as it causes a gradual loss in the superficial charge of the droplets and reduces the thickness of the interfacial layer.[11] In the micrographs of the cellulose ether emulsions after the gastric phase (Fig. 4), the dilution effect was more visible than after the oral phase, the size of the oil droplets remained almost unchanged (Fig. 4) and flocculation mechanisms were absent. Bellesi *et al.*[11] found that after few minutes of gastric digestion, HPMC-coated droplets showed a slight change in particle size distribution, which remained almost constant for the rest of the gastric digestion time. The authors explained that this was because of the lower number of ionizable groups reported for the HPMC compared to the proteins (soy and whey proteins) and because the pepsin had no effect on fats and carbohydrates. Moreover, Gallier *et al.*[43] observed that a non-ionic

surfactant was not affected by the drop in pH in the stomach and thus Tween-oil emulsions remained stable under gastric conditions. The results corroborate the fact that cellulose ether emulsions are more resistant under gastric conditions than protein emulsions.

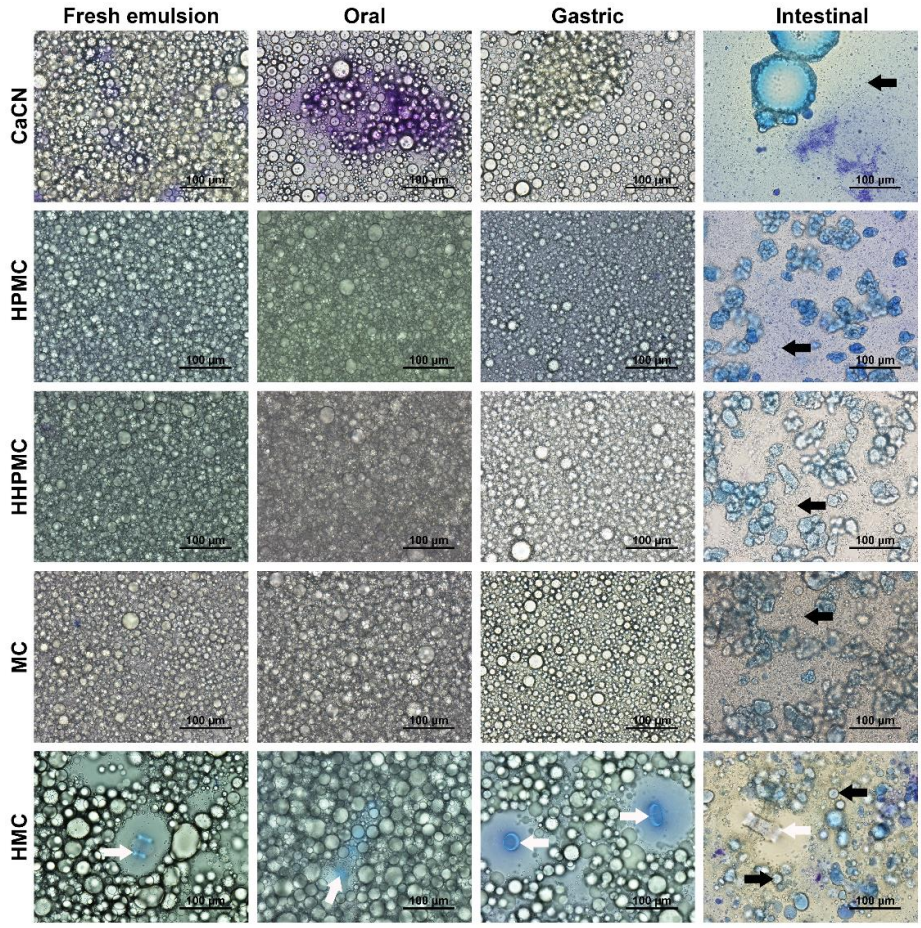

Fig. 4. Light microscopy micrographs of emulsions stabilised by different emulsifiers before (fresh emulsion) and after oral, gastric and intestinal digestion phases. Magnification 20x. The scale bars measure 100 µm. White and black arrows show cellulose ethers and oil droplets respectively. CaCN: calcium caseinate emulsion; HPMC: hydroxypropyl methylcellulose emulsion; HHPMC: high hydroxypropyl methylcellulose emulsion; MC: methylcellulose emulsion; HMC: high methylcellulose emulsion.

After the intestinal phase (Fig. 4 and 5A), the micrographs showed that large changes had occurred in all the emulsions, especially in the CaCN emulsion. Very small oil droplets (with a mean diameter of 2.2 ± 0.9 μm for CaCN emulsion, 3.1 ± 1.0 μm for HPMC emulsion, 2.7 ± 1.1 μm for HHPMC emulsion, 2.3 ± 0.6 μm for MC emulsion, and 4.5 ± 2.8 μm for HMC emulsion) and the formation of new kinds of aggregates were observed, which were very large (around 130–140 μm) in the case of the CaCN emulsion (Fig. 4). However, the HMC emulsion exhibited smaller changes (Fig. 4 and 5A), as several oil droplets with different sizes were still observed and aggregates did not seem to have been formed. The oil droplet size distribution of the HMC emulsion (Fig. 5B) showed a wide peak around 3–4 μm near the peaks of the other emulsions, but also another peak around 8 μm and a tail around 10–14 μm. These results matched those obtained for the percentage of FFA release. On the one hand, the CaCN emulsion was the one with highest values of FFA release, indicating that it was the most-digested sample, and this emulsion showed the smallest oil droplets (with a mean diameter of 2.2 ± 0.9 μm at the end of the intestinal phase). On the other hand, the HMC emulsion was the least-digested (it showed lower %FFA values) likely due to the largest oil droplets (with a mean diameter of 4.5 ± 2.8 μm) exhibited after *in vitro* gastrointestinal digestion. Bellesi *et al.*[11] observed that irrespective of the composition/structure of the emulsions, the initial surface area determined the initial rate of lipolysis. Therefore, the lower release of FFA from the HMC emulsion could be due to a wide variety of reasons. Firstly, its higher droplet size compared to the other cellulose ether emulsions. Hence, this emulsifier provided a smaller initial interfacial area for the lipase to attach to, with the possibility of hydrolysing lipids at a lower rate and to a smaller extent, as Torcello-Gómez and Foster[2] observed in different HPMC emulsions. In this regard, the inhibition of lipid digestion could be expected to increase as the flocs

size rose and as the packing of droplets and polymers within the flocs grew, since these factors would reduce the ability of lipase molecules to diffuse rapidly through the whole of the flocs.[1] Nevertheless, although the CaCN emulsion could present a smaller initial interfacial area due to the formation of flocs at the gastric phase (Fig. 4), it was the most-digested sample. Secondly, the highest bulk viscosity of the HMC emulsion, which implies a physical impediment for the lipase to reach the interface. Thirdly, the possible thermal gelation of the continuous aqueous phase at 37 °C,[17] which could make even more difficult the access of the enzyme to the substrate.

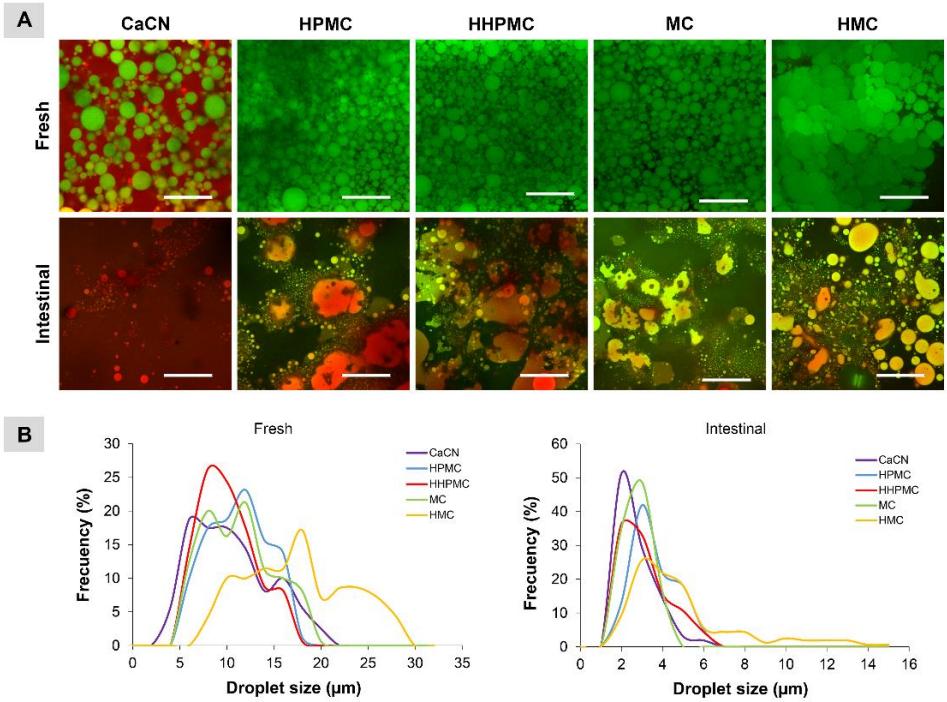

Fig. 5. A) Confocal micrographs of emulsions stabilised by different emulsifiers before (fresh emulsion) and after intestinal digestion phase. Magnification 60x. The scale bars measure 60 µm. **B)** Droplet size distribution of emulsions before (fresh) and after intestinal digestion phase. CaCN: calcium caseinate emulsion; HPMC: hydroxypropyl methylcellulose emulsion; HHPMC: high hydroxypropyl methylcellulose emulsion; MC: methylcellulose emulsion; HMC: high methylcellulose emulsion.

Pizones Ruiz-Henestrosa *et al*.[16] concluded that the difference in the rate and extent of lipolysis found in their results could mainly be attributed to the molecular events occurring at the interface upon bile salt adsorption, rather than to differences in the molecular weight/viscosity or the size/surface area available for the action of lipase/colipase. Hydrophobic interactions have been postulated to take place between cellulose ethers and bile salts[44] and both methyl and hydroxypropyl groups can bind or "sequester" bile salts.[16] Specifically, the hydrophobic faces of bile salt molecules adsorb to the hydrophobic portions of cellulose ethers.[45] In the case of methylcelluloses (which only have methyl group substituents), although bile salts would adsorb to the methyl groups, other methyl groups would be still available for hydrophobic association for cellulose molecule self-assembly.[45] In the case of hydroxypropyl methylcelluloses (HPMCs), the adsorption of bile salts onto the larger hydroxypropyl groups would hinder the hydrophobic association to a larger extent due to steric effects and because hydroxypropyl groups are more "difficult" to incorporate within ordered structures than methylcelluloses.[44] Therefore, in the case of HPMCs the lower methyl group content and the presence of more polar and larger hydroxypropyl groups that inhibit intermolecular association leads to the formation of a more untangled system than with MC and explains why HPMCs would be more affected by bile salts.[44,45] In this context, bile salts interacting with the hydrophobic groups of the cellulose backbone would impart a negative charge that would increase the repulsion between the cellulose molecules, thus decreasing their tendency to aggregate or self-assemble. As the self-assembly or aggregation tendency of HPMCs was more hindered by bile salts than that of the other cellulose ethers, as described above, this would provoke more untangling of the cellulose molecules at the interface, making more sites available for lipase adsorption and resulting in more extensive lipolysis.[16] Therefore, a thicker adsorbed interfacial layer

formed by cellulose ethers and/or an interfacial arrangement with more entanglements, which could be the case of HMC emulsion due to higher methyl substitution, and thus stronger hydrophobic interactions, could possibly be less susceptible to disruption by intestinal components (mainly bile salts as described above).[2] This could be why the HMC emulsion exhibited the lowest percentage of FFA release, besides its larger initial droplet size and its higher consistency. Consequently, according to the findings of the present study, the physical barrier effect of the cellulose ethers on the droplet interfaces, the increased viscosity in the continuous phase, the molecular events occurring at the interface as well as the droplet size could have a great impact on lipid digestibility.

4. Conclusions

This study has demonstrated that the use of cellulose ethers provided good physical and oxidative stability to o/w emulsions. This seemed to be due to the ability of the cellulose ethers to form a physical barrier on the interface, allowing the lipid substrate to be separated from the pro-oxidants, as well as to a thickened aqueous phase that would slow down the diffusion of these pro-oxidants.

Also, the use of these cellulose ethers with thermo-gelling ability, specifically HMC, made it possible to obtain o/w emulsions with high consistency even during gastric digestion, which could slow down gastric emptying and increase gastric distension, thus increasing fullness and satiety perceptions.

Moreover, this study has shown that cellulose ethers, in particular HMC, delay lipid digestion of o/w emulsions compared to a conventional food emulsifier (calcium caseinate).

Therefore, considering the results as a whole, it was concluded that controlling the structure of emulsions and their digestion behaviour could achieve emulsions that enhance satiating capacity and decrease lipid digestion, which could be used in weight management.

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How do different types of emulsifiers/stabilisers affect the *in vitro* intestinal digestion of o/w emulsions?

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Food Biophysics (en revisión)

Abstract

This study analyses the influence of different types of molecules (tween, lecithin, xanthan gum, and methylcellulose) on the physical properties (flow behaviour and particle size) and microstructure of oil-in-water (o/w) emulsions before and during *in vitro* intestinal digestion. The release of free fatty acids during a simulated intestinal stage has also been examined. The results show that various o/w emulsions present different rates and extents of lipolysis and that these differences are not primarily due to their rheological properties nor to the droplet size/surface area available for the action of lipase. Rather, the observed differences in the kinetics of lipolysis are most likely attributable to the nature and location of each type of molecule in their respective o/w emulsions as well as to their interactions with intestinal components. These results shed light on the mechanisms by which the interfacial layer controls lipid digestion, paving the way for a practical application of some of these emulsions in the production of foods used for regulating dietary lipid digestion in order to prevent and treat obesity and related disorders.

Keywords: tween, lecithin, xanthan gum, methylcellulose, microstructure, physical properties

1. Introduction

Although lipids are an essential component of our daily food intake, their overconsumption can lead to serious medical conditions. The challenge for the food industry thus lies in designing healthier foodstuffs, with a focus on reducing fat without compromising the organoleptic properties of the food. Unfortunately, low-fat foods do not generally meet consumers' sensorial expectations. In this context, the last decade has seen an upsurge in research on how food structure influences the digestion rates of macronutrients, with a major focus on lipid digestion (Singh & Sarkar, 2011).

Several previously published reviews provide a good overview of the biochemistry of human lipid digestion (Golding & Wooster, 2010; McClements, Decker, Park, & Weiss, 2008; Singh, Ye, & Horne, 2009). Briefly, fat digestion is an interfacial process dependent on the adsorption of lipolytic enzymes to the surface of emulsified fat droplets (Golding & Wooster, 2010). Foods containing lipids may either be ingested as oil-in-water emulsions or they may be converted into oil-in-water emulsions in the mouth, stomach, and/or small intestine through the mechanical stresses they experience during digestion. This process is also influenced by the presence of various endogenous and dietary surface-active agents (Mun, Kim, McClements, Kim, & Choi, 2017), which ensure an interface for lipase adsorption (Norton, Fryer, & Norton, 2013). In healthy humans, gastric lipase leads to the hydrolysis of between 10-30% of ingested triacylglycerides (TGs), monoacylglycerols (MGs), and free fatty acids (FFAs) (Singh et al., 2009), with the remainder (70-90%) being digested in the small intestine. The activity of pancreatic lipase primarily depends on the presence of colipase and bile salts (Hur, Lim, Decker, & McClements, 2011). Bile salts are surface-active molecules that adsorb

onto the oil-water interface and displace any existing emulsifier molecules, thus aiding lipase adsorption. Colipase binds to the bile salts' rich interface area and facilitates the activation of pancreatic lipase, which forms a complex with colipase (Gallier & Singh, 2012). In the aqueous phase, bile salts, together with endogenous phospholipids, also form mixed micelles and vesicles, which are capable of incorporating lipid digestion products and removing them from the lipid droplet surfaces (Hur et al., 2011).

In general, the human body has an excess capacity for fat digestion. This, combined with the fact that lipolysis is an interfacial-mediated process, means that the rate of digestion is controlled by the ability of lipase to bind to emulsion interfaces, a process which is mainly controlled by droplet size and interfacial characteristics (Golding & Wooster, 2010). The rate of lipid hydrolysis in the stomach and small intestine should actually increase as the mean lipid droplet size decreases, as this provides a larger surface area of exposed lipid to which gastric and pancreatic lipases can adhere (McClements et al., 2008). However, the rate of hydrolysis is also influenced by the interfacial characteristics (e.g. initial composition, thickness, and charge) surrounding the lipid droplets. These characteristics are, in turn, determined by the type of ingredients used to produce the food emulsion (McClements et al., 2008). For example, the rate of lipid digestion decreases in the presence of a sufficiently high concentration of highly surface-active molecules that adhere to the lipid droplet surface, thus blocking the adsorption of lipase and inhibiting its enzymatic activity (Hur, Decker, & McClements, 2009). Moreover, different hydrocolloids may influence the digestion of emulsified lipids by means of various mechanisms that may either inhibit or enhance the process by (i) binding to various intestinal components (e.g. bile salts, calcium ions, fatty acids, and lipase), (ii) altering the aggregation state of the oil droplets, (iii) forming protective coatings

around lipid droplets, or (iv) increasing the macroscopic viscosity of gastrointestinal fluids (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014; Qin, Yang, Gao, Yao, & McClements, 2016; Qiu, Zhao, Decker, & McClements, 2015). The choice of particle size and emulsifier are thus major factors to take into account when designing food to reduce or increase lipid digestion (Gallier & Singh, 2012). However, despite the fact that the initial size and interfacial composition of lipid droplets can often be controlled during the food manufacturing process by selecting the appropriate emulsifier type and homogenisation conditions, these properties normally undergo changes after ingestion due to a number of physicochemical processes that occur in the human gastrointestinal (GI) tract (McClements et al., 2008).

In the formulation of emulsion systems, a distinction is generally made between the emulsifier and the stabiliser (Li & Nie, 2016). Emulsifiers are surface-active molecules that lower the surface tension and prevent droplet flocculation through adsorption onto the droplet surfaces (Traynor, Burke, Frías, Gaston, & Barry-Ryan, 2013), while stabilisers confer long-term stability to emulsions via viscosity enhancement of the continuous phase. Polysaccharides are not considered to be classic emulsifiers, but fit into the stabiliser category due to their hydrophilicity, high molecular weight, and gelation behaviour (Li & Nie, 2016), all of which lead to the formation of a macromolecular barrier by increasing the viscosity of the aqueous phase and slowing down coalescence between dispersed droplets (Papalamprou, Makri, Kiosseoglou, & Doxastakis, 2005). It is worth noting that a limited number of polysaccharides can also act as emulsifying agents (Dickinson, 2018), including gum arabic, modified starches and celluloses, several types of pectin, and some galactomannans (Dickinson, 2009).

The present study focuses on tween (non-ionic) and lecithin (zwitterionic) because they are good emulsifying agents commonly used for the stabilisation of oil-in-water emulsions. Xanthan gum has also been included as it is a stabilising agent with the ability to form a weak gel-like structure in solution (Papalamprou et al., 2005). Rounding out the list is methylcellulose, a surface-active polymeric compound that can act as both an emulsifying and a stabilising agent (Karlberg, Thuresson, & Lindman, 2005).

Recent studies have suggested that lipid digestion may potentially be influenced by the following factors: (i) oil droplet size, which influences the surface area available for lipase adsorption, (ii) macroscopic viscosity of the systems, which affects mass transport, and (iii) the nature of the emulsifier/stabiliser used to form emulsions, which influences interfacial phenomena. This begs the question of what happens when several of these factors are present; does any one of them have more influence than the others on lipid digestion?

The aim of this study is to gain a better understanding of the mechanisms by which different molecules (tween, lecithin, xanthan gum, and methylcellulose) influence *in vitro* intestinal digestion of o/w emulsions. Specifically, we have tried to answer the following questions: (i) what factors lead to the differences observed in lipid digestion results, if any, when o/w emulsions are stabilised by different molecules (tween, lecithin, xanthan gum or methylcellulose)? (ii) does a single factor alone govern lipid digestion results? and (iii) what factor(s) exert(s) the greatest influence on lipid digestion?

2. Materials and methods

2.1. Materials

Sunflower oil (Koipe Sol, Deoleo SA, Burgos, Spain) and mineral water (Bezoya, Calidad Pascual SAU, Burgos, Spain) were purchased from a local market. Tween® 20 or polyoxyethylene sorbitan monolaurate (P1379) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lecithin (Emultop™) and xanthan gum (Satiaxane CX™ 911) were purchased from Cargill (Minnetonka, MN, USA). Methylcellulose (METHOCEL™ A4M; 30% methoxyl) was kindly supplied by The Dow Chemical Company (Bomlitz, Germany).

