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Interactions between Bile Acids and Plant Compounds – with Particular Reference to the Fractionation and Processing of Lupin Seeds (*Lupinus angustifolius* L.)

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Contents

Preliminary Remarks

The work presented in this thesis is a selection of papers published in international peer reviewed journals, which are listed below.

Full papers

1. Naumann, S., Schweiggert-Weisz, U., Bader-Mittermaier, S., Haller, D., Eisner, P. (2018). Differentiation of adsorptive and viscous effects of dietary fibres on bile acid release by means of *in vitro* digestion and dialysis. *The International Journal of Molecular Sciences*, 19, 2193, doi: 10.3390/ijms19082193.
   (Special Issue: Dietary Fibre: New Insights on Biochemistry and Health Benefits)

2. Naumann, S., Schweiggert-Weisz, U., Eglmeier, J., Haller, D., Eisner, P. (2019). *In vitro* interactions of dietary fibre enriched food ingredients with primary and secondary bile acids. *Nutrients*, 11, 1424, doi:10.3390/nu11061424.
   (Special Issue: Dietary Fibre and Human Health)

3. Naumann, S., Schweiggert-Weisz, U., Haller, D., & Eisner, P. (2019). Retention of primary bile acids by lupin cell wall polysaccharides under *in vitro* digestion conditions. *Nutrients*, 11, 2117, doi: 10.3390/nu11092117.
   (Special Issue: Grains and Human Health)

4. Naumann, S., Schweiggert-Weisz, U., Eisner, P. (2020). Characterisation of the molecular interactions between primary bile acids and fractionated lupin cotyledons (*Lupinus angustifolius* L.). *Food Chemistry*, 323, 126780, doi: 10.1016/j.foodchem.2020.126780.

5. Naumann, S., Schweiggert-Weisz, U., Martin, A., Schuster, M., Eisner, P. (2021). Effects of extrusion processing on the physiochemical and functional properties of lupin kernel fibre. *Food Hydrocolloids*, 111, 106222, doi: 10.1016/ j.foodhyd.2020.106222.

Reviews

1. Naumann, S., Haller, D., Eisner, P., Schweiggert-Weisz, U. (2020). Mechanisms of interactions between bile acids and plant compounds – a review. *The International Journal of Molecular Sciences*, 21, 6495, doi: 10.3390/ijms21186495.

Further scientific contributions resulted from the same period.

Oral presentations

1. Naumann, S., Schweiggert-Weisz, U., Bader-Mittermaier, S., Eisner, P. (2018): *In vitro* digestion, dialysis and kinetic analysis – Adsorptive and viscous effects of dietary fibre on bile acid release. 7th International Dietary Fibre Conference 'Fibre Diversity in Food, Fermentation and Health', 4-6 June 2018, Rotterdam, Netherlands.

2. Naumann, S., Schweiggert-Weisz, U., Eisner, P. (2019): Lupin cell wall polysaccharides – influence of sequential extraction on the interaction with primary bile acids. 15th International Lupin Conference 'Developing lupin crop into a modern and sustainable food and feed source', 18–21 March 2019, Cochabamba, Bolivia.

3. Naumann, S., Schweiggert-Weisz, U., Eisner, P. (2020): Extrusion processing – influence on the physiochemical and functional properties of lupin kernel fibre. 34th EFFoST International Conference, 'Bridging high-tech, food-tech and health: Consumer-oriented innovations', 10–12 November 2020, Online event.

Poster presentations

1. Naumann, S., Schweiggert-Weisz, U., Eisner, P. (2019): Adsorptive and viscous interactions of dietary fibre enriched food ingredients with primary and secondary bile acids. IFT 19 Annual Meeting & Food Expo 'Feed your Future', 2–5 June 2019, New Orleans, LA USA.

2. Naumann, S., Schweiggert-Weisz, U., Eisner, P. (2019): *In vitro* interactions of lupin cotyledon fractions with primary bile acids. IFT 19 Annual Meeting & Food Expo 'Feed your Future', 2–5 June 2019, New Orleans, LA USA.

Awards

1. 3rd prize winner of the 'PhD Student of the Year Award' at 34th EFFoST International Conference, 'Bridging high-tech, food-tech and health: Consumer-oriented innovations', 10–12 November 2020, Online event.

Summary

Bile acids contribute to a variety of key systemic functions in the human body. Through activation of various signalling pathways and interaction with gut microbiota, bile acids regulate not only their own synthesis and enterohepatic circulation, but also triglyceride, cholesterol, glucose, and energy homeostasis. In particular, the hepatic production of primary bile acids represents the main metabolic pathway to remove excess cholesterol from the body. Furthermore, faecal levels of secondary bile acids formed by the gut microbiota correlate with markers of colorectal cancer risk. Secondary bile acids, particularly desoxycholic acids, are known to accumulate in the bile acid pool of individuals on a 'Western diet'. The development towards this diet came along with the decreasing consumption of fibre-rich plant food sources like vegetables, whole grains or legumes. The interference of bile acid metabolism by plant compounds as potential contribution to disease prevention is thus receiving increasing attention. Plant-based foods rich in dietary fibres are reported to interact with bile acids preventing their reabsorption and promoting their excretion into the colon. Yet, the properties and the nature of this interaction have not been fully understood.

To bridge the gap between physiological effects and the responsible molecular mechanisms, suitable *in vitro* methods are needed. The initial part of this thesis (CHAPTER 1) thus included a comparison of a widespread *in vitro* approach based on centrifugation with an adapted model based on *in vitro* digestion and equilibrium dialysis. Most studies to explain the interaction of plant compounds and bile acids allow a classification into two possible mechanisms, which need to be covered and differentiated *in vitro*. Either increased viscosity during digestion results in reduced micellar mobility of bile acids or bile acids and plant compounds are associated or complexed at molecular level. The comparison of *in vitro* approaches revealed that the centrifugal approach may not be appropriate to take into account the effects of viscous digestive matrices. Yet, reduction of bile acid diffusion by viscous networks is suggested to be a core mechanism of interaction especially for plant-based foods rich in dietary fibre. On the other hand, diffusion kinetics of bile acids across a dialysis membrane were demonstrated to include both molecular interactions and viscosity effects. Applying this model the *in vitro* digesta are dialysed as a simplified absorption model of the unstirred water layer. First order diffusion kinetics are analysed and evaluated to differentiate between bile acid retention due to viscosity and permanent molecular binding forces.

In a second study (CHAPTER 2), the method established in CHAPTER 1 was extended to the analysis of primary and secondary bile acids, which are predominantly present in human bile. In this approach, correlations between bile acid interactions and bile acid chemistry were

examined to further elucidate mechanistic principles. Ten dietary fibre enriched ingredients derived from different plant origins were digested *in vitro* and interactions with glyco- and tauroconjugated cholic acids, chenodesoxycholic acids, and desoxycholic acids were analysed. Viscous interactions were detected for apple, barley, citrus, lupin, pea, and potato derived ingredients, which slowed the bile acid release rate by up to 80%. In viscous matrices, diffusion was slowed down most for desoxycholic acids, followed by chenodesoxycholic acids and cholic acids. This delayed diffusion in viscous fibre matrices was further associated with the micellar properties (critical micelle concentrations and aggregation numbers) of the bile acids. Bile acid adsorption of up to 4.7 µmol/ 100 mg dry matter were significant in barley, oat, lupin, and maize preparations. Bile acid adsorption was revealed to decrease in the order of desoxycholic acid > chenodesoxycholic acid > cholic acid. These results indicated that hydrophobic interactions are core to the molecular interactions of bile acids with plant compounds. As increased viscous and adsorptive interactions were revealed for hydrophobic bile acids, our results indicate that the consumption of plant compounds can contribute to the clearance of procarcinogenic and proinflammatory secondary bile acids from the bile acid pool.

In continuation of the above mentioned work, structure-function relationships of the revealed viscous retention of bile acid diffusion and molecular interactions with bile acids were examined for lupin seeds (*Lupinus angustifolius* L.). Increased viscosity during digestion is mainly attributed to specific dietary fibres. It is further frequently hypothesised that bile acids may be adsorbed by hydrophobic surfaces of insoluble dietary fibres. Thus, bile acid interactions after isolation and sequential extraction of fibres from lupin hull and cotyledon were compared (CHAPTER 3). Alcohol insoluble residues, which were obtained after de-oiling, protein hydrolysis, and repeated alcoholic extraction, were sequentially extracted yielding three fractions: pectin-like substances, a hemicellulose fraction, and a lignocellulosic fraction. Lupin hull consisted mainly of cellulosic polymers, which was consistent with low bile acid retention induced by viscosity effects and no significant bile acid adsorption. Bile acid adsorption observed for a lignin reference material was not evident in lupin hull or cotyledon due to low lignification. Sequential extraction and monosaccharide analysis revealed that pectin-like substances are mainly responsible for viscosity in lupin cotyledon digesta. These results suggest that the formation of entangled networks, causing predominantly elastic properties, majorly contributes to the increase of viscosity. In contrast to lupin cotyledon flour, extracted fibre fractions did not show a significant bile acid adsorption. As a direct contribution of the cell wall polysaccharides in the bile acid adsorption of lupins could thus be disproven, molecular interactions could be caused by components associated with the fibre fraction. Therefore, possible contributions of proteins and phytochemicals to bile acid interactions were evaluated (CHAPTER 4). For this purpose a pilot-scale procedure was applied to generate protein isolates containing α-, β-, and δ-conglutin (LPI-E) and others containing

γ-conglutin (LPI-F). Significant adsorption was found for LPI-E (2.1 µmol chenodesoxycholic acids/100 g dry matter), which was comparable to lupin cotyledon flour (1.6 µmol chenodesoxycholic acids/100 g dry matter). But no correlation of the adsorptive capacity with the abundance of protein in lupin could be found. Alcohol purification confirmed that bile acid adsorption is independent of protein structures. Moreover, high adsorption was observed with an alcohol extract (7.0 µmol chenodesoxycholic acids/100 g dry matter) that was rich in phytochemicals, such as flavonoids (1842 mg/100 g dry matter). These results suggest hydrophobic interactions between polyphenols and bile acids as core mechanism of bile acid adsorption by lupins.