Lipase from porcine pancreas Type II (L3126), bile extract (B8631), sodium phosphate (monobasic, monohydrate), nile red (72485), calcofluor white (18909), and agarose type IX (A5030) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-Fluorescein isothiocyanate (FITC) (15670) was purchased from Electron Microscopy Science (Hatfield, PA, USA). Calcium chloride, sodium chloride, sodium hydroxide, sodium phosphate (dibasic, dodecahydrate), and toluidine blue O (C.I. 52040) were purchased from Panreac Química (Barcelona, Spain).

2.2. Emulsion preparation

Tween, lecithin, or methylcellulose (2 g/100 g emulsion) was dispersed in the oil phase (10 g/100 g emulsion under magnetic stirring at room temperature for 30 min. The lecithin oil phase solution was heated at 35 °C to ensure proper dispersion (Mezdour, Lepine, Erazo-Majewicz, Ducept, & Michon, 2008). Xanthan gum (2% w/w) was dispersed in the water phase (88 g/100 g emulsion) under magnetic stirring at room temperature for 30 min. Then, both oil and aqueous phases were

emulsified using a high speed homogenizer (Ultraturrax T18, IKA, Germany) by drop-wise addition of the aqueous phase (at room temperature for tween and lecithin emulsions and at 4-5 °C for methylcellulose emulsion) into the oil phase or vice-versa (for xanthan emulsion) under stirring at 6400 rpm. Homogenisation was continued at 14 000 rpm for 1 min and subsequently at 18 000 rpm for another 1 min. The resulting high viscosity of both polysaccharide emulsions inhibited the disruption of the dispersed oil phase into sufficiently small droplets. Consequently, the xanthan (X) and methylcellulose (MC) emulsions were further homogenized at 20 000 rpm for another 1 min in order to obtain emulsions with an oil droplet size (CLSM visualisation) similar to that in the tween (T) and lecithin (L) emulsions.

2.3. *In vitro* digestion model and mathematical modelling of digestion profiles

An *in vitro* digestion model that simulated the small intestine was used, following a slightly modified version of Li, Hu, & McClements' (2011) method. The samples (5 g) were mixed with phosphate buffer at a 1:3 ratio. The mixture was incubated at 37 °C for 10 min with continuous stirring at 760 rpm (pre-digestion step). Then, 5 mL of bile extract solution (275 mg of bile extract dissolved in phosphate buffer, pH 7.0) and 1 mL of electrolyte solution (30.5 mg of $CaCl_2$ and 244.1 mg of NaCl dissolved in phosphate buffer, pH 7.0) were added to the samples and the mixture was adjusted to pH 7.0 (at this step, the withdrawn aliquot corresponds to t = 0 min). Afterwards, 1.5 mL of freshly prepared lipase suspension (200 mg lipase powder dispersed in phosphate buffer; 1:2.5 enzyme/substrate ratio) was added to the above mixture and the titration started. The mixture was maintained at 37 °C and 760 rpm for 2 h to mimic conditions in the small intestine

(0.1 mL was withdrawn at different times of the small intestine stage: t = 30, 60, and 120 min). The final composition of the sample in the reaction cell was 500 mg lipid, 10 mg/mL bile extract, 19 mg/mL lipase, 10 mM $CaCl_2$, and 150 mM NaCl. A pH-stat automatic titration unit (Mettler-Toledo DL 50, Greinfensee, Switzerland) was used to automatically monitor the pH and maintain it at pH 7.0 by titrating appropriate amounts (mL) of NaOH solution (0.5 M). The volume of NaOH added to the sample was recorded and used to calculate the concentration of free fatty acids (FFAs) generated by lipolysis, that is, the number of moles of NaOH required to neutralise the FFAs (assuming 2 FFAs produced per 1 triacylglyrecol molecule). The measurement was carried out in triplicate.

A mathematical model was developed by Li & McClements (2010) to describe the %FFA *versus* time profiles obtained by the pH-stat method. The percentage of free fatty acid release (Φ) as a function of digestion time (t) measured by the pH-stat method is characterized by the following equation (1):

$$\Phi = \phi_{max}\left(1 - \left(1 + \frac{3kMt}{2d_0\rho_0}\right)^{-2}\right) \quad (1)$$

Here, Φ_{max} provides a measure of the total *extent* of digestion (*i.e.* the maximum percentage of FFAs released at the end of lipid digestion), k provides a measure of the *rate* of digestion (*i.e.* mols of FFA released per unit of droplet surface area per unit of time (μmol s^{-1} m^{-2})), d_0 is the initial droplet mean diameter (m), ρ_0 is the density of the oil (≈ 913 kg m^{-3}), and M is the molecular weight of the triglyceride oil (≈ 0.830 kg mol^{-1}). By fitting this model to the experimental data it is possible to extract one parameter related to the rate of digestion (k) and another that is related to the extent of digestion (Φ_{max}). Hence, these two parameters were calculated by minimizing the difference between the data and the model. In this study, the solver

tool included in the Microsoft Office excel 2016 software package was used to determine the values of Φ_{max} and k.

2.4. Microstructure

2.4.1. Confocal laser scanning microscopy (CLSM)

Prior to making the emulsion samples, nile red fluorescent dye was dispersed into sunflower oil at a concentration of 0.3 mM. Twenty μl of the samples (fresh emulsions and aliquots withdrawn at the end of each digestion step: pre-digestion, t = 0, 30, 60, 120 min) were placed on the central microscope slide and the cover slide was carefully positioned to exclude air pockets. A Nikon confocal microscope C1 unit fitted onto a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) was used. A Helium-Neon laser line (543 nm) was employed as the light source to excite nile red fluorescent dye and the emitted light was collected at 570 nm. The images were obtained and stored at 1024 x 1024 pixels using the microscope software (EZ-C1, version 3.40, Nikon, Tokyo, Japan).

2.4.2. Bright-field microscopy

Each fresh emulsion and the corresponding samples at t = 0 min of lipid digestion were mixed with 3% (w/v) gelling agarose (kept at 30 °C) at a ratio 1:4 in a glass petri dish (diameter: 40 mm). After cooling (24 h at 4 °C), the gelled samples were cut into blocks (length: 3 mm; height: 2 mm; depth: 1 mm) and fixation, staining, dehydration, and embedding was carried out following the procedure used by Hernández-Carrión, Hernando, Sotelo-Díaz, Quintanilla-Carvajal, & Quiles (2015). Sections (1.0 μm-thick) were prepared using an ultramicrotome (Leica

Mycrosystems®, Wetzlar, Germany) and stained with 1% toluidine blue. The samples were examined with the aid of a Nikon Eclipse 80i® light microscope (Nikon, Tokyo, Japan) and the images were captured and stored at 1280 x 1024 pixels using the microscope software (NIS-Elements F, Version 4.2, Nikon, Tokyo, Japan).

2.4.3. Fluorescence microscopy

For methylcellulose (MC) and xanthan (X) emulsions, the sections obtained were also stained with specific fluorophores, calcofluor (0.1 %) and FITC (0.1%) respectively. The dyes were detected using a mercury arc lamp as the light source. The excitation and emission filters for calcofluor were 370/36-25 nm and 440/40-25 nm, while for FITC they were 482/35 nm and 536/40 nm. The samples were examined with the aid of a Nikon Eclipse 80i® light microscope (Nikon, Tokyo, Japan) and the images were captured and stored at 1280 x 1024 pixels using the microscope software (NIS-Elements F, version 4.2, Nikon, Tokyo, Japan).

2.5. Particle size measurement

Particle size distribution was determined by means of laser light scattering (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). The instrument was set up as follows: refractive index of sunflower oil: 1.47, refractive index of the dispersant (water): 1.33, assumed absorbance of sunflower oil: 0.01. Measurements were performed in duplicate.

2.6. Rheological measurement

Rheological measurements were carried out with a dynamic shear rheometer (Kinexus Pro+ Rheometer, Malvern Instruments Ltd, Worcestershire, UK) using a parallel-plate geometry (diameter: 35 mm, plate gap: 1 mm). The samples were loaded onto the rheometer measurement plate and allowed to equilibrate at 25 °C (for fresh samples) or 37 °C (for withdrawn aliquots at t = 0 min of the lipid digestion) for 5 min before the beginning all experiments. Flow curves were obtained recording the shear stress values when shearing the samples with a linearly increasing shear rate from 0.1 to 100 s^{-1} for a period of 150 s. The measurements were performed in triplicate. Flow behaviour of emulsion samples was described by fitting the experimentally measured shear stress-shear rate data to Herschel-Bulkley model (equation 2).

$$\sigma = \sigma_0 + K \cdot \dot{\gamma}^n \quad (2)$$

Where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency index (Pa s^n), and n is the flow behaviour index (dimensionless). These parameters were calculated by minimizing the difference between the experimental data and the model using the solver tool of Microsoft Office Excel 2016.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was applied to study the differences between formulations in all the experiments; for this purpose, XLSTAT statistical software (Addinsoft, New York, NY, USA) was used. The least significant differences (LSD) were calculated with Fisher's test ($P < 0.05$).

3. Results and discussion

3.1. Effect of droplet size on lipid digestion

First, the influence of oil droplet size in emulsions stabilised by a specific type of emulsifier (tween® 20) on lipid digestion was studied. For this purpose, two emulsions were prepared: an initial coarse emulsion (T), which was then further sonicated (40% of amplitude during 3 min, Vibra-Cell 100 W, Sonics & Materials, Inc., USA) to obtain a fine emulsion (T-US). Fig. 1A shows CLSM images at t = 0 min of lipid digestion while Fig. 1B shows the curves of free fatty acid (FFA) release during lipid digestion from both the T and T-US emulsions. The fine emulsion (T-US) exhibited smaller droplets at t = 0 min (surface mean diameter ($D_{3,2}$) ≈ 0.32 μm; specific surface area (SSA) ≈ 18.75 μm^2) than the coarse emulsion T ($D_{3,2}$ ≈ 3.98 μm; SSA ≈ 1.51 μm^2) (Fig. 1A), indicating that the former has a larger surface area for the binding of pancreatic lipase. Consequently, the differences observed in the digestion of the two emulsions were significant ($P < 0.05$), with the fine emulsion (T-US) being digested at a greater rate and to a greater extent than the coarse emulsion (T) (Fig. 1B).

We can thus conclude that when two emulsions differ only in oil droplet size, this factor significantly influences lipid digestion. Several previously published studies (Li et al., 2011; Li & McClements, 2010; McClements & Li, 2010; Qiu et al., 2015; Sarkar, Ye, & Singh, 2016; Torcello-Gómez & Foster, 2016) also reported the influence of lipid droplet size on the rate and extent of lipid digestion when working with different emulsions. However, when emulsions also differ with regard to the type of emulsifier or stabiliser used and/or their rheological properties, does the particle size remain the sole factor affecting the lipolysis results? What is the role of other factors; how do they influence this process and to what extent?

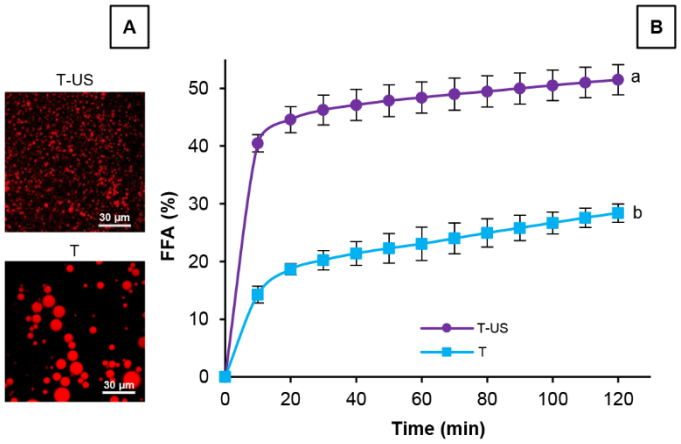

Fig. 1. Confocal laser scanning microscopy (CLSM) images at t = 0 min of lipid digestion (after the addition of bile extract and electrolytes solutions but before lipase addition) (A) and free fatty acid (FFA) release under *in vitro* small intestinal conditions (B) from T and T-US emulsions. T: tween emulsion; T-US: sonicated tween emulsion. The error bars represent standard deviations. Different lowercase letters (a; b) indicate significant differences ($P < 0.05$) at t = 120 min.

3.2. Influence of molecule type on lipase activity in o/w emulsions

In order to study the influence of molecule type on the rate and extent of lipid digestion, four emulsions with similar droplet size were prepared using different emulsifiers/stabilisers: tween, lecithin, xanthan gum, and methylcellulose. All the emulsions exhibited similar profiles: there was an initial rapid release of FFAs during the first 10-20 min, followed by a more gradual increase over a longer period of time until a relatively constant final value was attained (Fig. 2). The fact that a fairly constant amount of fatty acids had been released from the emulsions after a given time, even despite the fact that not all of the lipids had been digested, may be

due to the inhibition of lipase activity by the released fatty acids themselves (Mun, Decker, & McClements, 2007).

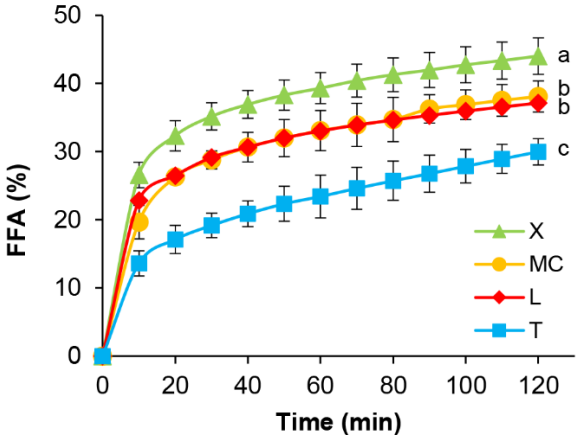

Fig. 2. Influence of molecule type (tween, lecithin, xanthan gum, and methylcellulose) on free fatty acid (FFA) release under *in vitro* small intestinal conditions from o/w emulsions. T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion. The error bars represent standard deviations. Different lowercase letters (a; b; c) indicate significant differences ($P < 0.05$) at t = 120 min.

Because these acids are surface-active molecules, they tend to adsorb to oil droplet surfaces (Mun et al., 2007). At a sufficiently high concentration, they can displace lipase from the oil-water interface, thus preventing it from coming into close contact with the emulsified lipids (Mun et al., 2007).

The amount of FFAs released during the digestion process over time (Fig. 2) was fitted with the mathematical model (Eq. (1)) in order to describe the kinetics of the FFA release (rate (k) and extent (Φ_{max}) parameters) and to obtain a clearer comparison between the samples (Table 1). The results showed that emulsions

stabilised with different molecules exhibited different rates and extents of lipid digestion (Fig. 2 and Table 1).

Table 1. Parameters describing the rate (k) and extent (Φ_{max}) of lipid digestion in o/w emulsions.

Sample	k (μmol s^{-1} m^{-2})	Φ_{max} (%)	R^2
T	6.77	29.02	0.999
L	10.72	35.42	0.999
X	23.59	42.84	0.999
MC	10.23	37.77	0.999

T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion

Emulsion T exhibited the lowest rate and smallest extent of lipid digestion, whereas emulsion X presented the highest levels. Previous studies have reported that surfactants that adsorb more strongly to the droplet surfaces are more effective at inhibiting lipid digestion, presumably because they inhibit the binding of bile salts and lipase (McClements, 2018). Thus, in agreement with the results reported by McClements (2018), the tween molecules in our study may have inhibited lipid digestion through a number of mechanisms: (i) inhibition of lipase adsorption to the lipid droplet surfaces; (ii) binding to and denaturation of the lipase molecules; and (iii) alterations in the aggregation state of the lipid droplets. Conversely, polysaccharide coatings are not very effective barriers for preventing lipase action (Pilosof, 2017). This may explain why emulsion X was significantly ($P < 0.05$) the most-digested sample (Fig. 2). Still, not only can polysaccharides stabilise or destabilise emulsions, thus changing the available surface area for lipase action, they can also bind biosurfactants to calcium, which is necessary for the lipolysis reaction,

thereby affecting the rate and extent of lipolysis (Pilosof, 2017). Sequestration of calcium ions and/or bile salts can inhibit lipid digestion since they play an important role in removing lipolysis products from lipid droplet surfaces, which enables the continuation of lipid digestion (Qin et al., 2016; Sarkar et al., 2016). However, as our results showed emulsion X to be the most-digested sample, it is unlikely that this polysaccharide interacts strongly with intestinal components. Emulsions L and MC presented similar ($P > 0.05$) rates and extents of lipid digestion (Fig. 2 and Table 1), in between those observed for emulsions X and T.

All the emulsions were initially prepared with oil droplets of similar size in order to minimise the influence of the interfacial area available for lipase adsorption on lipid digestion. Nevertheless, it must be noted that even more important than the initial size of the lipid droplets is their size in the small intestine (McClements, 2018). Therefore, the different rates and extents of lipid digestion obtained in the emulsions may be related to other factors, including (i) structural changes, (ii) different rheological properties before lipase addition, or (iii) different grade of interaction between various intestinal components (e.g. bile salts, lipase molecules, calcium, lipolysis products) and the different molecules.

3.3. Influence of molecule type on the microstructure and particle size of o/w emulsions during *in vitro* intestinal digestion

The confocal microscopy images (Fig. 3A-D) showed that all the fresh emulsions presented either small and large fat droplets, which were homogeneously distributed throughout the sample in emulsions T and L (Fig. 3A and B) but heterogeneously distributed in emulsions X and MC (Fig. 3C and D). The lower fat droplet density observed in emulsions X and MC (Fig. 3C and D) could be due to

the polysaccharides forming an extended network in the continuous phase, thereby increasing emulsion viscosity and making it difficult to visualise most of the oil droplets in the same plane. Accordingly, the particle size distribution results indicated that fresh T and L emulsions (Fig. 4A) exhibited a primarily bimodal distribution, with a first peak around 5 μm and a second peak around 25 μm. In contrast, fresh X and MC emulsions exhibited a mainly monomodal distribution, with a peak around 25 μm, but fat droplets with smaller size were also detected around 1-5 μm. In general, the fresh emulsions contained similarly sized droplets (around 25 μm).

After the pre-digestion phase (samples diluted with phosphate buffer and stirred for 10 min at 37 ºC) (Fig. 3E-H), the main change observed was due to the dilution effect, with emulsions T and L (Fig. 3E and F) presenting lower fat droplet density than in their fresh emulsion forms (Fig. 3A and B).

After the addition of bile extract and electrolyte solutions (t = 0 min) (Fig. 3I-L), emulsions T and MC (Fig. 3I and L) remained almost unchanged; however, emulsions L and X (Fig. 3J and K) presented flocculation and coalescence phenomena, respectively. This suggests that changes in oil droplet size likely occur when these emulsions are exposed to intestinal conditions (Fig. 4B). Li & McClements (2010) and Chang & McClements (2016) obtained similar results with different o/w emulsions, observing that the droplets flocculated or coalesced when simulated small intestinal fluid was added. Another study concluded that the size of the oil droplets reaching the small intestine may vary widely, depending on the nature of the ingested food and the physicochemical processes occurring within the gastrointestinal tract, e.g., droplet digestion, flocculation, coalescence, and disruption (Li et al., 2011).