Finally, the effects of food processing on bile acid interactions were investigated. This was investigated exemplified by thermo-mechanical extrusion processing of lupin kernel fibre (CHAPTER 5). Lupin kernel fibre was extruded at various temperatures, feed moistures, and screw speeds. The physiochemical properties were investigated focusing on changes in fibre composition, water and oil binding capacities. To evaluate fibre functionality, extrudates were digested *in vitro* and digesta were characterised with emphasis on rheological properties and interactions with bile acids. These assessments revealed that after extrusion processing the soluble fraction of lupin fibre was increased from 1.9 g/100 g dry matter to up to 37.7 g/100 g dry matter. Concurrently, water binding was increased by up to 95% and oil binding was significantly decreased. Viscosity of *in vitro* digesta was increased for most extrudates. Accordingly, diffusion of bile acids was significantly decelerated. Moisture content, followed by barrel temperature were identified as the most relevant extrusion parameters to modify functionality. Our results indicate that extrusion processing caused solubilisation of pectic polymers, which exhibit high hydration capacities and thus increase the physiological viscosity. These findings suggest that extrusion could be a practical technology to enhance health benefits of lupin kernel fibre.

In summary, the findings of this thesis provided a deeper understanding of interaction mechanisms between bile acids and plant compounds focusing on lupin seeds. Mechanistic principles of interactions were linked with bile acid chemistry and micellar properties. Structure-function relationships were elucidated by targeted isolation and fractionation studies. These results will be helpful to clarify the complex interplay between the interaction of plant compounds and bile acids, the microbial changes of bile acids, the fermentation of indigestible plant compounds and the consequences on the gut microbiome–bile acid axis. Influences of fractionation and processing technologies on biofunctional properties were recognised, which in turn support the integration of the results in development of food products with increased health benefits.

Zusammenfassung

Gallensäuren nehmen eine Vielzahl systemischer Schlüsselfunktionen im menschlichen Körper ein. Durch die Aktivierung verschiedener Signalwege und die Interaktion mit den Mikroorganismen im Dickdarm steuern Gallensäuren nicht nur ihre eigene Synthese und regulieren den enterohepatischen Kreislauf, sondern tragen zudem zur Homöostase von Triglyceriden, Cholesterin, Glukose und zur Aufrechterhaltung des Energiehaushaltes bei. Insbesondere herauszustellen ist, dass die Produktion von primären Gallensäuren in der Leber den wichtigsten Stoffwechselweg zur Entfernung von überschüssigem Cholesterin aus dem Körper darstellt. Darüber hinaus besteht ein direkter Zusammenhang zwischen den von Mikroorganismen im Dickdarm gebildeten sekundären Gallensäuren mit Markern für das Darmkrebsrisiko. Es ist bekannt, dass sich sekundäre Gallensäuren, insbesondere Desoxycholsäuren, im Gallensäurepool von Personen mit "westlicher Ernährungsweise" anhäufen. Die Entwicklung hin zu dieser Ernährungsweise ging mit dem abnehmenden Verzehr von ballaststoffreichen pflanzlichen Nahrungsquellen wie Gemüse, Vollkorngetreide oder Hülsenfrüchten einher. Die Beeinflussung des Gallensäure-Stoffwechsels von Pflanzenstoffen als potentieller Beitrag zur Krankheitsprävention findet daher zunehmend Beachtung. Es wird berichtet, dass ballaststoffreiche, pflanzliche Nahrungsmittel, mit Gallensäuren interagieren, ihre Resorption verhindern und ihre Ausscheidung in den Dickdarm fördern. Die Art und Mechanismen dieser Wechselwirkung sind jedoch noch nicht vollständig verstanden.

Um die Lücke zwischen physiologischen Wirkungen und den verantwortlichen molekularen Mechanismen zu schließen, werden geeignete *in vitro*-Methoden benötigt. Der erste Teil dieser Arbeit (KAPITEL 1) befasste sich daher mit dem Vergleich einer weit verbreiteten *in vitro* Methode zur Analyse der Gallensäurebindung basierend auf Zentrifugation mit einem angepassten Modell, das aus einem *in vitro*-Verdau mit anschließender Gleichgewichtsdialyse besteht. Die meisten Studien zur Erklärung der Wechselwirkung zwischen Pflanzenstoffen und Gallensäuren erlauben eine Einteilung in zwei mögliche Mechanismen, die *in vitro* analysiert und differenziert werden müssen. Einerseits, könnte eine erhöhte Viskosität während der Verdauung zu einer verminderten mizellaren Mobilität der Gallensäuren führen. Andererseits, könnten Gallensäuren und Pflanzeninhaltsstoffe auf molekularer Ebene assoziiert oder komplexiert sein. Der Vergleich der verschiedenen *in vitro*-Ansätze ergab, dass die auf Zentrifugation basierende Methode nicht geeignet ist, um die viskositätsbedingte Verlangsamung der Gallensäurefreisetzung zu berücksichtigen. Die Verringerung der Gallensäurendiffusion durch erhöhte Viskosität wird jedoch als ein Kernmechanismus der Interaktion ballaststoffreicher, pflanzlicher Lebensmitteln angesehen. Andererseits konnte

gezeigt werden, dass die Analyse der kinetischen Freisetzung von Gallensäuren über eine Dialysemembran sowohl molekulare Wechselwirkungen als auch Viskositätseffekte umfasst. Unter Anwendung dieses Modells werden die *in vitro* verdauten Proben dialysiert, was als vereinfachtes Absorptionsmodell des sogenannten unstirred water layers betrachtet werden kann. Die Diffusion wird anhand einer Kinetik erster Ordnung bewertet, um zwischen viskositätsbedingter Verlangsamung der Gallensäurefreisetzung und permanenten molekularen Wechselwirkungen zu unterscheiden.