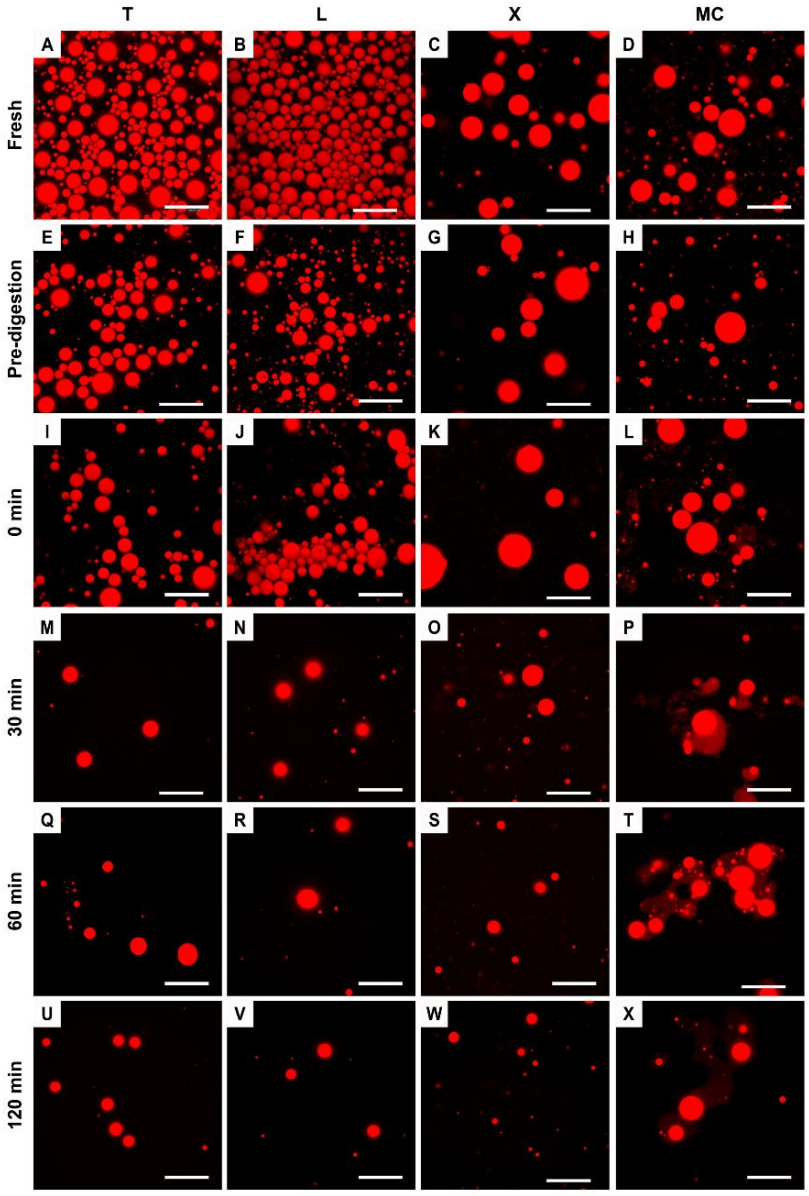

Fig. 3. Confocal laser scanning microscopy (CLSM) images from the different o/w emulsions during *in vitro* small intestine digestion. Pre-digestion: samples diluted with phosphate buffer at 37 ºC for 10 min; 0 min: after the addition of bile extract and electrolyte solutions (before lipase addition); 30 – 120 min: lipase digestion. T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion. The scale bars measure 30 μm.

In our study, the particle size distribution from emulsions T and L shifted from bimodal (Fig. 4A) to monomodal, with a wide peak between ~1.5 and 50 μm (Fig. 4B). Moreover, the main peak from emulsion X became larger (around 50 μm) due to coalescence phenomenon, as can be seen in Fig. 3K, although small droplets (around 3-10 μm) were also observed. The particle size distribution observed in the fresh MC emulsion remained almost unchanged at t = 0 min. On the one hand, the changes observed in the particle size distribution may be due to the presence of bile salts and mineral ions. For example, bile salts may have fully or partially displaced the original emulsifier molecules from the lipid droplet surface, with cationic sodium and calcium ions subsequently promoting droplet flocculation through charge neutralisation and bridging effects (Li & McClements, 2010). On the other hand, xanthan gum is a known depletion flocculant (Dickinson, 2009; Papalamprou et al., 2005), which makes any subsequent coalescence more likely.

After 30 min of lipase digestion, all the emulsions (Fig. 3M-P) exhibited an important decrease in the number of fat droplets present. This can be mainly attributed to the digestion of the lipid droplets by lipase. Moreover, some fat droplets seemed to decrease in size, mainly in emulsion X (Fig. 3O). It is worth noting that between 30 and 120 min (Fig. 3Q-X), no important changes were observed, just a decrease in the size of the fat globules due to the progression of lipid digestion. This suggests that the main structural changes take place during the first 30 minutes.

In summary, after the addition of bile extract and electrolyte solutions, the emulsions underwent physicochemical changes that gave rise to differently-sized oil droplets before the addition of lipase molecules.

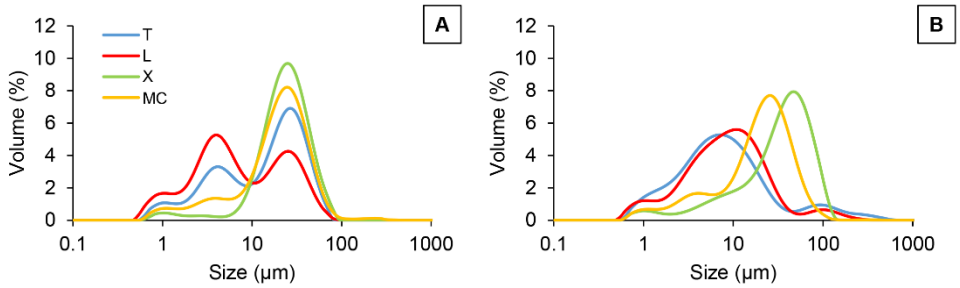

Fig. 4. Droplet size distribution from fresh o/w emulsions (A) and before lipase addition (at t = 0 min of the intestinal digestion) (B). T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion.

Therefore, and in accordance with the possible impact of droplet size on lipid digestion results, emulsions T and L should have been the most digested samples (because they contained the smallest oil droplets before lipase addition), whereas emulsion X should have been the least digested sample (as it had the largest oil droplets before lipase addition). However, our results showed that the particle size measured at t = 0 min of lipid digestion was not, in fact, inversely correlated to the %FFA released. In their study, Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof (2016) found that the maximum %FFA released from the emulsions they examined (β-lactoglobulin, soy protein isolate, and hydroxypropyl methylcellulose oil/water emulsions) likewise showed no correlation to the initial specific surface area (SSA) of the emulsions, nor to the values of the SSA at the end of gastric digestion. These findings indicate that the structural characteristics of the interfacial films may be a key factor in explaining the differences observed in FFA release. We thus hypothesized that factors other than oil droplet size may have a greater impact on the rate and extent of lipid digestion, as described in section 3.1.

3.4. Influence of molecule type on rheological properties of o/w emulsions

Fig. 5 shows the flow behaviour curves obtained from the fresh emulsions (Fig. 5A) and after the addition of bile extract and electrolyte solutions corresponding to t = 0 min of lipid digestion (Fig. 5B). The rheograms from the fresh emulsions (Fig. 5A) indicate that various types of molecules influence the rheological properties, thus varying the flow behaviour of o/w emulsions. The emulsions stabilised by tween and lecithin molecules (emulsions T and L) exhibited a Bingham plastic behaviour, whereas emulsions X and MC exhibited a shear-thinning behaviour with and without a yield stress, respectively. After the addition of bile extract and electrolyte solutions (Fig. 5B), emulsions T and L remained almost unchanged. In contrast, emulsion X exhibited the same flow behaviour, but with sharply decreased shear stress values, while emulsion MC became a Bingham plastic fluid with very low shear stress values.

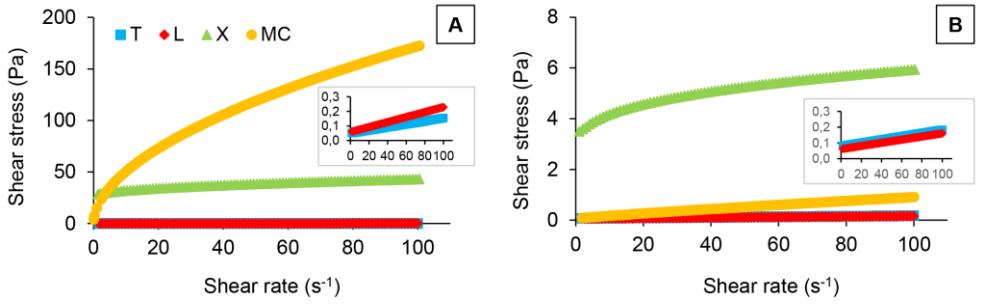

Fig. 5. Flow behaviour from fresh o/w emulsions (A) and before lipase addition (at t = 0 min of the intestinal digestion) (B). T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion.

The flow behaviour index (n), yield stress (σ_0), consistency index (K), and viscosity at 100 s^{-1} (η_{100}) values for the fresh emulsions and at t = 0 min of lipid digestion were obtained after fitting the flow curves to the equation of the Herschel-Bulkley model, which includes the Bingham model (n = 1 and K = η) and the power law model ($\sigma_0 = 0$) (Table 2). As can be observed in Fig. 5A, fresh T and L emulsions exhibited a Bingham behaviour (n = 1; σ_0 = 48.5 and 58.6 mPa respectively) and presented the lowest viscosity values, with no significant differences between them ($P > 0.05$). At t = 0 min, the rheological parameters from fresh T and L emulsions remained almost unchanged. The fresh MC emulsion exhibited a shear-thinning behaviour (n < 1; σ_0 = 0 mPa) and presented significantly higher consistency index and viscosity values than the other emulsions ($P < 0.05$). After the addition of bile extract and electrolyte solutions (at t = 0 min of lipid digestion), emulsion MC became a Bingham plastic fluid (n = 1; σ_0 = 98 mPa) and its viscosity exhibited a sharp decrease becoming significantly lower than the one of emulsion X ($P < 0.05$).

The decreases observed in the consistency index and viscosity values from all the emulsions could be attributed to the different temperatures used for the rheological analyses (25 °C for fresh emulsions and 37 °C for t = 0 min of lipid digestion samples). However, the decreases in rheological parameter values from emulsions X and MC could be mainly attributed to the dilution effect due to the addition of phosphate buffer solution in the pre-digestion step and then subsequent addition of bile extract and electrolyte solutions at t = 0 min, as water is the main ingredient in all of these solutions. In contrast, because fresh T and L emulsions exhibited low viscosity values, the addition of the aforementioned solutions would have a lesser effect on their rheological characteristics.

Table 2. Rheological parameters obtained from the Herschel-Bulkley model and the viscosity at 100 s^{-1} (η_{100}) for o/w emulsions.

Sample	Fresh					t = 0 min				
	n	σ_0 (mPa)	K (mPa s^n)	η_{100} (mPa s)	R^2	n	σ_0 (mPa)	K (mPa s^n)	η_{100} (mPa s)	R^2
T	1[a,*]	48.5[a] ± 0.8	1.05[a] ± 0.13	1.05[a] ± 0.13	0.983	1[a,*]	70.1[a] ± 1.0	1.077[a] ± 0.004	1.077[a] ± 0.004	0.979
L	1[a,*]	58.6[a] ± 1.4	1.71[a] ± 0.15	1.71[a] ± 0.15	0.991	1[a,*]	60[a] ± 6	1.0[a] ± 0.2	1.0[a] ± 0.2	0.977
X	0.48[a] ± 0.07	26306[b] ± 87	1969[a] ± 679	432[b] ± 13	0.997	0.34[b] ± 0.02	2667[b] ± 188	694[b] ± 73	59[b] ± 2	0.997
MC	0.53[a] ± 0.05	0[a,**]	14986[b] ± 3948	1725[c] ± 148	0.997	1[a,*]	98[a] ± 8	8.3[a] ± 0.3	9.1[c] ± 0.2	0.999

Mean values ± standard deviations. Values with different letters within the same column are significantly different ($P < 0.05$) according to the LSD multiple range test.
*Bingham model (the Herschel-Bulkley model was adjusted with n = 1 and K = η); **Power law model (the Herschel-Bulkley model was adjusted with $\sigma_0 = 0$)
T: tween; L: lecithin; X: xanthan gum; MC: methylcellulose emulsion; t = 0 min: after the addition of bile extract and electrolyte solutions (before lipase addition)

In addition, the decrease in rheological parameter values observed in emulsion MC at t = 0 min may also be due to the interactions between MC molecules and bile salts that take place in the aqueous phase and/or at the o/w interface (Pizones Ruiz-Henestrosa, Bellesi, Camino, & Pilosof, 2017; Torcello-Gómez, Fernández Fraguas, Ridout, Woodward, Wilde, & Foster, 2015; Torcello-Gómez & Foster, 2014). In fact, these same authors found that the presence of bile salts decreased the gel viscoelasticity of MC solutions by interfering with the hydrophobic association of methyl groups between different bundles.

In conclusion, after the addition of bile extract and electrolyte solutions, emulsions X and MC underwent physicochemical changes that resulted in reduced consistency and viscosity, although these values were still higher than those for emulsions T and L. Previous studies have reported that changes in the rheological properties of the gastrointestinal digesta may have an impact on the rate and extent of lipid digestion (Espinal-Ruiz et al., 2014; Qin et al., 2016; Zhang, Zhang, Zhang, Decker, & McClements, 2015). An increase in the macro-viscosity of a sample may thus influence the intimate mixing of the sample with the digestive components, which could, in turn, inhibit the ability of lipase to reach the lipid droplet surfaces. Consequently, polysaccharide may decrease the rate and extent of lipid digestion (Espinal-Ruiz et al., 2014; Pilosof, 2017). However, Pizones Ruiz-Henestrosa et al. (2017) found that o/w emulsions prepared with a cellulose ether that presented the highest viscosity (E4M) also underwent the highest lipolysis. Likewise, in the present study, the higher consistency and apparent viscosity of emulsion X at t = 0 min did not seem to play a major role in reducing lipid digestion, as it turned out to be the most digested sample. Therefore, either the rheological properties of the intestinal digesta was unable to appreciably inhibit the ability of lipase to reach the lipid droplet surfaces, or other factors had a greater impact on the rate and extent of

lipid digestion. As our results seem to indicate that the different rates and extents of lipolysis are neither attributable to differences in the rheological properties of the o/w emulsions, nor to the droplet size/surface area available for the action of lipase, we concluded that the observed differences most likely depend on the nature of the interfacial layer surrounding the lipid droplets.

3.5. Location of the different molecules in o/w emulsions

Fig. 6 shows the microstructure of the o/w emulsions, both in their fresh forms (Fig. 6A-D') and at t = 0 min of lipid digestion (Fig. 6E-H'). All samples were stained with toluidine blue. In addition, emulsions X and MC were also stained with specific fluorophores (FITC and calcofluor, respectively) in order to improve xanthan gum and methylcellulose visualisation in the o/w emulsion. In the fresh o/w emulsions, tween molecules were observed at the interface of the oil droplets in blue (white arrows Fig. 6A) due to blue toluidine staining, whereas lecithin molecules were observed both at the interface of the oil droplets and forming aggregates in the aqueous continuous phase (white arrows Fig. 6B). In emulsion X, blue staining (Fig. 6C) or green fluorescence (Fig. 6C') were only observed in the aqueous phase (white arrows Fig. 6C and C'), suggesting that xanthan molecules were only present in this phase. This is most likely due to the fact that xanthan molecules are not considered to be surface-active at the o/w interface (Bouyer, Mekhloufi, Huang, Rosilio, & Agnely, 2013; Papalamprou et al., 2005). In emulsion MC, solely the aqueous phase was observed bluish (Fig. 6D). Nevertheless, when this emulsion was stained with a specific fluorophore (calcofluor), methylcellulose molecules were observed at the interface of the oil droplets forming an intense fluorescent interface, as well as in the aqueous continuous phase (white arrows Fig. 6D'). Jia et al. (2015) and Shen, Guo,

Wu, Zhang, & Abid (2016) obtained similar results in o/w emulsions stabilised by amorphous cellulose and microcrystalline cellulose, respectively. They observed fluorescent surfaces as well as a fluorescent signal from the external phase, suggesting that cellulose was adsorbed on the drop surfaces and was also present in the continuous phase.

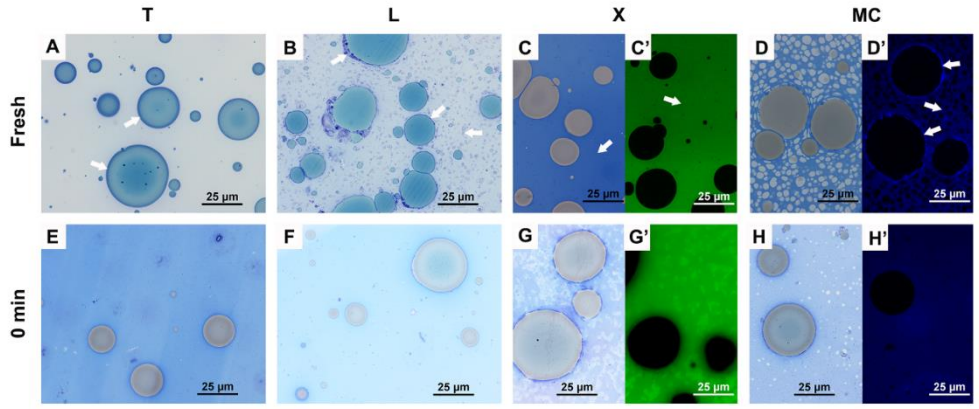

Fig. 6. Location of the different molecules (white arrows) in fresh o/w emulsions (A-D') and microstructure of these o/w emulsions before lipase addition (at t = 0 min of the intestinal digestion) (E-H'). A-H: bright-field microscopy. C', D', G' and H': fluorescence microscopy. A and E: tween; B and F: lecithin; C, C', G and G': xanthan gum; D, D', H and H': methylcellulose emulsion.

After the addition of the bile extract and electrolyte solutions, all the emulsions exhibited a blue/purple interface due to blue toluidine staining (Fig. 6E-H). Previous studies have found that bile salts occupy the oil-water interface extremely quickly (high surface activity) due to a combination of their strong affinity for hydrophobic phases and their flat conformation (Maldonado-Valderrama, Wilde, Macierzanka, & MacKie, 2011). These salts may displace, either completely or partially, some of the original emulsifier molecules from the droplet surface,

interpenetrate between the existing emulsifier molecules, or form an interfacial complex with the original emulsifier molecules (Mun et al., 2007). Thus, the slim blue/purple interfaces at t = 0 min (Fig. 6E-H) are probably mainly composed of intestinal components (mostly bile salts), along with some remnants of the existing adsorbed molecules. In emulsion T, the intense blue interface observed in the fresh emulsion (Fig. 6A) seemed to become thinner at t = 0 min (Fig. 6E) whereas the continuous phase became bluish, indicating that some tween molecules may have been displaced from the o/w interface by bile salts. Mun et al. (2007) reported that despite the fact that only some tween molecules may be displaced by bile salts from the droplet surfaces, these are much more surface-active than lipase, meaning that this small surfactant could form an interfacial layer around the droplets. This, in turn, prevents the enzyme from coming into close proximity with the emulsified lipids, thereby inhibiting lipase hydrolysis. This could explain why emulsion T was the least-digested sample in our study. In emulsion L, the lecithin aggregates observed at the o/w interface and in the aqueous phase in the fresh emulsion (white arrows Fig. 6B) seem to have been disaggregated at t = 0 min (Fig. 6F). This can be explained by the fact that the adsorption of bile salts to the phospholipid monolayer disrupts the structural organisation (phospholipid packing) at the interface, resulting in a mixed phospholipid-bile salt interface (Maldonado-Valderrama et al., 2011). The break-up of the well-packed phospholipid interface was postulated to allow greater binding of lipase to the interface, which enhanced enzyme activity (Maldonado-Valderrama et al., 2011). This explains why emulsion L was digested to a greater extent than emulsion T. However, the phospholipids dispersed in the aqueous phase could also bind with pancreatic lipase, which would tend to impair lipase adsorption on the surface of the emulsion droplets (Ye, Cui, Zhu, & Singh, 2013). This is why emulsion L was not the most-digested sample in our study. In

emulsion X, the blue aqueous phase observed in its fresh form (Fig. 6C) seemed to become lighter at t = 0 min (Fig. 6G), likely due to the dilution effect, whereas the interface became bluish due to bile salt adsorption, as commented above. In addition, this emulsion exhibited almost the same fluorescent aqueous phase at t = 0 min (Fig. 6G') as in its fresh form (Fig. 6C'), suggesting that xanthan molecules remained in the aqueous phase. Very recent research has highlighted the key role of the interaction of bile salts with surface active or non-surface active polysaccharides, a process that can decrease unadsorbed bile salt content, potentially delaying or inhibiting lipolysis (Pilosof, 2017). This could be attributed to insufficient removal of lipolysis products in mixed micelles, which would inhibit lipid digestion (Sarkar et al., 2016). Concretely, unadsorbed bile salts could prevent the accumulation of inhibitory lipolysis products on the interface, thereby enabling the continuation of lipid digestion (Sarkar et al., 2016). Therefore, although some polysaccharides may interact with intestinal components in the aqueous phase, affecting both the rate and extent of lipolysis (Pilosof, 2017), our results showed that emulsion X was the most-digested sample. In this case, then, if these interactions did indeed take place, they most likely occurred to a lesser extent than those reported in emulsion L. Since neither the large particle size of emulsion X at t = 0 min, nor its high viscosity, nor the possible interactions with bile salts in the aqueous phase seem to play a key role in the %FFA results, we propose that xanthan molecules do not hinder bile salt and lipase access to the oil droplet surfaces. Finally, in emulsion MC, the interface was observed in blue/purple (Fig. 6H) as in the other emulsions. However, the fluorescent interface (Fig. 6D') disappeared while the fluorescent aqueous phase remained almost unchanged (Fig. 6H'). These results suggest that methylcellulose molecules may have been displaced by bile salts. However, Torcello-Gómez & Foster (2014) reported a coexistence of both cellulose and bile salts at the o/w interface. In

addition, these authors reported that interactions between bile salt and cellulose ethers are taking place in the aqueous phase. This could explain why emulsion MC, despite bile salt displacement, was not the most-digested sample and exhibited intermediate %FFA results.