In einer zweiten Studie (KAPITEL 2) wurde die in KAPITEL 1 etablierte Methode um die Analyse der in der menschlichen Galle vorwiegend enthaltenen Gallensäuren erweitert. Auf diese Weise wurden mögliche Korrelationen zwischen Gallensäure-Wechselwirkungen und der chemischen Zusammensetzung der Gallensäuren untersucht, um daraus mechanistische Prinzipien abzuleiten. Zehn ballaststoffreiche Faserpräparate unterschiedlicher pflanzlicher Herkunft wurden *in vitro* verdaut und die Wechselwirkungen mit glyko- und taurokonjugierten Cholsäuren, Chenodesoxycholsäuren und Desoxycholsäuren analysiert. Viskositätsassoziierte Wechselwirkungen wurden für Apfel-, Gersten-, Zitrus-, Lupinen-, Erbsen- und Kartoffelfaserpräparate festgestellt, die die Freisetzungsrate der Gallensäure um bis zu 80% verlangsamten. In viskosen Matrices wurde die Diffusion von Desoxycholsäuren am stärksten verlangsamt, gefolgt von Chenodesoxycholsäuren und Cholsäuren. Diese verzögerte Diffusion in viskosen Medien konnte mit den mizellaren Eigenschaften der Gallensäuren (kritische Mizellbildungskonzentrationen und Anzahl der Moleküle in den Mizellen) in Verbindung gesetzt werden. Eine signifikante Adsorption von Gallensäuren von bis zu 4,7 µmol/100 mg Trockensubstanz war in Gersten-, Hafer-, Lupinen- und Maispräparaten messbar. Es zeigte sich, dass die Adsorption in Abhängigkeit der Gallensäurestruktur für Desoxycholsäure > Chenodesoxycholsäure > Cholsäure abnahm. Diese Ergebnisse legen nahe, dass hydrophobe Wechselwirkungen den zugrundeliegenden Mechanismus der molekularen Wechselwirkungen von Gallensäuren mit Pflanzenstoffen darstellen. Da für hydrophobe Gallensäuren erhöhte viskositätsassoziierte und adsorptive Wechselwirkungen festgestellt wurden, deuten die Ergebnisse darauf hin, dass der Verzehr pflanzlicher Faserpräparate zur Ausschleusung von prokanzerogenen und proinflammatorischen sekundären Gallensäuren aus dem Gallensäurepool beitragen kann.

In Fortsetzung der Arbeiten wurden Struktur-Wirkungs-Beziehungen der aufgedeckten viskositätsassoziierten und molekularen Wechselwirkungen mit Gallensäuren anhand von Lupinensamen (*Lupinus angustifolius* L.) untersucht. Die Erhöhung der Viskosität während der Verdauung wird hauptsächlich auf spezifische Ballaststoffe zurückgeführt. Darüber hinaus wird häufig vermutet, dass Gallensäuren an hydrophoben Oberflächen von unlöslichen Ballaststoffen adsorbiert werden könnten. Daher wurden die Gallensäure-Interaktionen nach

Isolierung und sequentieller Extraktion der Lupinenballaststoffe aus der Schale und den Kernen der Samen untersucht (KAPITEL 3). Alkoholunlösliche Rückstände, die durch Entölung, Proteinhydrolyse und wiederholte alkoholische Extraktion gewonnen wurden, wurden mehrfach extrahiert, so dass drei Fraktionen gewonnen wurden: Pektin-ähnliche Substanzen, eine Hemicellulose-Fraktion und eine Lignocellulose-Fraktion. Die Lupinenschale bestand zum größten Teil aus Cellulose, was im Einklang mit einer niedrigen gemessenen Gallensäure-Retention und fehlenden Gallensäure-Adsorption stand. Die bei Lignin als Referenzsubstanz beobachtete Adsorption von Gallensäuren war in der Schale und den Kernen von Lupinen aufgrund deren geringer Lignifizierung nicht nachweisbar. Die Ergebnisse der sequentiellen Extraktion und einer Analyse der Monosaccharid-Zusammensetzung zeigten, dass vorwiegend Pektin-ähnliche Substanzen für die Viskosität von *in vitro* verdauten Lupinen-Kernen verantwortlich sind. Diese Substanzen bilden vermutlich verzweigte Netzwerke, die ein vorwiegend elastisches rheologisches Verhalten bedingen und die Viskosität erhöhen. Im Gegensatz zu Mehl aus Lupinenkernen zeigten die extrahierten Ballaststofffraktionen keine signifikante Gallensäureadsorption. Da eine direkte Beteiligung der Zellwandpolysaccharide an der Gallensäureadsorption von Lupinen somit widerlegt werden konnte, könnten molekulare Wechselwirkungen durch assoziierte Komponenten in der Faserfraktion verursacht worden sein. Daher wurden mögliche Beiträge von Proteinen und sekundären Pflanzenstoffen zur Gallensäure-Interaktion evaluiert (KAPITEL 4). Hierfür wurden zwei Proteinisolate, die vorwiegend α-, β- und δ-Conglutin (LPI-E) bzw. γ-Conglutin (LPI-F) bestanden, im Pilotmaßstab hergestellt. Für LPI-E wurde eine signifikante Adsorption (2,1 µmol Chenodesoxycholsäuren/100 g Trockensubstanz) gefunden, die mit der für das Mehl aus Lupinenkernen nachgewiesenen Adsorption (1,6 µmol Chenodesoxy-cholsäuren/100 g Trockensubstanz) vergleichbar war. Es bestand jedoch keine Korrelation zwischen der gemessenen Adsorption und dem Proteingehalt der Lupinenfraktionen. Durch alkoholische Aufreinigung konnte schließlich belegt werden, dass die Adsorption von Gallensäuren nicht durch in der Lupine enthaltene Proteine bedingt wird. Darüber hinaus wurde eine hohe Adsorption in dem gewonnenen alkoholischen Extrakt (7,0 µmol Chenodesoxycholsäuren/100 g Trockensubstanz) nachgewiesen. Dieser Extrakt enthielt vorwiegend sekundäre Pflanzenstoffe, insbesondere Flavonoide (1842 mg/100 g). Die Ergebnisse deuten darauf hin, dass hydrophobe Wechselwirkungen zwischen Polyphenolen und Gallensäuren den zugrundeliegenden Mechanismus der Adsorption von Gallensäuren durch Lupinensamen darstellen.

Im letzten Abschnitt der Arbeit wurde der Einfluss der Lebensmittelverarbeitung auf die Gallensäure-Interaktionen erforscht. Dies wurde am Beispiel der thermo-mechanischen Extrusion von Lupinenfasern untersucht (KAPITEL 5). Die Lupinenfasern wurde bei verschiedenen Temperaturen, Feuchtegehalten und Schneckendrehzahlen extrudiert. Die

physiochemischen Eigenschaften wurden untersucht, wobei der Schwerpunkt der Untersuchungen auf Veränderungen der Faserzusammensetzung sowie der Wasser- und Ölbindungskapazität lag. Zur Bewertung der Biofunktionalität wurden die Extrudate *in vitro* verdaut und der Speisebrei charakterisiert, wobei die rheologischen Eigenschaften und die Wechselwirkungen mit Gallensäuren analysiert wurden. Es zeigte sich, dass die lösliche Fraktion der Lupinenfaser nach der Extrusion von 1,9 g/100 g Trockensubstanz auf bis zu 37,7 g/100 g Trockensubstanz erhöht wurde. Gleichzeitig wurde die Wasserbindung um bis zu 95 % erhöht und die Ölbindung signifikant verringert. Die Viskosität des *in vitro*-Speisebreis wurde bei den meisten Extrudaten erhöht. Dementsprechend wurde die Diffusion von Gallensäuren signifikant verlangsamt. Der Feuchtigkeitsgehalt, gefolgt von der Extrusionstemperatur, wurden als die relevantesten Extrusionsparameter zur Beeinflussung der Biofunktionalität identifiziert. Unsere Ergebnisse deuten darauf hin, dass die Extrusion zu einer Solubilisierung von pektinähnlichen Polymeren führte. Diese weisen hohe Hydratationskapazitäten auf und erhöhen somit die physiologische Viskosität. Diese Ergebnisse legen nahe, dass die Extrusion eine anwendungsnahe Technologie darstellen könnte, um die gesundheitlichen Vorteile der Lupinenfaser zu verbessern.

Zusammenfassend ist festzuhalten, dass die Ergebnisse dieser Arbeit ein tieferes Verständnis der Mechanismen der Wechselwirkung zwischen Gallensäuren und Pflanzenstoffen, insbesondere von Lupinensamen, ermöglichen. Mechanistische Prinzipien der Wechselwirkungen wurden mit der Gallensäurenchemie und den mizellaren Eigenschaften der Gallensäuren in Verbindung gebracht. Struktur-Wirkungs-Beziehungen wurden durch gezielte Isolations- und Fraktionierungsstudien aufgeklärt. Diese Ergebnisse sind hilfreich, um das komplexe Zusammenspiel zwischen der Interaktion von Pflanzenstoffen und Gallensäuren, mikrobiellen Veränderungen der Gallensäuren, der Fermentation unverdaulicher Pflanzenstoffe und den Auswirkungen auf die Darmmikrobiom-Gallensäuren-Achse zu klären. Zudem wurden Einflüsse von Fraktionierungs- und Verarbeitungs-technologien auf die gesundheitsfördernden Eigenschaften der Pflanzenstoffe erarbeitet, was die Integration der Ergebnisse in die Entwicklung von Nahrungsmitteln mit erhöhter Biofunktionalität ermöglicht.