A schematic representation of the proposed mechanisms involved in the lipolysis of the different emulsions is presented in Fig. 7 in order to facilitate a better understanding of the explanations given above. The lesser extent of digestion of tween emulsions may be related to the higher surface activity of tween molecules – only observed at the o/w interface — which provides an interfacial layer resistant to bile salt displacement. As a result, the adsorption of lipase is hindered, thus inhibiting lipid digestion. With regard to emulsions L and MC, lecithin and methylcellulose molecules were present both at the interface and in the aqueous phase. However, both types of molecules seemed to have been displaced from the o/w interface by bile salts, thereby providing more sites for lipase molecules to access in comparison with emulsion T. Moreover, several interactions seem to have taken place in the aqueous phase between lecithin or methylcellulose and the intestinal components (bile salts and lipase), thus delaying lipid digestion. This would explain why these emulsions exhibited intermediate %FFA values. Finally, xanthan molecules exhibited neither surface activity (which was only observed in the aqueous phase), nor a strong interaction with intestinal components. Therefore, xanthan molecules did not hinder the access of bile salts and lipase molecules to lipid droplets, making emulsion X the most-digested sample.

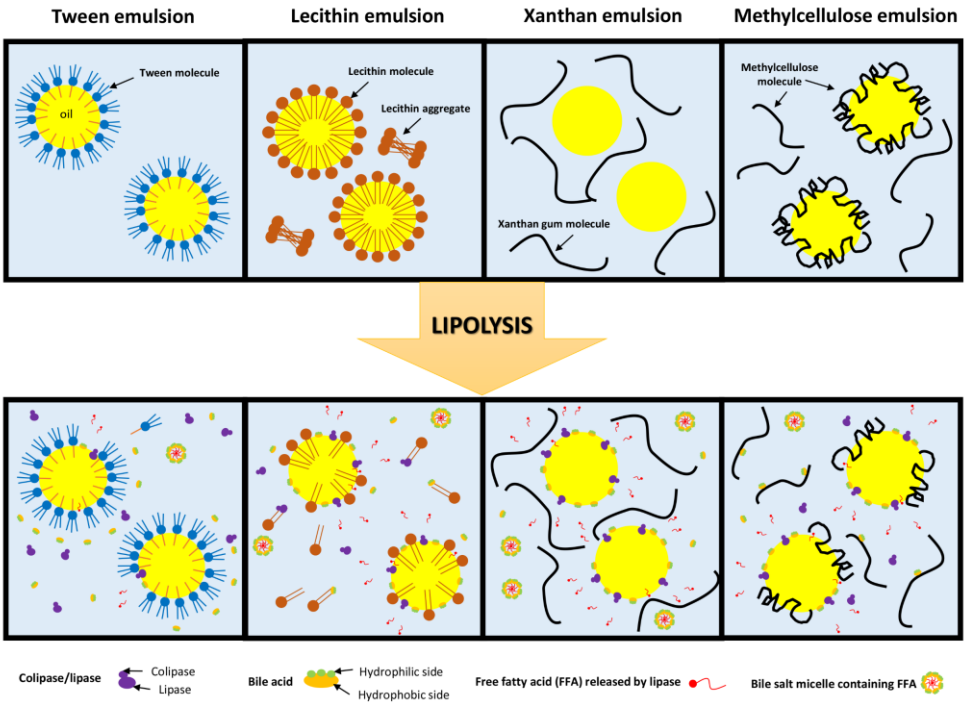

Fig. 7. Schematic representation of the proposed mechanisms involved in the modulation of lipolysis of the different emulsions.

4. Conclusions

Fresh o/w emulsions (T, L, X and MC) with similar droplet size presented different flow behaviours and viscosities depending on the type of molecule used to stabilise them. After the addition of intestinal fluids (bile extract and electrolyte solutions without lipase), the o/w emulsions underwent several changes. Specifically, the droplet size increased in emulsion X whereas the viscosity of the emulsions stabilised by polysaccharides (emulsions X and MC) sharply decreased. Consequently, at t = 0 min of lipid digestion (before lipase addition), emulsion X exhibited the highest droplet size and viscosity values while emulsion T exhibited

the smallest values for both parameters. Surprisingly, emulsion X was the most-digested sample and emulsion T was the least-digested sample. Therefore, the different kinetics of lipolysis observed in our results were not primarily related to differences in the droplet size/surface area available for the action of lipase, nor to the viscosity of the o/w emulsions.

Indeed, the differences found in the lipolysis kinetics can most likely be attributed to the nature and location of each type of molecule in the o/w emulsions. Different types of emulsifiers offer different resistance to bile salts (BS) displacement (T > L and MC > X). Once BS are adsorbed at the o/w interface, they aid lipase adsorption (X > L and MC > T), thereby enhancing lipid digestion. Nevertheless, some interactions take place between lecithin or methylcellulose molecules and intestinal components (BS and lipase molecules) in the aqueous phase, preventing them from reaching the o/w interface and/or inhibiting sufficient removal of lipolysis products in mixed micelles. This, in turn, can delay lipid digestion. Finally, xanthan gum molecules neither hinder the access of BS and lipase molecules to the o/w interface, nor interact with them, and thus, emulsion X was the most-digested sample, exhibiting a high release of FFAs. The overall results in this study have shown that the type of molecule used in the preparation of o/w emulsions, as well as their interactions with intestinal components, both play a key role in controlling lipid digestion of these emulsions.

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New hydrocolloid-based emulsions for replacing fat in panna cottas: a structural and sensory study

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Abstract

BACKGROUND: Dairy desserts are popular traditional products, but because of their high calorie or fat content, they can be unsuitable for people who have certain dietary requirements. The aim of this study was to design panna cottas with similar organoleptic and textural properties to the traditional ones but with a lower fat content, by replacing part of the cream with new emulsions prepared with hydrocolloids (cellulose ethers), namely methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC).

RESULTS: Incorporating the MC and HPMC emulsions modified the textural properties (firmness and stiffness) of the panna cottas. Regarding the sensory results, the panna cottas prepared with the MC and HPMC emulsions were considered lumpy and soft respectively.

CONCLUSION: Considering the results as a whole, the cellulose type and the amount of cream are factors to take into account. Although the texture and taste of the control panna cotta are better than those of the panna cottas prepared with the MC and HPMC emulsions, it is possible to replace 75% of the cream in traditional panna cottas with HPMC emulsion and obtain good consumer acceptance and purchase intention. The panna cottas with 75% substitution by HPMC emulsion were described as creamy, with smooth appearance and moist mouth feel.

Keywords: dairy dessert, reduce fat, cellulose ether emulsion, texture, microstructure, sensory properties

1. Introduction

In recent years, extensive scientific evidence has emerged which indicates that dietary patterns have specific health or disease outcomes.[1] However, the public often prefers particular foods, sometimes of a markedly traditional character, even though their composition is inadvisable. In this regard, reformulating traditional products by replacing unhealthy ingredients with others that have similar technological functions and retain the sensory characteristics of these foods is a promising alternative for producing healthier products.[1] Achieving such changes in composition without altering their organoleptic properties and overall acceptability to consumers requires a very great and highly complex research effort.[2-4] Designing healthier foods gives rise to new opportunities and challenges for the food industry, which, in today's competitive market, needs to pursue growth through constant innovation in products that consumers find attractive.[5, 6]

Dairy desserts are popular traditional products in Europe. As well as their sensory properties, their being easy to consume and easily swallowed makes them particularly suitable and convenient foods for the population in general. Nevertheless, some of them may be unsuitable for those with particular dietary requirements, owing to their high calorie and fat contents. Panna cotta (Italian for 'cooked cream') is a typical dessert from the Piedmont region of Italy that is largely composed of cream, sugar and gelatine, so as well as being high in calories, it also has a high saturated fat content. Saturated fat is associated with the appearance of numerous disorders such as obesity, cancer, cholesterol and coronary diseases.[7] The quantity and type of fat in the composition of a food have become a subject of concern for many consumers who want to maintain healthy habits.[8, 9] Reducing the fat content of a food alters its composition and structure, giving rise to perceptible

changes in colour, flavour and texture[10] that influence its sensory acceptability. To compensate for this effect, different types of fat replacers that have similar physical, chemical and sensory properties to those of fats but add few or no calories are habitually used.[11] The choice of these replacements depends on the composition and characteristics of each food.[12-14] Fat replacers are classified into three basic types according to their chemical composition: carbohydrate-based, protein-based and fat-based.[11, 15]

Fats in foods can be replaced by emulsions.[16] In the present study, to reduce the amount of fat, new emulsions were designed based on cream, water and two types of hydrocolloid: a methylcellulose (MC) and a hydroxypropyl methylcellulose (HPMC). MC and HPMC are hydrocolloids which are obtained from cellulose through chemical derivatisation.[7] These cellulose ethers produce gel networks that can modify the texture, increase the viscosity and contribute dietary fibre.[17] Additionally, they have been used to replace saturated fats as stabilisers of oil-in-water emulsions, since cellulose ethers adsorb to the emulsion interface.[18, 19] Some studies have shown that when the fat phase is dispersed in an aqueous medium, the same organoleptic result can be achieved with less initial fat[11] and, irrespective of the energy content of the emulsion, the fat droplet size falls and the sensation of creaminess increases, leading to increased satiation.[20, 21]

The aim of this study was to design panna cottas with similar organoleptic and textural properties to those of the traditional product but with lower fat and calorie contents, by replacing the cream with new emulsions based on cellulose ethers (MC and HPMC). To this end, it examined the effects of substituting the emulsions for different proportions of the cream on the texture, microstructure

(using light microscopy and confocal laser scanning microscopy) and sensory properties of the panna cottas.

2. Materials and methods

2.1. Emulsion preparation

The emulsions were prepared with two different cellulose ethers with thermo-gelling ability (METHOCEL™ MX and F4M, both supplied by The Dow Chemical Company, Bomlitz, Germany). MX is a methylcellulose (MC) and F4M is a hydroxypropyl methylcellulose (HPMC). Their viscosity was 50 000 and 4000 mPa s respectively (in a 20 g kg^{-1} aqueous solution at 20 °C, measured by The Dow Chemical Company following reference methods ASTM D1347 and ASTM D2363).

The ingredients of the emulsions were liquid cream (500 g kg^{-1}) with 350 g kg^{-1} of fat (Pascual, Calidad Pascual SAU, Burgos, Spain), drinking water (490 g kg^{-1}) (Bezoya, Calidad Pascual SAU) and the different cellulose ethers (10 g kg^{-1}). The emulsions were prepared according to Sanz *et al*.[22] with some modifications. Briefly, the cellulose ethers were dispersed in previously warmed cream (microwaved at 500 W for 30 s) using a stirrer (RZR 1, Heidolph, Schwabach, Germany) at 120 × g for 5 min. The mixture was then hydrated by gradually adding the water at 1 °C while stirring with a homogenizer (Ultraturrax T18, IKA, Staufen, Germany) at 3390 × g for 30 s and subsequently at 7458 × g for 60 s.

2.2. Panna cotta preparation

A control panna cotta was prepared with reconstituted skimmed milk powder (Central Lechera Asturiana, Corporación Alimentaria Peñasanta SA, Siero, Spain) in mineral water, liquid cream with 350 g kg^{-1} of fat, κ-carrageenan (Satiagel™ ME5, Cargill France SAS, Saint-Germain-en-Laye, France), sodium alginate (MANUCOL DMF, FMC Biopolymer, Philadelphia, PA, USA) and liquid sweetener (Consum, Krüger GmbH & Co. KG, Bergisch Gladbach, Germany) (Table 1). Four different panna cottas (P75M, P100M, P75H and P100H) were prepared by replacing 75 or 100% of the liquid cream in the control panna cotta formulation with the MC emulsion (P75M or P100M) or the HPMC emulsion (P75H or P100H). Because the emulsions were also prepared with cream, the final cream content was 465.3 g kg^{-1} in panna cottas P75M and P75H and 372.3 g kg^{-1} in panna cottas P100M and P100H (Table 1). Therefore, the cream content reduction compared with the control panna cotta was 37.5 and 50% respectively.

The panna cottas were prepared according to Borreani *et al.*,[23] then placed in silicone moulds (truncated cone: base circle 6.3 cm, height 3.7 cm, top circle 4.5 cm) and cooled to ambient temperature. The samples were stored at 4–5 °C in a refrigerator until they were analysed.

Table 1. Panna cotta formulations (g kg^{-1}) with new emulsions as fat replacers.

Ingredients (g kg^{-1} panna cotta)	**Panna cottas**				
	Control	**P75M**	**P100M**	**P75H**	**P100H**
Skimmed milk powder	41.4	41.4	41.4	41.4	41.4
Water	206.8	206.8	206.8	206.8	206.8
κ-carrageenan	3.3	3.3	3.3	3.3	3.3
Sodium alginate	2.5	2.5	2.5	2.5	2.5
Sweetener	1.6	1.6	1.6	1.6	1.6
Cream	744.5	186.1	-	186.1	-
MC emulsion	-	558.4	744.5	-	-
HPMC emulsion	-	-	-	558.4	744.5
Total cream content	***744.5***	***465.3***	***372.3***	***465.3***	***372.3***
Fat content	***261.0***	***163.2***	***130.7***	***163.2***	***130.7***

MC: methylcellulose. HPMC: hydroxypropyl methylcellulose. P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

2.3. Texture analysis

A penetration test was performed according to Borreani *et al*.[23] with a penetration distance of 15 mm. The firmness of the panna cotta was defined as the maximum force (N) measured during sample penetration[24] and the stiffness (N s^{-1}) as the slope of the curve before the rupture point. The texture analysis was performed in triplicate.

2.4. Microstructure analysis

An aliquot of both the MC and HPMC emulsions and a slim section of each panna cotta sample were placed on glass slides and observed at 60× magnification according to Borreani *et al*.[25] for light microscopy (LM) and according to Borreani *et al*.[23] for confocal laser scanning microscopy (CLSM).

2.5. Sensory analysis

The sensory analysis was carried out with 117 consumers (64 women and 53 men) recruited among the employees and students of the University. The samples (control, P75M, P100M, P75H and P100H) were analysed in a sensory laboratory equipped with individual booths.[26] Each consumer tasted the four reformulated panna cottas and the control sample, presented monadically at a single session following a balanced complete block experimental design. The samples were served in small plastic cups coded with random three-digit numbers. The samples were served at room temperature in random order. Water was supplied to clean the consumers' mouths between each sample.

2.5.1. Acceptability test

The consumer acceptance test was performed using a nine-point hedonic scale (1 = dislike extremely to 9 = like extremely). For each panna cotta, the consumers scored their degrees of liking in the following order: 'appearance', 'aroma', 'texture', 'taste' and 'overall acceptability'. The consumers also rated their probability of purchasing each sample on a five-point hedonic scale ranging from 1 = 'I would definitely not buy it' to 5 = 'I would definitely buy it'.

2.5.2. CATA questionnaire

For each sample, the participants answered a CATA (check-all-that-apply) questionnaire featuring 22 attributes: lumpy appearance, smooth appearance, thick appearance, odd flavour, pleasant flavour, sweet flavour, cream flavour, tasteless, aftertaste, lumpy in mouth, soft in mouth, thick in mouth, dry mouth feel, moist mouth feel, mouth coating, creamy, gummy, nutritious, satiating, healthy, low in calories, high in calories. The terms had been previously selected on the basis of the available literature about similar products. The following instruction was given to participants: 'Check all the characteristics you consider represent this sample'.

2.6. Statistical analysis

All data analyses were performed with XLSTAT statistical software (Addinsoft, New York, NY, USA).

2.6.1. Texture and acceptability tests

One-way analysis of variance (ANOVA) was applied to study the differences between formulations in the texture and consumer acceptance tests. The honest significant differences were calculated by Tukey's test ($P < 0.05$).

2.6.2. CATA questionnaire

Firstly, the frequency of use of each CATA term was determined by counting the number of consumers that selected that term to describe each sample. Cochran's Q test[27] was then carried out to identify the significant differences between the

samples for each of the sensory terms. The variability in frequencies of attribute selection which were significant according to the Cochran test was analysed with a correspondence analysis. Finally, in order to assess the relationship between the CATA questionnaire responses and the panna cotta acceptability scores, a multi-factor analysis was performed on the frequencies of mention in the CATA questionnaire.

3. Results and discussion

3.1. Texture analysis

The firmness values of the different panna cottas analysed in this study ranged from 0.765 N (P100H) to 1.172 N (P100M) (Table 2). The control sample was significantly different ($P < 0.05$) from the other panna cottas, with a firmness value that lay between those of the samples prepared with the MC emulsion and with the HPMC emulsion. The panna cottas prepared with the MC emulsion (P75M and P100M) presented the greatest firmness, with no significant differences between them ($P > 0.05$). Conversely, those prepared with the HPMC emulsion were the least firm, with the P100H panna cotta presenting significantly ($P < 0.05$) the lowest firmness value (0.765 N). Although using cellulose ethers can contribute viscosity to some systems,[19, 28] in the present case it was found that replacing cream with the HPMC emulsion decreased the firmness values of samples P75H and P100H compared with those of the control. This could be due to the lower consistency of the HPMC emulsion than that of the cream used in the control panna cotta. Consequently, the panna cottas prepared with the HPMC emulsion presented less consistency than the control panna cotta, and therefore the higher the percentage of HPMC emulsion used, the lower was the firmness value obtained. In contrast, the

MC emulsion gave the P75M and P100M samples higher firmness values than both the HPMC emulsion samples and the control panna cotta. This is probably due to the fact that the gel strength of aqueous MC and HPMC solutions is strongly dependent on their concentration and methoxyl content.[29] Indeed, when oil-in-water emulsions were prepared with MC and HPMC by Sanz *et al.*,[22, 30] those with MC developed a stronger consistency than when HPMC was used. This could explain why the MC panna cottas (P75M and P100M) were firmer than those prepared with the HPMC emulsion (P75H and P100H). It is well known that self-assembly of cellulose ethers is driven by hydrophobic interactions between the hydrophobic substituents (methyl groups).[31] The higher the methyl substitution, the stronger are the hydrophobic interactions, so stronger consistencies are obtained. Therefore, in the case of HPMC, the lower methyl group content and the presence of the more polar and larger hydroxypropyl groups that inhibit intermolecular association lead to the formation of a weaker consistency system than with MC.[32, 33] In short, the firmness of the panna cottas appeared to depend on the type of emulsion and therefore on the type of cellulose employed in the formulation, but in the case of the panna cottas prepared with the HPMC emulsion, it also depended on the percentage of cream replaced (75 or 100%).

As regards the stiffness values of the different panna cottas investigated in this study, they varied between 1.446 N s^{-1} (P100H) and 2.297 N s^{-1} (control), with significant differences ($P < 0.05$) among all formulations. The stiffest sample was the control panna cotta. The panna cottas prepared with the MC emulsion were significantly stiffer ($P < 0.05$) than those prepared with the HPMC emulsion, at both replacement levels (75 and 100%).

Table 2. Influence of cream substitution percentage and cellulose ether type on the texture of the panna cottas.

Sample	Firmness (N)	Stiffness (N s^{-1})
Control	1.078 ± 0.042 [a]	2.297 ± 0.072 [a]
P75M	1.167 ± 0.027 [b]	2.127 ± 0.081 [b]
P100M	1.172 ± 0.032 [b]	2.033 ± 0.115 [c]
P75H	0.801 ± 0.027 [c]	1.678 ± 0.100 [d]
P100H	0.765 ± 0.022 [d]	1.446 ± 0.074 [e]

Mean values ± standard deviations (n = 15). Values with different letters within the same column are significantly different ($P < 0.05$) according to the HSD multiple range test.
P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

Consequently, it would seem that the stiffness values, like the firmness values, depend on the type of emulsion used and consequently on the type of cellulose used. Indeed, it has been reported that the stiffness depends on the level of methoxyl and hydroxypropyl substitution, where an increase in hydroxypropyl substitution diminishes the stiffness of the resulting gel.[34] Regarding the cream content, the control panna cotta had the highest cream content and exhibited the highest stiffness value, as mentioned above. Moreover, the panna cottas prepared with replacement of 75% of the cream by the MC emulsion (P75M), which had a higher cream content than the panna cottas with 100% cream replacement (P100M), exhibited stiffness values that were significantly ($P < 0.05$) higher than those of the latter (P100M) and approached those of the control panna cotta. This was also observed in the panna cottas prepared with the HPMC emulsion, where P75H exhibited significantly ($P < 0.05$) higher stiffness values than P100H. Consequently, the proportion of cream replaced by the MC or HPMC emulsion appeared to have a directly proportional influence on the stiffness of the panna cottas. The higher the cream content, the

greater was the stiffness. In short, the stiffness of the panna cottas seemed to depend both on the type of emulsion employed (MC or HPMC) – and consequently on the type of cellulose employed – and on the percentage of cream that was replaced.

3.2. Microstructure analysis

The structures of the MC and HPMC emulsions that replaced part or all of the cream in the original panna cotta formulation employed in the control can be observed in Fig. 1A and B and Fig. 1C and D respectively. In the LM images (Fig. 1A and C) the protein and the carbohydrates in the emulsions are stained purple and the fat is unstained, while in the CLSM images (Fig. 1B and D) the protein and the carbohydrates are stained red and the fat is stained green. In the MC emulsion (Fig. 1A and B) the fat can be seen both as fat globules of different sizes very close to each other, forming a dense network, and as large, dense clusters (pointed to by the white arrows), possibly due to flocculation and/or coalescence among some of them. In contrast, although flocculation and/or coalescence can also be seen in the HPMC emulsion (Fig. 1C and D), the fat seems to be distributed in a more dispersed and homogeneous way throughout the sample and to form smaller clusters. These results were in accordance with Sanz *et al*.,[22] who observed that the fat globule size was larger in emulsions with MC than in emulsions with HPMC, when both emulsions were made with vegetable oil, and that the MC emulsion presented fat globule coalescence and a gel texture. Consequently, the more compact structural appearance of the MC emulsions compared with the HPMC emulsions could be related to the textural findings, as the samples prepared with these emulsions presented higher and lower firmness values respectively.

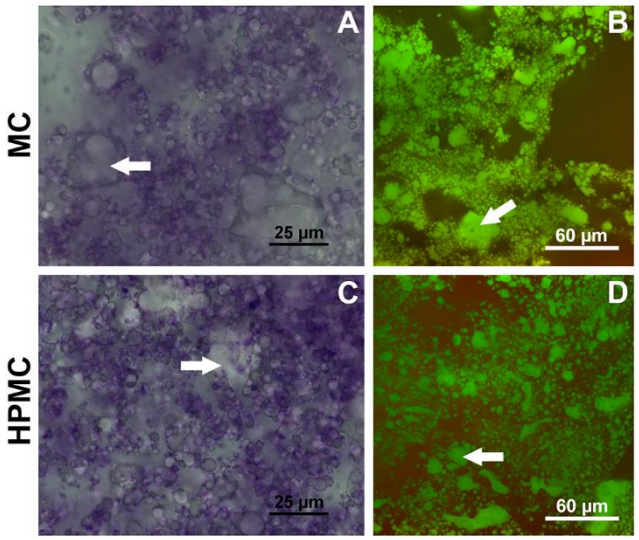

Fig. 1. (A, C) Light microscopy and (B, D) confocal laser scanning microscopy images of the MC (A, B) and HPMC (C, D) emulsions. Arrow: fat. Toluidine blue stained proteins and carbohydrates in purple, Nile Red stained fat in green and Rhodamine B stained proteins and carbohydrate in red. Magnification 60x.

Figure 2 shows the LM (Fig. 2A–E) and CLSM (Fig. 2F–J) images of the different panna cottas examined in this study. The fat of the control sample (Fig. 2A and F) can be seen both as small globules and as clusters of different sizes dispersed through the protein network, which is stained red (Fig. 2F). The higher fat content probably favoured coalescence formation in the control sample, giving rise to large clusters of fat. As in the control panna cotta, in sample P75M (Fig. 2B and G) the fat was also found to be surrounded by the protein network (stained purple in Fig. 2B and red in Fig. 2G). The fat also seemed to present a similar appearance to that observed in the MC emulsion. In other words, it formed large, compact, dense clusters as well as globules of different sizes. The appearance of the P100M panna cotta (Fig. 2C and H) was similar to that of P75M. However, it seemed to have a lighter, less compact/dense structure, probably because the fat clusters were smaller

and more dispersed. This agrees with the texture analysis results, which found that the higher the fat content, the greater was the stiffness value. Of the two panna cottas formulated with MC, P75M was the more similar in appearance to the control. It was also the one with the closest firmness and stiffness values to those of the control. In the P75H (Fig. 2D and I) and P100H (Fig. 2E and J) panna cottas, as in the MC ones, a homogeneously distributed protein network surrounded the fat. Sample P75H (Fig. 2D and I) appeared not to retain the structure of the HPMC emulsion, since homogeneously distributed fat globules were not observed and there were mainly large fat clusters (Fig. 2I). As the HPMC emulsion had a lower consistency from the start than the MC emulsion, it could be that the conditions in which the samples were prepared affected it more than they did the MC emulsion. Nevertheless, P100H did appear to maintain the structure observed in the HPMC emulsion, possibly because it contained a higher proportion of the emulsion than the P75H panna cotta.

In general, the panna cottas prepared with the HPMC emulsion (P75H and P100H) appeared to present smaller fat globules and less flocculation and/or coalescence, giving them a lighter structure than those prepared with the MC emulsion (P75M and P100M). This agrees with the texture findings, as the panna cottas prepared with the HPMC emulsion had lower firmness and stiffness values than those prepared with the MC emulsion.

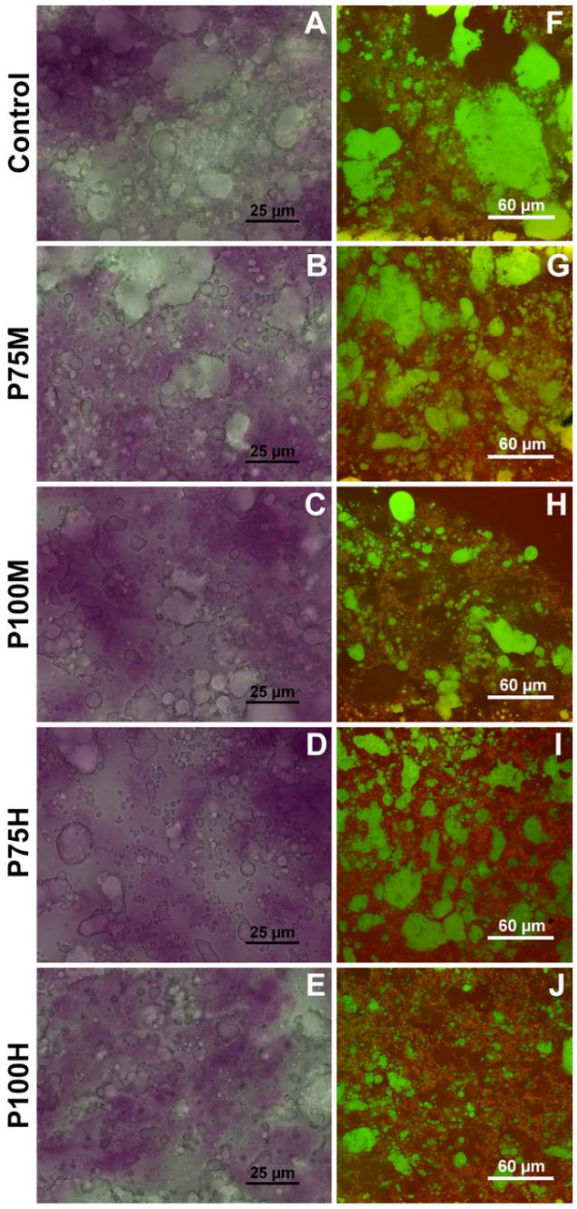

Fig. 2. Light microscopy (A - E) and confocal laser scanning microscopy (F - J) images of the five panna cottas. P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion. Toluidine blue stained proteins and carbohydrates in purple, Nile Red stained fat in green and Rhodamine B stained proteins and carbohydrates in red. Magnification 60x.

3.3. Sensory analysis

3.3.1. Acceptability study

Table 3 shows the results of the consumer acceptability test for the different panna cottas. The attributes assessed in this test were appearance, aroma, texture, taste and overall acceptability. Although no significant differences between the samples were found for appearance and aroma ($P > 0.05$), a slight decrease in aroma was observed between the control panna cotta and those containing the MC and HPMC emulsions, as well as between the panna cottas with 75 and 100% cream replacement. This could be due to the decrease in fat content. Fewer of the lipophilic compounds that influence flavour release and perception were present in panna cottas prepared with fat replacement than in the control panna cottas.

As regards the acceptability of the panna cotta textures, the control and P75H were rated the best, with no significant differences between them ($P > 0.05$). Nor were significant differences ($P > 0.05$) found between P75H, P75M and P100H. Although the firmness values of the panna cottas prepared with the MC emulsion were closer to those of the control sample (Table 2), the consumers rated the texture of the panna cottas prepared with the HPMC emulsion more highly. This could be due to the lighter structure of the panna cottas prepared with the HPMC emulsion, where the fat was distributed in a more homogeneous way throughout the sample, than the panna cottas prepared with the MC emulsion, as seen in the microstructure results (Fig. 2). Consequently, the HPMC emulsion gave rise to a soft texture in the panna cottas (see 'Differences between samples described by the CATA questionnaire', where the attributes 'soft in mouth', 'creamy' and 'moist mouth feel' were more often mentioned for panna cottas prepared with the HPMC emulsion).

Moreover, the consumers rated the softer texture of the P75H and P100H panna cottas more highly (Table 3).

Table 3. Liking for appearance, flavour, texture and taste and overall acceptability scores of the five panna cottas.

Sample	Appearance	Aroma	Texture	Taste	Overall acceptability
Control	6.75 ± 1.56 ^{a}	5.63 ± 1.60 ^{a}	7.10 ± 1.43 ^{a}	6.99 ± 1.93 ^{a}	6.85 ± 1.65 ^{a}
P75M	6.24 ± 1.69 ^{a}	5.61 ± 1.64 ^{a}	5.86 ± 2.15 ^{bc}	5.35 ± 2.27 ^{b}	5.62 ± 1.96 ^{bc}
P100M	6.22 ± 1.47 ^{a}	5.31 ± 1.36 ^{a}	5.48 ± 2.06 ^{c}	4.42 ± 2.10 ^{c}	5.19 ± 1.80 ^{c}
P75H	6.56 ± 1.58 ^{a}	5.58 ± 1.50 ^{a}	6.43 ± 1.87 ^{ab}	5.64 ±2.04 ^{b}	5.94 ± 1.84 ^{b}
P100H	6.44 ± 1.59 ^{a}	5.42 ± 1.56 ^{a}	5.90 ± 2.22 ^{bc}	5.05 ± 1.94 ^{bc}	5.47 ± 1.72 ^{bc}

Mean values ± standard deviations (n = 117). Values with different letters within the same column are significantly different ($P < 0.05$), according to the HSD multiple range test.
P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

With regard to taste, the control sample was rated most highly, by a significant difference ($P < 0.05$) compared with all other panna cottas. The following panna cottas in the taste rankings were P75M, P75H and P100H. In the same way as for texture, P100M scored lowest, although there was no significant difference ($P > 0.05$) between this sample and P100H. It would appear that the acceptability of the taste was in line with the differences in cream content. Samples with a higher percentage of cream (P75M and P75H) obtained better results than those with a lower percentage of cream (P100M and P100H), and the control panna cotta (which had the highest cream content) obtained the highest score.

Lastly, as regards the overall acceptability of the panna cottas, the scores followed the same trend as for taste. In other words, the most acceptable sample was the control panna cotta, followed by samples P75H and P75M. Consequently, according to these findings, it may be concluded that taste and texture are the attributes that seem to have determined the overall acceptability of the panna cottas, and that these depended to a certain extent on the cream content and the type of emulsion employed. The higher the cream content – and therefore the lower the cellulose emulsion content – the higher was the overall acceptability (better texture and taste). In accordance with these results, Arancibia *et al*.[35] reported that adding food thickeners is believed to increase texture and decrease aroma and taste intensity, although these effects seem to be dependent on thickener type.

As regards the likelihood of purchasing the panna cottas, over half the consumers (54.9%) would buy the control. Of the panna cottas formulated with the emulsions, the 75% replacement samples achieved a purchase intention of nearly 30% (29.4% of the consumers would buy P75H and 26.8% would buy P75M). The panna cottas with the lowest purchase likelihood rates were P100H and P100M at 17.7 and 15.0% respectively. These results could indicate a direct relationship between the quantity of cream and the purchase likelihood, as the lower the cream content of the panna cotta formulation, the lower was the likelihood of purchase. They agree with the overall acceptability rating and texture analysis, where P100M scored lowest and presented the highest firmness.

3.3.2. Differences between samples described by the CATA questionnaire

Cochran's non-parametric test found significant differences in 14 of the 22 CATA attributes used to describe the samples (Table 4). This demonstrates that

differences in the consumers' sensory perception of the panna cottas could be detected with the CATA questionnaire.

Table 4. Frequency of mention of CATA terms and p-value of Cochran's Q test for differences between the five panna cotta formulations.

Attributes	p-value (Cochran test)	Frequency of mention				
		Control	P75M	P100M	P75H	P100H
Thick appearance	0.057[a]	30	34	37	26	20
Mouth coating	0.011	27	34	31	42	45
Satiating	0.642[a]	33	31	26	30	27
High in calories	0.168[a]	28	37	27	27	32
Low in calories	0.521[a]	7	8	11	5	9
Nutritious	0.420[a]	8	11	9	8	13
Healthy	0.498[a]	7	3	7	7	7
Aftertaste	0.650[a]	12	17	18	16	17
Pleasant flavour	<0.0001	57	25	15	34	22
Odd flavour	0.003	7	20	26	22	19
Tasteless	<0.0001	10	35	48	30	42
Cream flavour	0.000	70	53	40	53	54
Sweet flavour	<0.0001	49	18	13	30	14
Moist mouth feel	0.036	14	7	9	15	19
Dry mouth feel	0.001	5	16	23	17	22
Gummy	0.038	8	18	21	11	18
Thick in mouth	0.069[a]	19	27	24	14	15
Creamy	<0.0001	64	54	37	64	63
Soft in mouth	<0.0001	65	31	22	40	41
Lumpy in mouth	0.002	10	13	22	10	5
Smooth appearance	0.002	35	26	22	44	40
Lumpy appearance	<0.0001	15	22	28	7	4

[a]Attributes that do not present significant differences according to the Cochran test (n = 117).
P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

Figure 3 shows the diagram resulting from a correspondence analysis (CA) of these 14 attributes. The first two dimensions of the CA graph explained 95.5% of all the variability in the data: 71.2% corresponded to the first dimension and 24.3% to the second dimension. It will be seen that terms such as pleasant flavour and creamy, which were associated with the control sample and with P75H, appeared on the positive side of the *X* axis. P75M, P100M and P100H, associated with negative terms such as lumpy appearance, tasteless and mouth coating, appeared on the negative side of the *X* axis. Moreover, the attributes can roughly be divided into five groups. The first group consisted of positive taste attributes and is where the control panna cotta was located. Opposite the 'positive taste' group, the 'negative taste' group consisted of negative taste attributes such as tasteless and odd flavour, as well as negative in-mouth attributes (gummy and dry mouth feel), and is where the panna cottas P75M and P100M were placed close together. The 'soft' group consisted of attributes related to soft texture and sensations (creamy, moist mouth feel and smooth appearance) and contained the P75H panna cotta, which exhibited low firmness and stiffness values (Table 2). Opposite the 'soft' group was the 'lumpy' group, where the P75M and P100M panna cottas were also found close to each other. The negatives attributes found in the 'negative taste' and 'lumpy' groups for these samples (P75M and P100M) were related to the presence of the MC emulsion, which provided high firmness and stiffness values and a worse taste to the panna cottas than the HPMC emulsion. The last group only consisted of the mouth coating attribute, where the P100H panna cotta was located. To summarize, the attributes of the panna cottas seemed to be organized along two 'sensory dimensions', as de Wijk *et al*.[36] also observed in custard desserts.

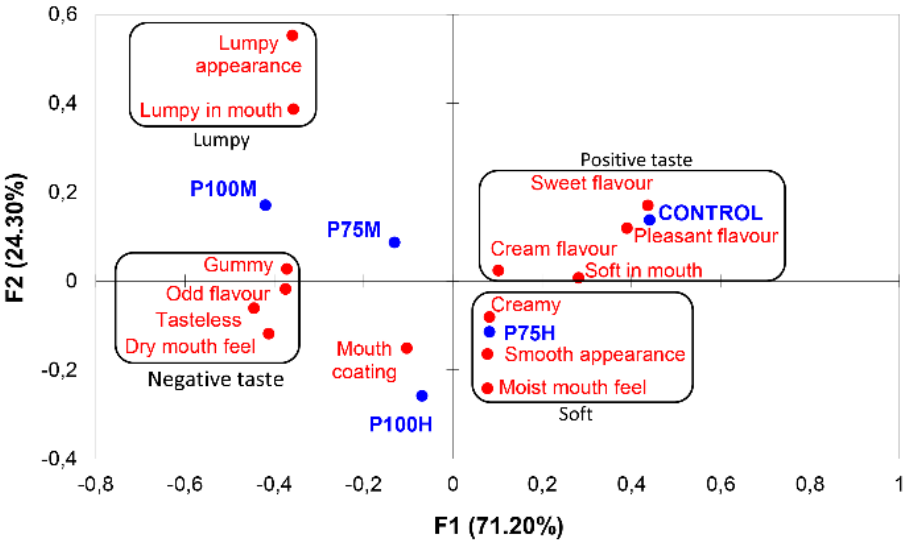

Fig. 3. Correspondence analysis of the check-all-that-apply (CATA) questions. P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

To gain a better understanding of which sensory characteristics were most highly related to the overall acceptability of the samples, multi-factor analysis (MFA) was used to examine a combination of frequency of mention of the significant attributes in the CATA questionnaire (Table 4) and the overall acceptability test results (Table 3). Figures 4A (attributes) and 4B (samples) show the results of this analysis. The first two dimensions of the MFA explained 93.83% of all the variability in the results: 64.44% corresponded to the first dimension and 29.39% to the second dimension. In Fig. 4A it can be seen that the overall acceptability was related to soft in mouth, cream flavour, pleasant flavour and sweet flavour, so it would appear that flavour was the main attribute that decided overall acceptability, together with

texture, although to a lesser extent. Indeed, it may be seen that the control, which was closely related to the positive flavour attributes, was the sample that obtained the highest overall acceptability score. Additionally, the frequency of mention of creamy (Table 4) was very high for the control sample, which will certainly also have influenced its overall acceptability. Nevertheless, sample P75H, related to moist mouth feel, smooth appearance and creamy, which refer to its texture, was also relatively close to the concept of overall acceptability (Fig. 4A). This confirms that texture attributes also influenced the overall acceptability of the samples, although to a lesser extent than flavour. In fact, like the control sample, P75H also obtained high frequencies for mentions of cream flavour and creamy (Table 4). The two samples prepared with the MC emulsion (P75M and P100M), which presented high firmness and stiffness values, were related to the negative texture attributes of gummy, lumpy in mouth and lumpy appearance, as may be seen in Fig. 3. For this reason, these samples scored worst in the acceptability test and were negatively correlated with the concept of overall acceptability (Fig. 4). Finally, sample P100H was related to mouth coating and was also placed at a distance from overall acceptability.

This analysis confirms that of the samples prepared with cellulose emulsions, the P75H panna cotta was the most acceptable to consumers. This could be because it scored higher on flavour - and texture-related attributes than the other samples formulated with emulsions and consequently presented similar attributes to those described for the control sample.

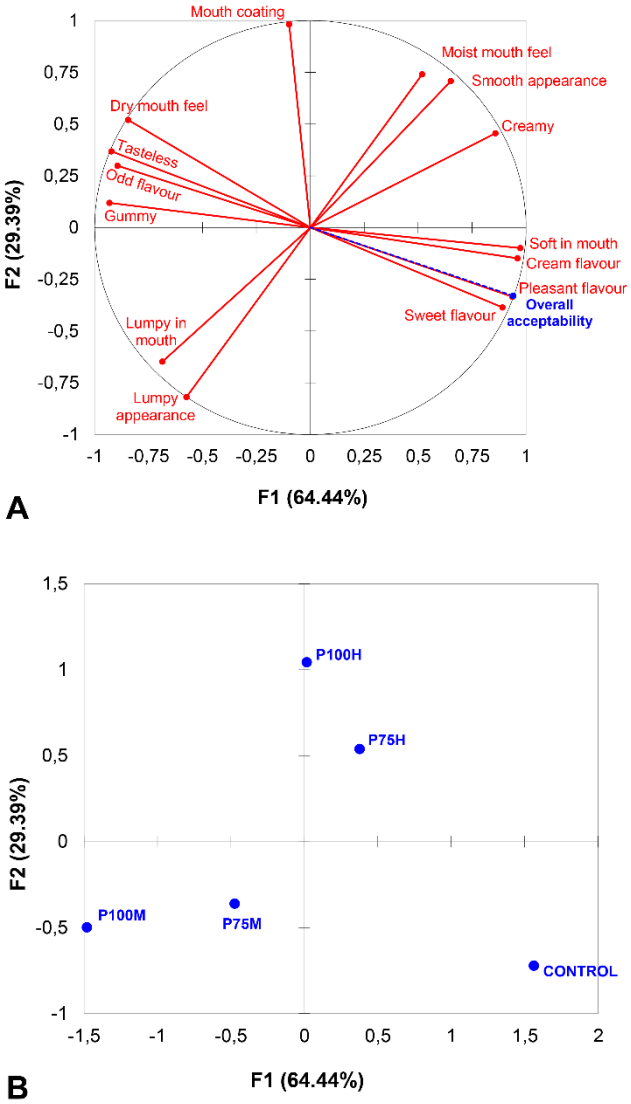

Fig. 4. A) Attribute map from the check-all-that-apply (CATA) questionnaire, and **B)** representation of the five panna cottas in the first two dimensions of the multiple factor analysis (MFA) of the CATA counts. P75M and P100M: respectively 75% and 100% of cream replaced by MC emulsion. P75H and P100H: respectively 75% and 100% of cream replaced by HPMC emulsion.