General Introduction

Bile acids are a family of molecules that contribute to a variety of key systemic functions in the human body. Bile acids act as detergents facilitating the digestion and absorption of lipids, cholesterol and fat-soluble vitamins. Recent research activities have shown that bile acids act as regulators of the gut microbiome and play a key role as signalling molecules by modulating cell proliferation, gene expression, lipid and glucose metabolism (Di Ciaula et al., 2017, Ridlon et al., 2014). Plant-based food components are considered to interact with bile acids during upper gastrointestinal digestion (Singh et al., 2019). By increasing the transfer rates of bile acids from the small intestine into the colon, these interactions may modulate the bile acid pool size and composition affecting metabolic processes involved in health and disease states. A further understanding of the interactions between bile acids and plant compounds is thus needed in order to recognise related changes of bile acid profiles as a measure of physiological homeostasis (Li and Chiang, 2014).

1. Bile acid metabolism and chemistry

Primary bile acids, cholic acid and chenodesoxycholic acid, are synthesised in the liver by the conversion of cholesterol, which involves 17 distinct enzymes and is accomplished via two different pathways (Figure 1).

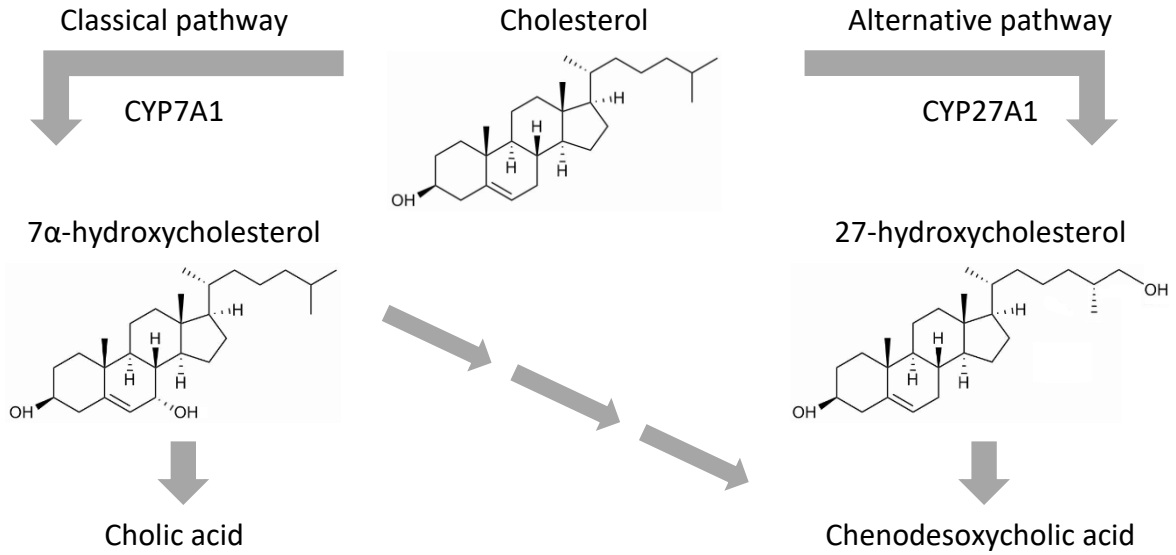


Figure 1: Schematic view of biosynthetic pathways of primary bile acids in the liver (CYP7A1: 7α-hydroxylase, CYP27A1: sterol-27-hydroxylase) adapted from Chiang (2013).

The first step of synthesis, described to be the rate-limiting step, is catalysed by cholesterol 7α-hydroxylase. The gene expression encoding cholesterol 7α-hydroxylase is known to be suppressed by a number of factors including insulin, protein kinase C activators, cytokines, steroid hormones and bile acids (Chiang, 2013, 2009). The feedback regulation of bile acid synthesis is realised in the liver and the intestine via the farnesoid X receptor acting as a bile acid sensor (Shin and Wang, 2019). The bile acid pool contains about 2.5–5 g of bile acids, which are conjugated either with taurine or glycine to form water-soluble bile salts (Russell and Setchell, 1992). Bile salts have different abundancies in bile, with glycoconjugates making up about 70% and tauroconjugates accounting for 30% of human bile salt mixtures (Parker et al., 2014). Bile salts are stored in the gall bladder, which is stimulated to contract and secrete the bile when food passes from the stomach into the duodenum (Mukhopadhyay and Maitra, 2004). Bile salts are steroidal detergents, which form mixed micelles with lipids, fats and/or cholesterol, and thus enable the digestion and absorption of fats and fat-soluble vitamins in the intestine. Conjugated bile acids are reabsorbed mainly by active transport mediated by the apical Na^+-dependent bile salt transporter, transported back to the liver via the portal circulation and then resecreted into the bile. During each cycle of the enterohepatic circulation (Figure 2) about 95% of the bile acids are recovered. The 5% of bile acids lost account for about 400 to 800 mg daily and become substrate to microbial transformation (Vlahcevic and Heuman, 1996).