4. Conclusions

There seems to be a direct relationship between the structure of the emulsions (MC and HPMC) and the texture values of the panna cottas. The MC emulsion, which has a compact/dense microstructure, gives greater firmness and stiffness to the panna cottas made with it, which were also described as lumpy. The HPMC emulsion gives a lighter structure and consequently less firmness and stiffness to the panna cottas made with it, which were described sensorially as being soft and smooth.

In the sensory study of the panna cottas, the most acceptable percentage of fat substitution was 75%. Specifically, the panna cotta with 75% of the cream replaced by the HPMC emulsion was rated the best by the consumers and presented similar attributes to those of the control sample. Consequently, although the texture and taste of the control panna cotta are better than those of the panna cotta prepared with 75% of cream substitution by HPMC emulsion, this emulsion could be used as a cream replacer to reduce the fat and calorie contents of a traditional panna cotta, with good consumer acceptability and purchase intention. In fact, the panna cottas with 75% substitution by HPMC emulsion were described as creamy, with smooth appearance and moist mouth feel.

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Cream replacement by hydrocolloid-stabilised emulsions to reduce fat digestion in panna cottas

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Abstract

The effect of emulsions based on different hydrocolloids (xanthan gum (XG), hydroxypropyl methylcellulose (HPMC) and methylcellulose (MC)) on the structural, textural and sensory properties and lipid digestibility of panna cottas was investigated. The hydrocolloid-based emulsions presented similar microstructures. However, the panna cottas formulated with these emulsions presented large microstructural differences, which therefore also led to different textural and sensory properties. The differences in the initial microstructure of the panna cottas, the microstructural changes that they exhibited at the beginning of lipid digestion and the mechanisms of emulsification imparted by the hydrocolloids led to different extents of digestion. The panna cotta prepared with the HPMC-based emulsion (PH) seemed to be well accepted by the consumers and it was the least-digested sample. These results may contribute to the manufacture of reduced lipid digestion foods which could be used in weight management.

Keywords: milk fat emulsion; xanthan gum; cellulose ethers; structure; sensory properties

1. Introduction

Maintaining a proportionate intake of fat is nutritionally necessary for health and wellbeing. However, overconsumption of dietary lipids can lead to an excessive daily energy intake, which is seen as a contributing factor to the obesity epidemic (Norton, Fryer, & Norton, 2013). Reducing the fat content of foods has commonly been proposed as a method for reducing the consumers' energy intake (Lett, Norton, & Yeomans, 2016) and still remains one of the most effective approaches that can be taken to minimise the impact of obesity and its related health issues (Norton et al., 2013).

However, reducing the fat content of foods usually decreases their desirable sensory qualities, because fat plays an important role in determining appearance, texture and taste (Li & Nie, 2016). Foods with reduced fat levels must therefore be carefully formulated to ensure that they maintain their desirable physicochemical, sensory and nutritional properties, as otherwise they will not be acceptable to consumers (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014).

An alternative approach that may maintain consumer satisfaction is to attempt to use other strategies associated with controlling fat digestion (Espinal-Ruiz et al., 2014). An understanding of the fate of lipids within the gastrointestinal human tract is important for designing functional foods to control the rate and extent of lipid digestion and absorption (Mun, Kim, McClements, Kim, & Choi, 2017). In general, the human body has excess capacity for fat digestion and the rate of fat digestion is controlled by the ability of lipase to bind to emulsion interfaces, which in turn is controlled by the emulsion size and interfacial composition (Golding & Wooster, 2010). The initial composition and structure of the interfacial layer

surrounding the lipid droplets in a food can be controlled by selecting specific emulsifier(s) and homogenisation conditions to prepare an emulsion (McClements, Decker, Park, & Weiss, 2008).

Hydrocolloids are used in dairy products for two reasons: because they improve the texture and protect against creaming or flocculation and because of their function as thickeners, gelling agents, stabilisers, etc. (Li & Nie, 2016). In addition, hydrocolloids are known to have an impact on the behaviour of lipids within the gastrointestinal tract, influencing lipid digestion through a variety of mechanisms: (i) they may bind to species that play a critical role in digestion, such as bile salts, phospholipids, enzymes or calcium; (ii) they may increase the viscosity of the intestinal phase, altering mass transport processes; (iii) they may form a protective coating around lipid droplets, thereby inhibiting lipase access; and (iv) they may promote lipid droplet aggregation, thereby changing the amount of lipid surface exposed to lipase, among other effects (Espinal-Ruiz et al., 2014).

For this study, three hydrocolloids (xanthan gum and two cellulose ethers) were selected because of their different characteristics. Xanthan gum is an anionic polysaccharide that thickens and stabilises emulsions due to its ability to modify the rheology of the continuous phase (Dickinson, 2009). Hydroxypropyl methylcellulose and methylcellulose are non-ionic hydrocolloids which present good emulsifying properties due to their surface activity and can also be used as thickeners and gelling agents due to their thermal gelation ability. These two cellulose ethers differ principally in their degree and type of chemical substitution. In addition, panna cottas were chosen for being a dairy dessert with high calorie and fat contents that could be occasionally consumed as an indulgent choice.

The aim of this study was to obtain dairy desserts (panna cottas) with reduced lipid digestion whilst maintaining their sensory qualities. To this end, milk fat emulsions stabilised with different hydrocolloids were used to replace all the dairy cream content in traditional panna cottas. The emulsions were formulated with the same fat content and type (butter) as dairy cream in order to maintain the sensory properties of the dairy dessert. Stabilising the emulsions with the hydrocolloids was expected to affect lipid digestion and so obtain an indulgent product (panna cottas) with reduced lipid digestion which could be used in weight management.

2. Materials and methods

2.1. Materials

Liquid cream (Pascual, Calidad Pascual SAU, Burgos, Spain) with a 35% fat content, butter (Consum, Iberleche SL, A Coruña, Spain), drinking water (Bezoya, Calidad Pascual SAU, Burgos, Spain), instant coffee (Carrefour, SEDA Outspan Iberia SL, Palencia, Spain) and liquid sweetener (12% sodium cyclamate and 1.2% saccharin) (Consum, Krüger GmbH & Co. KG., Bergisch Gladbach, Germany) were purchased from local supermarkets. Skimmed milk powder was kindly supplied by Central Lechera Asturiana (Siero, Spain). Xanthan gum (Satiaxane CX™ 911) and κ-carrageenan (Satiagel™ ME5) were obtained from Cargill France SAS (Saint-Germain-en-Laye, France). Sodium alginate (MANUCOL DMF) was purchased from FMC Biopolymer (Philadelphia, PA, USA). The cellulose ethers — hydroxypropyl methylcellulose (METHOCEL™ F4M; 6.8% hydroxypropyl, 29% methoxyl) and methylcellulose (METHOCEL™ A4M; 30% methoxyl) — were kindly supplied by The Dow Chemical Company (Bomlitz, Germany).

Lipase from porcine pancreas Type II (L3126), bile extract (B8631) and sodium phosphate (monobasic, monohydrate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcium chloride ($CaCl_2$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium phosphate (dibasic, dodecahydrate) and toluidine blue O (C.I. 52040) were purchased from Panreac Química (Barcelona, Spain).

2.2. Emulsion preparation

The emulsions were prepared with butter (42.68% w/w), drinking water (57.07% w/w) and one of the hydrocolloids (0.25% w/w): (xanthan gum (XG), hydroxypropyl methylcellulose (HPMC) or methylcellulose (MC)).

The aqueous phase for the emulsion prepared with XG was prepared by dispersing XG in water (80 °C) using a stirrer (RZR 1, Heidolph, Schwabach, Germany) at 283 rpm for 1.5 min and subsequently at 464 rpm for 2.5 min.

The aqueous phase for the emulsions prepared with cellulose ethers was prepared with the "hot/cold" technique (Espert et al., 2017), with some modifications. Each cellulose ether was dispersed in 2/3 of the total water (80 °C) at 283 rpm for 1.5 min, then the beaker with the dispersed cellulose was transferred to a cold water bath. The rest of the water (1/3) was added at 4 °C and the aqueous phase was stirred continuously at 464 rpm for 2.5 min.

The butter was heated at 60 °C and added to the aqueous phase. The mixture was homogenised (Ultraturrax T18, IKA, Staufen, Germany) for 1 min at each speed (6400, 10 000, 14 0000 and 18 000 rpm), obtaining the following emulsions: EX (emulsion with XG), EH (emulsion with HPMC), or EM (emulsion with MC).

2.3. Panna cotta preparation

The reformulated panna cottas (PX, PH or PM) were prepared by replacing all the liquid cream content in the control panna cotta formulation with the relevant emulsion (EX, EH or EM, respectively). All the panna cottas had the same fat content (25.98% w/w).

The panna cottas were prepared according to the method used by Borreani, Llorca, Quiles, & Hernando (2017) with some modifications. The different ingredients (reconstituted skimmed milk powder (4.12% w/w) in drinking water (20.58% w/w), κ-carrageenan (0.33% w/w), sodium alginate (0.25% w/w), liquid sweetener (0.25% w/w) and instant coffee (0.37% w/w)) were placed in a cooking device (Thermomix™ 31, Wuppertal, Germany) and heated to 70 °C with continuous stirring (700 rpm). After reaching this temperature, the liquid cream (74.10% w/w) or the emulsion (74.10% w/w) was added and the mixture was maintained under the same conditions (70 °C, 700 rpm) for 6 min, then placed in silicone moulds (truncated cone; base circle: 6.3 cm, height: 3.7 cm, top circle: 4.5 cm) and cooled to ambient temperature. The moulds were covered with aluminium foil and stored at 4-5 °C until they were analysed.

2.4. Microstructure analysis

2.4.1. Light microscopy (LM)

For light microscopy (LM) observation, one drop of the emulsion (cream, EX, EH or EM) or a slim section from a frozen (−20 °C for 24 h) cube of the panna cotta, cut with a razor blade (single edge carbon steel, Electron Microscopy Science,

Hatfield, PA, USA), was placed in the centre of the microscope slide. The samples were stained with 0.002 g mL^{-1} toluidine blue and were examined under a Nikon Eclipse E80i® light microscope (Nikon, Tokyo, Japan). The images were captured and stored at 1280 x 1024 pixels using the microscope software (NIS-Elements F, version 4.2, Nikon, Tokyo, Japan).

2.4.2. Cryo-Scanning Electron Microscopy (Cryo-SEM)

The samples were prepared and observed according to Hernández-Carrión, Hernando, Sotelo-Díaz, Quintanilla-Carvajal, & Quiles (2015). A Cryostage CT-1500C (Oxford Instruments Ltd., Witney, UK) coupled to a JSM-5410 scanning electron microscope (Jeol, Tokyo, Japan) was used. The samples were placed in the holder, frozen in slush nitrogen (–210 °C) and quickly transferred to the Cryostage where fracture of the sample took place. The samples were then etched (–90 °C) for 10 min and gold-coated (2 mbar and 2 mA). Observations in the scanning electron microscope were carried out at 15 kV and -130 °C, with a working distance of 15 mm.

2.4.3. Particle size measurement

The particle size (mean area of fat droplets and aggregates) of fresh, pre-digested and t = 0 min panna cottas was determined from LM and cryo-SEM images with the microscope software (NIS-Elements D, version 4.2, Nikon, Tokyo, Japan).

2.5. Macrostructure analysis

Digital photographs (Olympus E-510, Tokyo, Japan) of the samples (emulsions and panna cottas) were taken after their preparation.

2.6. Texture analysis

A penetration test was performed in accordance with Borreani, Hernando, Salvador, & Quiles (2017). The firmness of the panna cotta was defined as the maximum force (N) measured during sample penetration and the stiffness ($N\ s^{-1}$) as the slope of the curve before the rupture point. The texture analysis was performed in triplicate.

2.7. Sensory analysis

The sensory analysis was carried out with 70 consumers (43 men and 27 women) recruited among the employees and students of the University. The samples (control, PX, PH and PM) were analysed in a sensory laboratory equipped with individual booths (ISO, 2007), according to the method described by Borreani et al. (2017). Each consumer tasted the control sample and the three reformulated panna cottas, presented monadically at a single session following a balanced complete block experimental design. The samples were served at room temperature, in small plastic cups coded with random three-digit numbers in random order. Water was supplied to clean the consumers' mouths between each sample.

The consumer acceptance test was performed using a nine-point hedonic scale (from 1 = dislike extremely to 9 = like extremely). For each panna cotta, the

consumers scored their degrees of liking in the following order: "appearance", "texture", "taste" and "overall acceptability".

2.8. *In vitro* intestinal model

An *in vitro* digestion model that simulated the small intestine was used, following the method of Li, Hu, & McClements (2011) with some modifications. The panna cotta samples (5 g) were mixed with phosphate buffer at a 1:3 ratio. The mixture was incubated at 37 °C for 10 min with continuous stirring at 760 rpm (pre-digestion step), then 5 mL of bile extract solution (275 mg of bile extract dissolved in phosphate buffer, pH 7.0) and 1 mL of salt solution (30.5 mg of $CaCl_2$ and 244.1 mg of NaCl dissolved in phosphate buffer, pH 7.0) were added to the samples and the mixture was adjusted to pH 7.0 (at this step the withdrawn aliquot corresponds to t = 0 min). Afterwards, 1.5 mL of freshly prepared lipase suspension (522 mg lipase powder dispersed in phosphate buffer; 1:2.5 enzyme/substrate ratio) were added to the mixture and titration started. The mixture was maintained at 37 °C and 760 rpm for 2 h to mimic conditions in the small intestine, withdrawing aliquots at intervals during the small intestine stage (t = 30, 60 and 120 min). The final composition of the sample in the reaction cell was 1305 mg of lipid, 10 mg mL^{-1} of bile extract, 19 mg mL^{-1} of lipase, 10 mM $CaCl_2$ and 150 mM NaCl. A pH-stat automatic titration unit (Mettler-toledo DL 50, Greinfensee, Switzerland) was used to automatically monitor the pH and maintain it at pH 7.0 by titrating appropriate amounts (mL) of NaOH solution (0.5 M). The volume of NaOH added to the sample was recorded and used to calculate the concentration of free fatty acids (FFA) generated by lipolysis, in other words, the number of moles of NaOH required to neutralise the FFA (assuming 2

FFA produced per 1 triacylglycerol molecule). The measurement was carried out in triplicate.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to study the differences between formulations in all the experiments, employing XLSTAT statistical software (Addinsoft, NY, USA). The least significant differences (LSD) were calculated by Fisher's test ($P < 0.05$).

3. Results and discussion

3.1. Microstructure analysis

3.1.1. Emulsions

The microstructure of the commercial cream and of emulsions EX, EH and EM is shown in Fig. 1. In all the emulsions, the fat was observed as circular globules distributed homogeneously throughout the sample. The fat globules in the commercial cream (Fig. 1A) were smaller and more homogeneous in size than those of the other emulsions. This was probably due to the different homogenisation processes used in preparing the commercial cream (high-pressure homogeniser) and the EX, EH and EM emulsions (ultraturrax). Moreover, the higher protein content in the commercial cream than in milk fat emulsions (EX, EH and EM) could be an additional explanation to the smaller droplet size in the commercial cream. The microstructure of emulsions EX, EH and EM (Fig. 1B-D) was similar and few differences in globule size were observed between them.

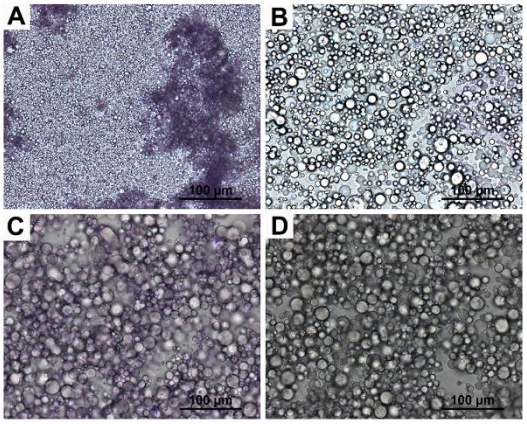

Fig. 1. Light microscopy images of the emulsions employed to prepare the panna cottas. A: commercial liquid cream; B: emulsion with XG (EX); C: emulsion with HPMC (EH); D: emulsion with MC (EM). The scale bars measure 100 μm.

3.1.2. Panna cottas

The microstructure of the control and of the reformulated panna cottas (PX, PH and PM) can be observed in Fig. 2. The control panna cotta presented a homogeneous microstructure (Fig. 2A) with the fat (uncoloured) embedded in the continuous network formed by proteins and polysaccharides (purple). In panna cottas PX and PH (Fig. 2B and C), well-defined round fat globules (black arrows) homogeneously distributed inside the continuous network were observed. However, these panna cottas (PX and PH) also presented some fat aggregates (red arrows in Fig. 2B and C), which seemed to be smaller in PX than in PH. In contrast, panna cotta PM (Fig. 2D) exhibited a heterogeneous microstructure in which most of the fat was observed to have flocculated and/or coalesced, resulting in clumps of fat.

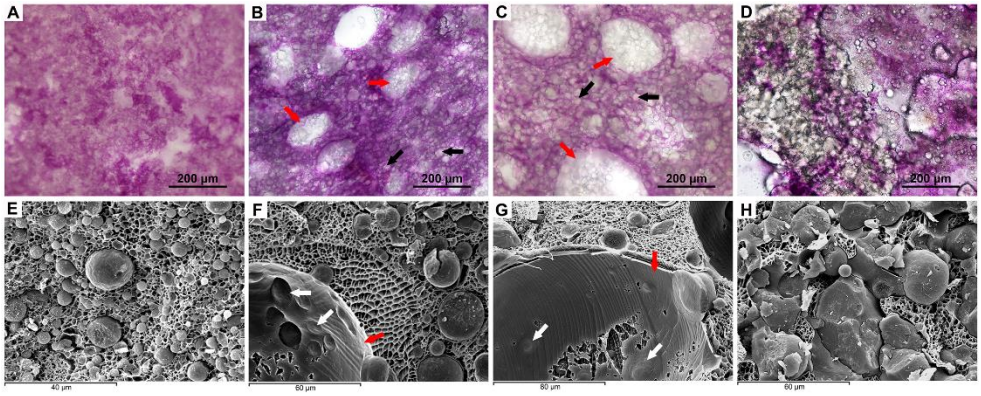

Fig. 2. Light microscopy (A-D) and cryo-SEM (E-H) images of the panna cottas. A and E: control; B and F: PX; C and G: PH; D and H: PM. The scale bars measure 200 µm (A-D), 40µm (E), 60 µm (F, H) or 80 µm (G). Red arrows show emulsion fragments, black arrows show fat globules embedded in the protein and polysaccharide network and white arrows show fat globules in the emulsion fragments.

The cryo-SEM microscopic technique provided more detail on the microstructure of the panna cottas (Fig. 2E-H). The fat in the control panna cotta was observed as round, well-defined fat globules, homogeneously distributed through the network (Fig. 2E) and smaller and more homogeneous in size than those in panna cottas PX and PH (Fig. 2F and G). This was due to the commercial cream having a smaller initial droplet size than in emulsions EX and EH, as previously observed in Fig. 1. The fat aggregates of panna cottas PX and PH (red arrows in Fig. 2B and C) seemed to be fragments of the emulsions (EX or EH, respectively) embedded in the continuous network formed by proteins and polysaccharides (red arrows in Fig. 2F and G). In these fragments of emulsions, fat globules (white arrows) remained trapped in the entangled hydrocolloid network. Because of xanthan gum possesses stable viscosity within a wide range of temperatures (Katzbauer, 1998) and HPMC possesses thermal gelation around 70 °C, emulsions EX and EH could have

maintained their structure during the preparation of panna cottas PX and PH respectively and thus, some fragments of these emulsions formed by stirring were present. The heterogeneous aspect of panna cotta PM in Fig. 2D was due to the undefined shape of the fat and the disrupted network (Fig. 2H). Therefore, emulsion EM seemed to have been destabilised during the preparation of panna cotta PM and thus, some flocculation and coalescence occurred resulting in the presence of clumps of fat.

Although no major microstructural differences were observed between emulsions EX, EH and EM (Fig. 1B-D), great differences were observed between their panna cottas, respectively PX, PH and PM (Fig. 2B-D and F-H). This shows that each emulsion was affected differently by the panna cotta preparation conditions and/or that different interactions took place between the emulsions and the other ingredients. Therefore, the use of different hydrocolloids leads to different complex matrix structures in the panna cottas.

3.2. Macrostructure analysis

3.2.1. Emulsions

The macroscopic appearance of the four emulsions (commercial cream, EX, EH and EM) is shown in Fig. 3A-D. The commercial cream (Fig. 3A) exhibited a smooth white appearance. Emulsion EX (Fig. 3B), also had a smooth appearance, but with a yellowish colour due to the use of butter, as also observed in the other emulsions EH and EM (Fig. 3C and D). However, the emulsions formulated with cellulose ether, EH and EM, both presented a lumpy appearance, which was more pronounced in the case of emulsion EM. As no great differences were found between

emulsions EX, EH and EM at microscopic level (Fig. 1B-D), the lumpy appearance of emulsions EH and EM could be due to the use of the cellulose ethers.

3.2.2. Panna cottas

The macroscopic appearance of the four panna cottas (control, PX, PH and PM) is shown in Fig. 3E-H. The control panna cotta (Fig. 3E) appeared smooth – except for the presence of some air bubbles – and had a slightly lighter colour than the other panna cottas, probably due to the whitish colour of the cream. Since emulsion EX, which had a smooth appearance (Fig. 3B), imparted the same characteristics to panna cotta PX (Fig. 3F), while panna cottas PH (Fig. 3G) and PM (Fig. 3H) exhibited a lumpy appearance, which was more pronounced in the case of the PM panna cotta, the macroscopic appearance of the emulsion appears to influence that of the panna cotta. The PM panna cotta's having a lumpier appearance than the PH panna cotta was mainly due to the presence of methylcellulose, which also gave this appearance to emulsion EM (Fig. 3D), as noted in the previous subsection.

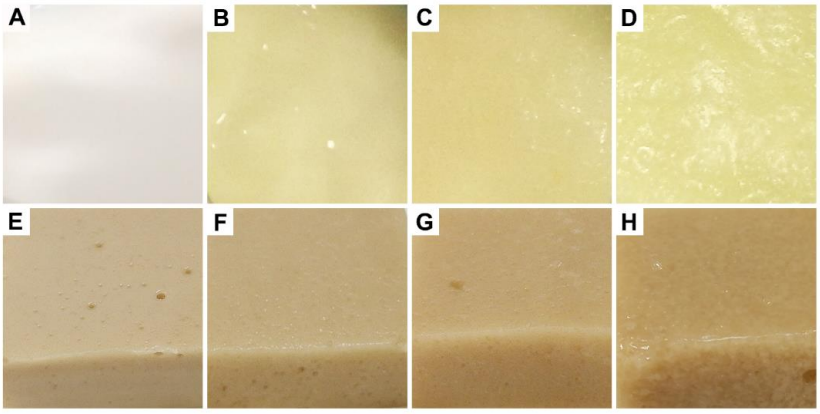

Fig. 3. Macroscopic structure of the emulsions (A-D) and panna cottas (E-H). A: commercial cream; B: EX; C: EH; D: EM; E: control; F: PX; G: PH; H: PM.

3.3. Texture analysis

The firmness and stiffness values obtained in the puncture test are shown in Table 1. The firmness values of the different panna cottas analysed in this study ranged from 0.80 N (PM) to 0.85 N (control). Panna cottas PH and PM exhibited significantly ($P < 0.05$) lower firmness values than the control and PX panna cottas. As regards the stiffness values, the control was significantly ($P < 0.05$) stiffer than all the other panna cottas.

Table 1. Influence of cream replacement by milk fat emulsions on the texture of the panna cottas.

Sample	Firmness (N)	Stiffness (N s^{-1})
Control	0.85 ± 0.02 [a]	2.05 ± 0.10 [a]
PX	0.84 ± 0.03 [a]	1.76 ± 0.10 [b]
PH	0.81 ± 0.05 [b]	1.82 ± 0.13 [b]
PM	0.80 ± 0.05 [b]	1.80 ± 0.09 [b]

Mean values ± standard deviations. Values with different letters within the same column are significantly different ($P < 0.05$) according to the LSD multiple range test.

The differences in firmness and stiffness values could be attributed to the different hydrocolloids used and/or to the distribution of the fat in the complex matrix of the panna cottas. On the one hand, the xanthan gum improved the firmness in comparison with the cellulose ethers, due to their different chemical structures and thus to their different thickening properties. Xanthan gum forms a high-viscosity pseudoplastic material and possesses stable viscosity within a wide range of pH values, temperatures and salt contents (Katzbauer, 1998), whereas

cellulose ether solutions undergo a sol−gel transition upon heating and return to a solution on cooling (Sanz, Falomir, & Salvador, 2015). The temperature at which the gelation process starts and the strength of the gel formed depend on the type and degree of cellulose substitution, the molecular weight and the presence and concentration of electrolytes (Sanz et al., 2015). As a result, the low temperature of the panna cottas (stored at 4-5 °C until texture analysis) may have reduced gel network formation in the panna cottas prepared with the cellulose ethers, which could be the reason for their exhibiting lower firmness values than those prepared with xanthan gum. Moreover, these results were in accordance with the microstructure results (Fig. 2), where panna cotta PX presented a more compact network than the cellulose ether panna cottas. On the other hand, since the PX panna cotta contained 0.19% w/w of hydrocolloid (XG) due to the incorporation of emulsion EX, it was expected to exhibit higher firmness than the control panna cottas, yet obtained almost the same ($P > 0.05$) firmness values. Therefore, the microstructure of the panna cottas also contributed to their texture characteristics. The small, well-defined fat globules homogeneously distributed in the continuous network of the control panna cotta (Fig. 2A and E) could provide a higher degree of cross-linking of protein molecules than in panna cotta PX, which exhibited larger fat globules and fragments of emulsion, and, especially, than in panna cotta PM, where the fat formed clumps (Fig. 2D and H). The higher degree of protein molecule cross-linking could result in three-dimensional networks with a more compact structure that would exhibit higher resistance to deformation (Lobato-Calleros et al., 2007) and thus provide higher firmness and stiffness values. Moreover, the high protein content of the control panna cotta, due to using cream in the formulation, could lead to the formation of a harder network surrounding the fat globules.

3.4. Sensory analysis

The consumer acceptability results for the different panna cottas are shown in Table 2. The control and PX panna cottas obtained the same appearance score. This could be due to the smooth appearance of both, as observed at macroscopic level in Fig. 3A-B. Despite the slightly lumpy appearance of panna cotta PH, shown in Fig. 3H, and thus its lower appearance score, no significant differences ($P > 0.05$) were observed between the control and PH panna cottas. As expected, panna cotta PM obtained significantly ($P < 0.05$) the worst result due to having the lumpiest appearance.

As regards the texture acceptability results, the control panna cotta was rated significantly ($P < 0.05$) the best. Surprisingly, no significant differences were found between panna cottas PX, PH and PM. Therefore, the lumpy macrostructure of panna cotta PM observed in Fig. 3H did not seem to have a significant effect on the consumers' texture acceptability ratings.

Table 2. Liking scores for appearance, texture, taste and overall acceptability of the panna cottas.

Sample	Appearance	Texture	Taste	Overall acceptability
Control	7.1 ± 1.2 ^{a}	7.3 ± 1.4 ^{a}	6.8 ± 1.8 ^{a}	6.9 ± 1.6 ^{a}
PX	7.1 ± 1.5 ^{a}	6.2 ± 1.7 ^{b}	4.8 ± 2.1 ^{b}	5.2 ± 1.9 ^{b}
PH	6.7 ± 1.3 ^{a}	6.4 ± 1.7 ^{b}	5.9 ± 2.0 ^{c}	6.0 ± 1.7 ^{c}
PM	6.1 ± 1.8 ^{b}	6.0 ± 1.7 ^{b}	5.6 ± 1.8 ^{c}	5.5 ± 1.6 ^{bc}

Mean values ± standard deviations. Values with different letters within the same column are significantly different ($P < 0.05$), according to the LSD multiple range test.

Replacing the cream with milk fat emulsions (EX, EH or EM) significantly affected ($P < 0.05$) the taste of the panna cottas. The control obtained the best score and PX the worst score, while PH and PM, the panna cottas formulated with cellulose ethers, obtained intermediate scores. On the one hand, Lett, Yeomans, Norton, & Norton (2016) found that flavour intensity significantly increased with decreasing droplet size. Thus, in line with this finding, the control panna cotta with the smallest droplet size (Fig. 2A and E) could lead to increased flavour intensity in comparison with the other panna cottas. The increased contact between the sample and the surface of the mouth could have enhanced flavour intensity (Lett, Yeomans, et al., 2016). On the other hand, the results demonstrated that the taste of the panna cottas seems to be dependent on hydrocolloid type, as Arancibia, Castro, Jublot, Costell, & Bayarri (2015) observed in their study on dairy desserts. It is generally believed that texture influences flavour perception and that an increase of viscosity, for example due to addition of hydrocolloids, generally leads to a decrease in aroma and taste perception (He, Hort, & Wolf, 2016; Tournier, Sulmont-Rossé, & Guichard, 2007). This is attributed to lowering diffusion rate of tastant molecules from the interior of the sample to the taste receptors on the tongue due to the increased solution viscosity. Nevertheless, some studies indicated that the impact of hydrocolloids on flavour perception appeared to be related to the physicochemical properties of the taste and aroma compounds (Bylaite, Adler-Nissen, & Meyer, 2005; Tournier et al., 2007), as well as to the nature of hydrocolloids (He et al., 2016). Therefore, the decrease of aroma and taste perception induced by hydrocolloids may also be the result of specific molecular interactions between the flavour compounds and the hydrocolloids (Bylaite et al., 2005). It was reported that xanthan decreases sweetness perception (He et al., 2016; Tournier et al., 2007), as well as aroma intensity of butyric acid and dimethyl sulphide (Tournier et al., 2007). In the present study,

texture acceptability scores did not show significant differences ($P > 0.05$) between panna cottas PX, PH and PM. Therefore, we hypothesised that the worse taste found in PX compared with panna cottas PH and PM, could be due mainly to different interactions (hydrophobic binding, hydrogen bonding, and/or entrapment) between xanthan gum and aroma and tastant compounds more than to different textures.

The overall liking scores for the different samples showed that the consumers preferred the control panna cotta, followed by PH, PM and finally PX. However, no significant differences ($P > 0.05$) were found between panna cottas PX and PM, or between panna cottas PH and PM.

In conclusion, although the fat type and content was maintained in the reformulated panna cottas (PX, PH and PM), the incorporation of different hydrocolloids significantly affected the sensory properties of the panna cottas. However, panna cotta PH, the best-rated among the reformulated panna cottas, seemed to be well accepted by the consumers.

3.5. *In vitro* intestinal digestion

It is important to take into account that food digestion is a sequential process, from mouth to stomach and from there to the intestine. Therefore, a multiple step model mimics the entire digestion process more accurately than a single step model. Nevertheless, the *in vitro* small intestine model is helpful for studying the factors that influence lipase activity during lipid digestion in a simpler and more reproducible way.

3.5.1. Microstructure

The microstructure of the panna cottas during *in vitro* small intestinal digestion is shown in Fig. 4. At the end of the pre-digestion step (samples diluted with phosphate buffer), the main change observed was the diluted appearance of all the panna cottas, as the fat was still embedded in the purple network.

After the addition of bile salts and electrolyte solutions (t = 0 min), important changes were observed in all the samples. The protein-hydrocolloid network seemed disrupted and many fat globules had coalesced, resulting in fat aggregates, and only a few small fat globules remained visible at this magnification. The coalescence phenomena were mainly due to the presence of bile salts and mineral ions. The bile salts may have fully or partially displaced the original emulsifier molecules from the lipid droplet surface, then the cationic sodium and calcium ions may have promoted droplet flocculation (Li & McClements, 2010) and thus the subsequent coalescence.

After 30 minutes of lipase digestion, well-defined fat globules were observed in all the panna cottas. This would suggest that the fat aggregates formed at t = 0 min as a result of coalescence phenomena had been attacked by the lipase molecules. From 30 to 120 min, no great changes were observed, only some coalescence phenomena and a lower number of fat globules due to the progress of digestion. This indicates that the main structural changes took place during the first 30 minutes and that the panna cottas, whatever their initial structure, underwent major changes during *in vitro* small intestinal digestion.

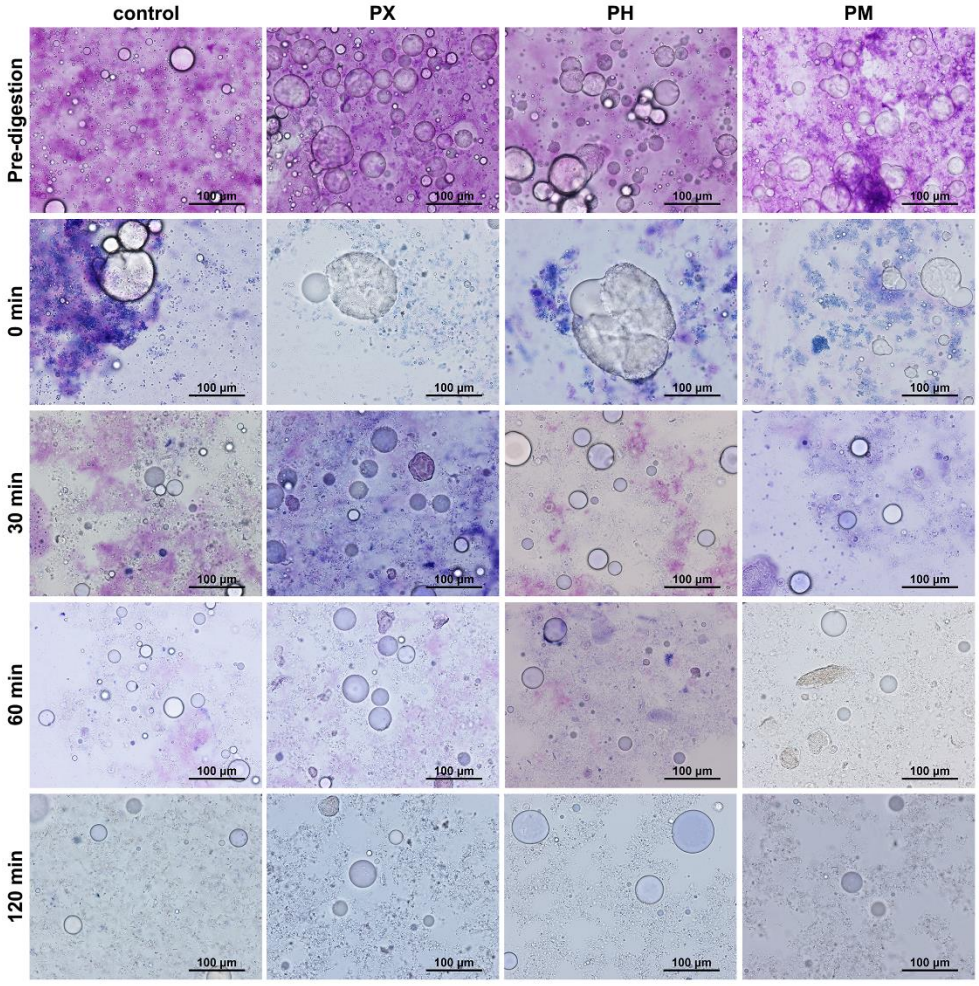

Fig. 4. Light microscopy images of the different panna cottas during the *in vitro* small intestine digestion. Pre-digestion: samples diluted with phosphate buffer during 10 min at 37 °C; 0 min: after the addition of bile salts and electrolytes solutions; 30 – 120 min: lipase digestion. The scale bars measure 100 µm.

3.5.2. FFA release

The lipid digestion profiles of all the panna cottas followed a similar pattern (Fig. 5). A rapid increase in FFA release was observed in the initial period (the first 10-20 min), followed by a more gradual increase over longer times. Therefore, lipid digestion seemed to occur mainly during the first 20 min, as observed in the microstructure findings (Fig. 4). The slower rate of FFA release after 10-20 min could be associated with an accumulation of lipolysis products at the droplet surface, which could compete with the lipase molecules for adsorption at the interface, reducing the lipase activity (Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016; Mun, Decker, & McClements, 2007).

To obtain a clearer comparison between the samples, the initial rate of FFA release was calculated from Fig. 5 by fitting a straight line to the first ten minutes, according to the Chang & McClements' (2016) method, and calculating the final extent of digestion at the end of 2 h incubation. These results are presented in Table 3.

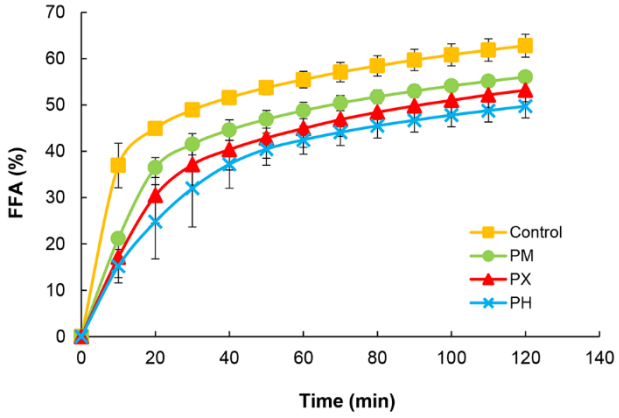

Fig. 5. Free fatty acid (FFA) released under *in vitro* small intestinal conditions from the different panna cottas. The error bars represent standard deviations.

Table 3. Influence of the emulsion structure and composition on the initial digestion rate and extent of final digestion of the panna cottas.

Sample	Initial rate (%FFA/min)	Extent (%FFA)
Control	3.69 ± 0.48 [a]	62.81 ± 2.47 [a]
PX	1.71 ± 0.44 [b]	53.19 ± 2.46 [bc]
PH	1.52 ± 0.36 [b]	49.72 ± 2.49 [b]
PM	2.12 ± 0.07 [b]	56.06 ± 1.25 [c]

Mean values ± standard deviations. Values with different letters within the same column are significantly different ($P < 0.05$), according to the LSD multiple range test.

The initial digestion rate was appreciably influenced by substituting milk fat emulsions for the cream. The control panna cotta was initially digested faster than the other panna cottas. This could be attributed to the smaller size of the lipid droplets in the control panna cotta (Figs. 2 and 4 at the pre-digestion step) and the slight coalescence phenomena observed before the addition of lipase (Fig. 4 at t = 0 min). As a result of its small droplet size, the control panna cotta presented an increase in surface area and therefore more sites for lipase molecules to bind to the lipid substrate, resulting in a relative increase in the rate of lipolysis. Although panna cotta PM at t = 0 min (Fig. 4) exhibited less extensive coalescence and therefore a higher initial digestion rate than panna cottas PX and PH, no significant ($P > 0.05$) differences between them were observed (Fig. 5 and Table 3). After the first 10 minutes, a clear differentiation between panna cottas PX, PH and PM was observed (Fig. 5), and the final extent of digestion of all the panna cottas showed the following order: control > PM ≥ PX ≥ PH (Fig. 5 and Table 3). The different digestion rate and extent results could be due mainly to the different initial structures of the panna cottas (observed in Fig. 2) and to the changes observed at the beginning of digestion

(at the pre-digestion step and at t = 0 min), but also to the different mechanisms of emulsification imparted by the hydrocolloids. The control panna cotta presented the smallest fat globule size initially (Fig. 2A and E, ≈ 54 μm^2) and the least extent of coalescence at t = 0 min (Fig. 4, ≈ 9 mm^2), providing more accessible sites for lipase molecules, and thus was the most-digested sample. Panna cottas PX and PH initially presented some fragments of emulsion (red arrows in Fig. 2) and larger fat globules (Fig. 2B and F, ≈ 500 μm^2 and Fig. 2C and G, ≈ 771 μm^2) than the control panna cotta (Fig. 2A and E, ≈ 54 μm^2), as well as larger fat aggregates (Fig. 4, ≈ 23 and 26 mm^2) at t = 0 min (due to coalescence phenomena), so the fat was less accessible to the lipase molecules and these were the least-digested samples. Moreover, the fragments of emulsion and the fat aggregates at t = 0 min were larger in panna cotta PH (Fig. 2C, ≈ 31 mm^2 and Fig. 4, ≈ 26 mm^2) than in panna cotta PX (Fig. 2B, ≈ 11 mm^2 and Fig. 4, ≈ 23 mm^2), consequently panna cotta PH was finally the least-digested sample. In addition, despite xanthan gum does not have surface activity, it has the ability to thicken the system, which could imply a physical impediment for the lipase to reach the interface, as well as it could interact with pre-adsorbed protein at the oil-water interface and thus hinder lipase to attach to. This could also explain why panna cotta PX exhibited lower digestion extent than the control and PM panna cottas. Moreover, HPMC has been reported to form a physical barrier on the interface, which is resistant to displacement by bile salts. Consequently, it is difficult for lipase to access the interface required for lipid digestion (Torcello-Gómez & Foster, 2016) and thus, this could also explain why panna cotta PH was the least-digested sample. In the case of the PM panna cotta, the clumps of fat (Fig. 2D and H) could present a smaller surface area than the small droplets in the control panna cotta (Fig. 2A and E) and thus, the PM panna cotta obtained a lower result for the extent of digestion than the control panna cotta. Moreover, because emulsion EM was destabilised

during the preparation of the relevant panna cotta (PM), methylcellulose molecules could have formed a weak protective layer around fat droplets; consequently, panna cotta PM was digested to a higher extent than panna cottas PX and PH.