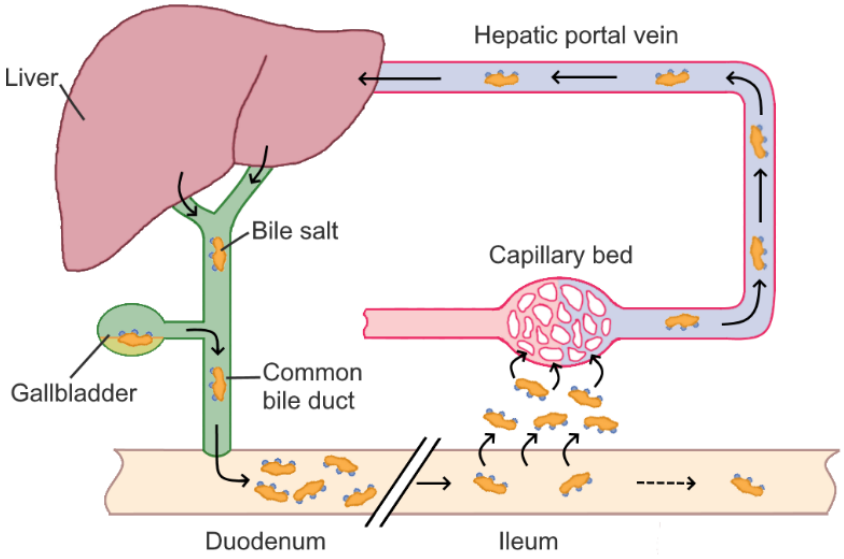


Figure 2: Enterohepatic circulation of bile acids.

By the action of anaerobic microbiota, primary bile acids are converted into secondary and tertiary bile acids. The most common secondary bile acids, resulting from deconjugation and dehydroxylation of primary bile acids, are desoxycholic acid and lithocholic acid (Ridlon et al., 2006). Due to its hydrophobicity, reabsorption rates into the enterohepatic circulation are

small for lithocholic acid (Hanafi et al., 2018). On the other hand, desoxycholic acid is reabsorbed in the colon and accumulates in the bile acid pool (Chiang, 2013). The human bile acid pool thus predominantly consists of cholic acids, chenodesoxycholic acids, and desoxycholic acids accounting for about 40%, 40%, and 20% of the bile acid pool, respectively (Chiang, 2009).

The basic chemical structure of all bile acids includes a rigid steroid nucleus and a short aliphatic side chain. Structural differences in the hydroxylation and conjugation of the most common primary and secondary bile acids are given in Figure 3. Bile acids contain hydrophobic and hydrophilic moieties, which makes them facially amphipathic (Mukhopadhyay and Maitra, 2004). The amphiphilic character of bile acids is explained by their rigid steroid backbone containing methyl groups oriented towards a hydrophobic face, whereas the hydroxyl groups and the amino group (taurine or glycine) are oriented towards a hydrophilic face (Torcello-Gómez et al., 2015). Due to their varying hydroxylation, bile acids differ in hydrophobicity, which decreases in the order of lithocholic acid > desoxycholic acid > chenodesoxycholic acid > cholic acid (Hanafi et al., 2018).

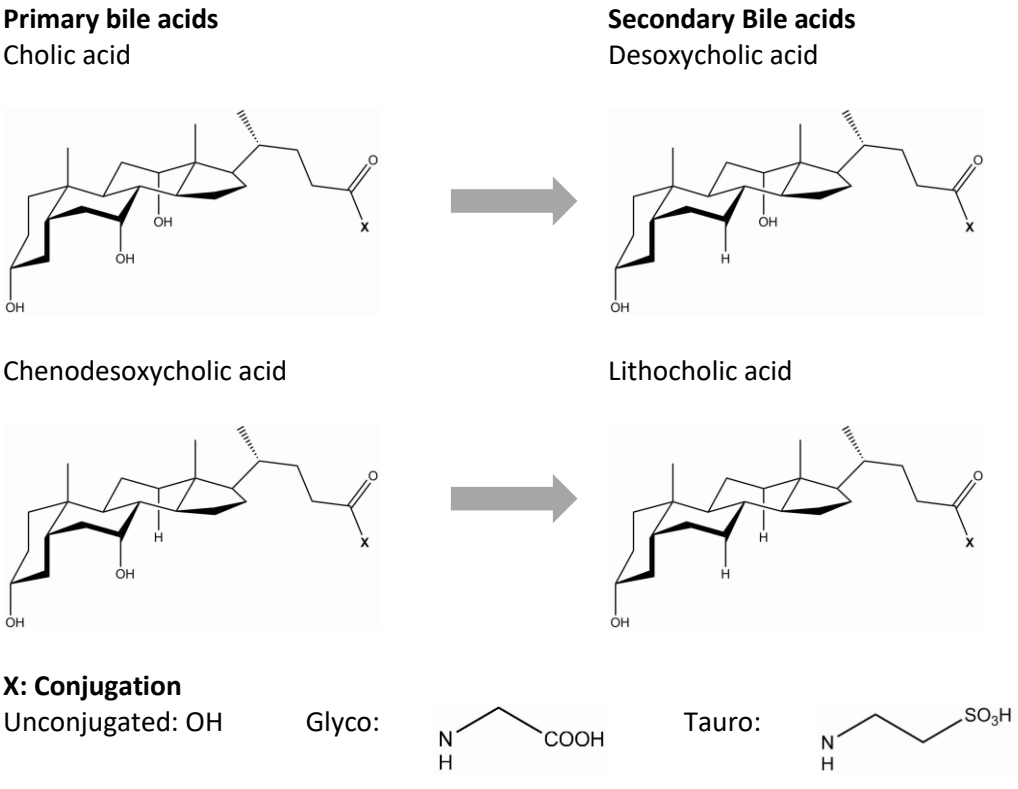


Figure 3: Chemical structure of primary bile acids, cholic acid and chenodesoxycholic acid, and secondary bile acids, desoxycholic acid and lithocholic acid.