4. Conclusions

On the one hand, this study shows that different hydrocolloids influence both the micro- and macrostructure of the panna cottas. Therefore, although the dairy fat type and content was maintained, the reformulated panna cottas possessed different textural and sensory properties from the control panna cotta. However, panna cotta PH obtained good scores for the attributes assessed and seemed to be well accepted by the consumers. On the other hand, this study shows that both the superficial area of the fat and the different mechanisms of emulsification imparted by the hydrocolloids have an impact on lipid digestion. Thus, because of panna cotta PH presented the fewest bind sites for the lipase molecules (high superficial area of the fat and strong surface-activity of HPMC molecules), it was the least-digested sample. These results may contribute to the manufacture of reduced lipid digestion foods which could be used in weight management.

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Discusión general de los resultados

La tesis abarca distintas estrategias para el estudio y diseño de alimentos de origen lácteo para incidir sobre el control de peso y tratamiento de la obesidad; por un lado, aumentando la capacidad saciante de los alimentos mediante la adición de ingredientes con alto poder saciante (proteínas lácteas y/o fibras solubles) y, por otro lado, reduciendo el aporte en grasa al organismo mediante la sustitución de ésta por emulsiones de menor contenido en grasa o de reducida digestibilidad lipídica. La matriz alimentaria elegida fue el postre lácteo panna cotta de alto contenido graso y proteico, el cual podría mantener altas expectativas hedónicas como producto indulgente, pero tras su reformulación aportaría además a los consumidores efectos saciantes o menor aporte en grasa, los cuales son beneficios para la salud y están relacionados con el control de peso.

Diseño de productos lácteos con capacidad saciante

En un primer estudio, la hipótesis de partida fue que la **adición de un hidrocoloide neutro (glucomanano konjac) o cargado negativamente (alginato sódico)** podría interactuar de diferente manera con los distintos tipos de proteínas lácteas (proteínas del suero y/o caseínas) empleadas en la formulación de **sistemas proteicos lácteos simples**. En consecuencia, sería interesante estudiar si esta interacción podría influir en la digestibilidad proteica de los sistemas lácteos y en su capacidad saciante.

El estudio de la **digestión de las proteínas lácteas** procedentes de diferentes fracciones proteicas de la leche (leche en polvo, concentrado de proteínas del suero o caseinato cálcico) reveló que las **proteínas del suero** permanecieron prácticamente **intactas** a lo largo de la digestión gástrica *in vitro*; mientras que las caseínas fueron rápidamente atacadas por la pepsina. En general, la **mayor velocidad de vaciado gástrico** de las proteínas del suero en el intestino y, de forma más inalterada (en

comparación con las caseínas), suele dar lugar a un **rápido y elevado incremento postprandial de la concentración de aminoácidos en plasma**, lo que se traduce en una mayor capacidad saciante a corto plazo.

La adición de **glucomanano konjac**, debido su gran capacidad de absorción y retención de agua, permitió obtener sistemas lácteos de elevada **viscosidad** durante la digestión gástrica *in vitro*; lo que podría dar lugar a un aumento de la **distención gástrica**, modulando así la capacidad saciante de los sistemas lácteos. No obstante, la adición de konjac no pareció afectar a la digestión de las proteínas en los diferentes sistemas, posiblemente debido a la ausencia de interacciones proteína-hidrocoloide en esos sistemas simples.

Por otra parte, a pesar de que el **alginato** formó agregados gelificados en presencia de calcio y a pH bajo, su adición no provocó un aumento en la viscosidad de los sistemas lácteos durante la digestión gástrica *in vitro*, presentando éstos, por tanto, valores similares a los sistemas formulados sin adición de hidrocoloides. Sin embargo, el alginato, por su carga negativa, interaccionó fuertemente con las proteínas formando grandes agregados resistentes a la acción de la pepsina, lo que retrasó la digestibilidad proteica de los batidos. La **prolongación de la digestión de las proteínas en el estómago** también podría considerarse una buena estrategia para prolongar la liberación de hormonas relacionadas con la saciedad.

En resumen, la adición de diferentes hidrocoloides permite modular la capacidad saciante de sistemas lácteos mediante diferentes mecanismos. Sin embargo, se debe tener en cuenta que tanto la distensión gástrica como la exposición intestinal de los nutrientes juegan un papel fundamental y temporalmente simultáneo en la regulación del apetito, por lo que es difícil saber si realmente un mecanismo tiene un mayor efecto sobre la saciedad que el otro.

En un segundo estudio, la hipótesis planteada fue que la **incorporación de cantidades extra de proteína**, así como la **eliminación de diferentes cantidades de grasa,** podrían provocar grandes cambios en las características estructurales de las **panna cottas**, lo que a su vez podría tener un efecto sobre la capacidad saciante.

Dependiendo del tipo de proteína láctea añadida a cada panna cotta, se obtuvieron matrices con diferentes grados de interacción entre sus componentes, y por lo tanto con distintos valores de firmeza. Concretamente, las panna cottas formuladas con un **contenido extra de proteínas** procedentes del **suero lácteo**, las cuales tienen especial habilidad para formar matrices compactas debido a la interacción proteína-proteína, fueron las que presentaron la matriz más densa y agregada y, por tanto, fueron las más **firmes**. Además, la **reducción del contenido en nata** favoreció el entrecruzamiento de la red proteica. Debido a que la textura parece ser la característica sensorial más estrechamente relacionada con la percepción de capacidad saciante, estas panna cottas son las más adecuadas para aportar **capacidad saciante** al consumidor.

A pesar de la desnaturalización de las proteínas del suero durante el tratamiento térmico empleado en la preparación de las panna cottas y de las posibles interacciones con los demás ingredientes, las **proteínas del suero** siguieron siendo **más resistentes a la proteólisis** durante la digestión gástrica *in vitro* que las caseínas, al igual que en los sistemas modelo lácteos simples, lo que también está relacionado con una **mayor capacidad saciante a corto plazo**.

En este sentido, tanto la mayor firmeza de las panna cottas formuladas con un contenido extra de proteínas del suero y con menor contenido en grasa, como la rápida y elevada liberación de hormonas relacionadas con la saciedad, podrían contribuir simultáneamente al aumento de la capacidad saciante.

En conclusión, para lograr un adecuado diseño de productos lácteos con elevada capacidad saciante, es importante comprender cómo afecta la adición de diferentes ingredientes, y sus posibles interacciones, sobre la estructura, textura y digestibilidad proteica de los productos alimentarios y su relación con los mecanismos de saciedad.

Uso de emulsiones para reducir el aporte en grasa al organismo

El uso de emulsiones aceite/agua permite no solo reducir el contenido en grasa y calorías de los alimentos, ya que parte de la grasa es reemplazada por agua, sino que, si se diseñan adecuadamente, pueden también reducir la digestibilidad lipídica.

En primer lugar, se diseñaron **emulsiones** con un alto contenido en grasa (47%) y con diferentes tipos de **derivados de celulosa** con el objetivo de obtener emulsiones con un elevado grado de consistencia para poder emplearlas como **sustitutos de grasa sólida**, como por ejemplo la mantequilla, manteca o grasa plástica ("shortening"), las cuales suelen presentar un contenido en grasa del 80% mínimo. Además, se estudió la digestibilidad lipídica de las diferentes emulsiones obtenidas.

La utilidad de los derivados de celulosa, **hidroxipropilmetilcelulosas** (HPMC) o **metilcelulosas** (MC), se basa fundamentalmente en que: 1) son espesantes eficientes, 2) tienen la capacidad de formar geles termorreversibles, 3) presentan actividad superficial y, por tanto, 4) tienen la habilidad de formar películas interfaciales. Gracias a estas propiedades, se pudo obtener emulsiones con **buena estabilidad fisicoquímica** durante el periodo de almacenamiento de un mes,

las cuales, además, **se digirieron casi un 50% menos** que una emulsión formulada con caseinato cálcico.

Concretamente, la emulsión formulada con el éter de **celulosa de alto metoxilo** fue la que presentó **mayor consistencia** antes y durante la digestión oral y gástrica *in vitro*, lo que podría influir en la distensión gástrica y, por tanto, en la **capacidad saciante**. Además, fue la emulsión que presentó el **menor grado de digestibilidad lipídica** al presentar una serie de características (mayor consistencia, mayor resistencia al desplazamiento por las sales biliares, mayor tamaño de glóbulos) que dificultaron el óptimo desarrollo de los procesos necesarios para la lipólisis. Por lo tanto, en este estudio se comprobó que tanto el tipo de emulsionante empleado, como el tamaño de los glóbulos de grasa y la consistencia de las emulsiones, son factores a tener en cuenta a la hora de elaborar emulsiones de reducida digestibilidad.

En segundo lugar, de acuerdo a los resultados obtenidos en el estudio anterior, se quiso ahondar más en el conocimiento de qué factor(es) tiene(n) un mayor impacto sobre la digestibilidad lipídica de las emulsiones. Para ello, se elaboraron una serie de **emulsiones estabilizadas con diferentes tipos de moléculas (polisorbato, lecitina, goma xantana o metilcelulosa)** y se analizó la influencia de cada una de ellas sobre las propiedades reológicas, de tamaño de partícula y microestructurales de las emulsiones antes y durante la digestión intestinal *in vitro.*

La caracterización reológica de las emulsiones reveló patrones de comportamiento al flujo muy diferentes dependiendo del tipo de emulsionante o estabilizante empleado, lo que influyó en la capacidad de obtención de glóbulos de grasa de tamaño similar durante la elaboración de las emulsiones. La **adición de los**

jugos intestinales, no solo afectó a las **características reológicas** disminuyendo drásticamente la consistencia de las emulsiones elaboradas con goma xantana o metilcelulosa, sino también al **tamaño de los glóbulos de grasa,** promoviendo fenómenos de coalescencia, y a la **estructura** de las emulsiones iniciales (cambios en la composición interfacial). A pesar de que las emulsiones elaboradas con polisorbato (Tween ® 20) presentaron menor consistencia y tamaño de glóbulos de grasa que las emulsiones estabilizadas con goma xantana, fueron las que finalmente presentaron el menor grado de digestibilidad lipídica. Esto se debe a que el polisorbato, debido a su elevada actividad superficial es capaz de resistir el desplazamiento por parte de las sales biliares y dificultar la adsorción de la enzima lipasa en la interfase de los glóbulos. Por el contrario, la goma xantana, al no presentar actividad superficial (ni siquiera al interaccionar con compuestos de los jugos intestinales), no dificultó el acceso de las sales biliares y de la enzima lipasa a los glóbulos, por lo que las emulsiones elaboradas con ese estabilizante presentaron el mayor grado de digestibilidad lipídica. Las emulsiones elaboradas con lecitina y metilcelulosa, presentaron un mayor grado de digestibilidad lipídica que las emulsiones formuladas con polisorbato. A pesar de presentar ambos emulsionantes (lecitina y metilcelulosa) buena actividad superficial, fueron parcial o totalmente desplazados por las sales biliares, facilitando el acceso a la enzima lipasa. Sin embargo, al interaccionar estos emulsionantes con los compuestos de los jugos intestinales, como son las sales biliares y la lipasa, en la fase acuosa y/o interfase, permitieron reducir y/o dificultar la digestibilidad lipídica, presentando así un menor grado de digestibilidad lipídica que las emulsiones elaboradas con goma xantana. En conclusión, a pesar de los cambios observados en cuanto al comportamiento reológico y al tamaño de los glóbulos de grasa –factores que también pueden influir en la digestibilidad lipídica-, en este estudio, las diferencias

encontradas en los resultados de lipólisis parecieron deberse principalmente al **tipo y localización de los emulsionantes** empleados (actividad superficial, propiedades fisicoquímicas) y a las **interacciones con compuestos de los jugos intestinales** como sales biliares y lipasa que pueden tener lugar en la fase acuosa y/o interfase durante la digestión intestinal.

En un tercer estudio del segundo capítulo, se reformularon las emulsiones con el objetivo de poder incorporarlas en la formulación del postre lácteo panna cotta para **reducir el contenido en grasa y minimizar posibles cambios en sus propiedades sensoriales**.

La sustitución de nata por emulsiones estabilizadas con **HPMC** permitió obtener panna cottas con una **microestructura ligera, poco compacta**, y por tanto fueron las más **blandas**. Concretamente, la panna cotta reformulada con un 75% de sustitución de nata por la emulsión HPMC fue caracterizada por los consumidores mediante atributos tales como "**apariencia lisa**", "**suave en boca**", "**cremosa**" y "**sensación húmeda en boca**", siendo finalmente la **panna cotta mejor valorada** tras el control, aunque no presentaron diferencias significativas entre sí en cuanto a la apariencia, aroma y textura. Por el contrario, la sustitución de nata por la emulsión estabilizada con **MC** hizo que las panna cottas presentaran una **estructura compacta y densa**, siendo las más **firmes** y, además. que se percibieran como "**grumosas**" tanto en apariencia como en boca, e "**insípidas**", características que hicieron que fueran las **peores valoradas**.

Por otra parte, tanto el estudio de la aceptabilidad global de las panna cottas como su caracterización mediante el método CATA permitieron establecer que el sabor y la textura son los atributos que más influyen en el consumidor a la hora de valorar un postre lácteo. En primer lugar, el sabor pareció estar relacionado con el

contenido en nata, siendo la panna cotta control la mejor valorada, seguida de las que presentaron un 75% de sustitución de nata y finalmente las que presentaron un 100% de sustitución. En segundo lugar, la textura pareció estar relacionada con el tamaño y distribución de los glóbulos de grasa en las panna cottas, con el tipo de hidrocoloide empleado en las emulsiones y con el porcentaje de nata. Este estudio muestra que es posible obtener panna cottas con un menor contenido en grasa (reducción del 37,5%) -al sustituir el 75% de la nata por una emulsión a base nata estabilizada con HPMC- y con una buena aceptabilidad sensorial.

Finalmente, en el último trabajo, se formularon **emulsiones a base de grasa láctea estabilizadas por diferentes hidrocoloides (HPMC, MC o goma xantana)** y se emplearon como sustitutos de grasa con el objetivo de **reducir la digestibilidad lipídica de las panna cottas**.

El uso de diferentes hidrocoloides para estabilizar emulsiones a base de grasa láctea empleadas como sustitutos de grasa provocó significantes cambios micro y macroestructurales, texturales y sensoriales en las panna cottas. Sin embargo, la panna cotta formulada con la emulsión estabilizada con **HPMC** fue la que obtuvo **mejores resultados en el análisis sensorial** tras la panna cotta control.

La **adición de los jugos intestinales** (en ausencia de la enzima lipasa) provocó **importantes cambios estructurales** en todas las muestras, lo que conllevó a **diferentes grados de digestibilidad lipídica** al añadir la enzima lipasa. No obstante, no solo la estructura inicial y los cambios sufridos fueron los únicos factores que afectaron a la digestibilidad lipídica, sino que también los mecanismos de emulsión impartidos por los diferentes hidrocoloides. La panna cotta **control** fue la **más digerida** por presentar mayor área superficial para la acción de la lipasa (glóbulos de grasa pequeños) y mayor desorción de las proteínas de la interfase. Por el

contrario, la **panna cotta formulada con la emulsión estabilizada con HPMC** fue la **menos digerida** por presentar menor área superficial para la acción de la lipasa (glóbulos grandes, fragmentos de emulsión, coalescencia), y mayor actividad superficial de la HPMC.

En resumen, la sustitución de grasa por emulsiones estabilizadas con diferentes hidrocoloides permite reducir la digestibilidad lipídica de las panna cottas. Sin embargo, aún se debe trabajar para mejorar la formulación de éstas y minimizar aún más el impacto de la sustitución de grasa sobre las propiedades sensoriales.

En conclusión, para lograr un adecuado diseño de productos lácteos con menor contenido en grasa o de reducida digestibilidad lipídica, es fundamental conocer la distribución e interacciones de los ingredientes en las matrices alimentarias ya que rigen tanto las propiedades texturales y sensoriales como la digestión de los productos alimentarios.

Conclusiones

- La adición de hidrocoloides a sistemas lácteos permite modular su capacidad saciante mediante diferentes mecanismos dependiendo de sus propiedades fisicoquímicas. Concretamente, la adición de un hidrocoloide neutro (goma konjac) permite incrementar la viscosidad durante la digestión gástrica *in vitro*, lo que podría incrementar la distensión gástrica, mientras que la adición de un hidrocoloide cargado negativamente (alginato) permite retrasar la digestibilidad proteica y podría prolongar la liberación de hormonas relacionadas con la saciedad. Este conocimiento es particularmente interesante para elegir adecuadas combinaciones en el diseño de productos lácteos con capacidad saciante.

- La adición extra de proteínas lácteas, en particular de proteínas del suero, y la reducción del contenido en grasa da lugar a panna cottas con una matriz más agregada y densa y, por tanto, una textura más firme que la panna cotta tradicional. Estos cambios en la formulación podrían ser un primer paso para la obtención de panna cottas de alta capacidad saciante.

- Las proteínas del suero lácteo son más resistentes a la acción de la enzima pepsina que las caseínas, tanto en sistemas modelo como en matrices lácteas más complejas tratadas a mayor temperatura. La resistencia de las proteínas del suero a la digestión gástrica está relacionada con una alta capacidad saciante.

- El uso de derivados de celulosa permite obtener emulsiones con buena estabilidad física y oxidativa, diferentes consistencias y grados de digestibilidad lipídica. Concretamente, el uso de metilcelulosa de alto metoxilo permite el diseño de emulsiones tipo “shortening” de elevada consistencia, incluso durante

la digestión gástrica *in vitro*, lo que puede favorecer los mecanismos postingestivos de saciedad (distensión gástrica); además de reducir la digestibilidad lipídica.

- Aunque el tamaño de los glóbulos de grasa y la consistencia de las emulsiones y sus posibles modificaciones durante la digestión (oral, gástrica e intestinal) son factores a tener en cuenta a la hora de diseñar adecuadamente emulsiones de reducida digestibilidad lipídica, también es fundamental la actividad superficial del emulsionante o estabilizante empleado, su localización en la fase acuosa o en la interfase y su capacidad de interaccionar con otros compuestos presentes en los jugos digestivos.

- Es posible obtener panna cottas con una reducción del contenido en grasa de hasta un 37,5%, con buena aceptabilidad global y bien valoradas por los consumidores mediante el uso de emulsiones estabilizadas con hidroxipropilmetilcelulosa (HPMC).

- El grado de digestibilidad lipídica de las panna cottas puede reducirse mediante la sustitución de nata por emulsiones estabilizadas por diferentes hidrocoloides, llegando hasta un 20% de reducción con el uso de HPMC. Estas panna cottas presentan un buen nivel de aceptabilidad sensorial.

- El uso de metodologías sensoriales, como los cuestionarios CATA y el análisis de aceptabilidad global, muestra que, tanto el sabor como la textura son los atributos más importantes para el consumidor a la hora de evaluar un postre lácteo, y que

estos atributos pueden verse perjudicados tras la reducción del contenido en nata y la incorporación de hidrocoloides. Sin embargo, las panna cottas formuladas con emulsiones estabilizadas con HPMC -empleadas para reducir el contenido en grasa o la digestibilidad lipídica de las panna cottas- se caracterizan mediante atributos positivos y presentan un buen nivel de aceptabilidad sensorial.