2. Mechanisms of interactions between bile acids and plant compounds

Most studies explaining the interaction of plant compounds and bile acids allow a classification of the interactions into two possible mechanisms. Either increased viscosity during digestion results in reduced micellar mobility of bile acids or bile acids or plant compounds are associated or complexed at the molecular level (Gunness and Gidley, 2010). These interactions are proposed to partially prevent bile acids from being reabsorbed into the enterohepatic circulation. Thus, an excess faecal bile acid excretion is considered as an indicator for bile acid interaction in *in vivo* studies. Accordingly, Ellegard and Andersson (2007) reported an increase in bile acid excretion and the activation of cholesterol 7α-hydroxylase after consumption of oat bran breakfast cereals in ileostomy subjects. Median excretion of bile acids was increased by 144% after a diet including native bran in comparison to a control diet using hydrolysed bran. Therefore, the authors concluded that an increase in bile acid excretion might be caused by the entrapment of bile acid micelles due to the increased viscosity by β-glucan. Similar results were reported for ileostomy bile acid excretion after consumption of highly viscous citrus pectin (Bosaeus et al., 1986).

Due to the alteration of intestinal viscosity, solubility, and molecular weight of viscous fibres are considered crucial parameters for bile acid interactions, as repeatedly and recently demonstrated for β-glucans (Thandapilly et al., 2018, Iaccarino et al., 2020, Marasca et al., 2020). Nevertheless, some studies fail to establish correlations between the molecular weight and the indicators for bile acid interaction. In particular, a recent study by Iaccarino et al. (2020) showed that a diet enriched in structurally different β-glucans increased faecal bile acid excretion *in vivo*. However, no significant differences were found between a commercial β-glucan preparation with a molecular mass of 100 kDa, and a β-glucan extract with a molecular mass of 530 kDa and a higher viscosity of the resulting feed preparation. On the other hand, Simonsen et al. (2009) described interactions between the same commercial β-glucan preparation and bile acids. The *in vitro* methodology applied in this study included a centrifugation step to measure bile acid adsorption. The results of Simonsen et al. (2009) thus indicate that molecular interactions may add to the viscous properties of the commercial β-glucan preparation. The increased bile acid excretion described by Iaccarino et al. (2020) could thus be explained by overlapping viscous and molecular effects. Potential interaction mechanisms on molecular level are further corroborated by a study investigating a different oat preparation after thermal treatment (Zacherl et al., 2011). Due to the thermal processing, the viscosity measured under simulated physiological conditions was almost completely lost. However, the authors reported a dose-dependent bile acid sequestering effect, which reached up to 26% of the bile acid adsorbing effect of the anionic agent cholestyramine. Similar results

were reported for oat and barley extracts and hydrolysates with low molecular weight and viscosity (Araki et al., 2001, Bae et al., 2010, Grundy et al., 2018).

Several studies report increased interaction of dihydroxy bile acids (chenodesoxycholic acid and desoxycholic acid) compared to trihydroxy bile acids (cholic acids), e.g., as described for potato peels, extrudates from barley and oat or pastry products enriched in buckwheat, chokeberry, and mulberry fractions (Camire et al., 1993, Huth et al., 2000, Drzikova et al., 2005b, Dziedzic et al., 2015). These studies indicate a positive correlation of bile acid sequestering effects with bile acid hydrophobicity. However, the exact mechanism of interaction remains to be fully elucidated. Furthermore, it remains unclear, whether the dependence on hydrophobicity can be linked to viscosity-related or molecular interactions with bile acids. This is mainly due to the fact that bile acid binding capacities are frequently stated without investigating the underlying mechanisms.

3. Analysis of bile acid interactions

The suitability and limitations of *in vivo* and *in vitro* studies for the detection and differentiation of viscous and molecular bile acid interactions are given in Table 1 and will be discussed in the following Sections 3.1 and 3.2.

3.1. *In vivo* approaches to study bile acid interactions

Due to the minimal bacterial degradation of bile acids, ileostomy studies are very useful in understanding the influence of food components on the ileal bile acid content (Ellegard and Andersson, 2007, Gunness and Gidley, 2010). However, these studies are very limited in use. As bile acids are altered by fermentation and are partially reabsorbed in the colon, excretion of bile acids in more readily available faecal samples can only be correlated to a certain extent with the interruption of the bile acid metabolism (Thandapilly et al., 2018). Recent studies further indicate that after an adaption phase to diet interventions, levels of total circulating bile acids are reduced, as demonstrated by β-glucan and arabinoxylan interventions in pig models (Gunness et al., 2016b, Gunness et al., 2016c). Thus, although reabsorption rates were decreased and relative faecal excretion rates were increased, an excess of total faecal bile acid excretion was not detected. These concurrent effects on the reduction of circulating bile acids may further hamper conclusions regarding interacting mechanisms. Moreover, the suitability of many animal models is limited for bile acid studies due to deviating bile acid profiles that reduce the transferability to mechanisms in humans (Thakare et al., 2018, Li and Dawson, 2019). For instance, the bile acid pool in mice consists mostly of hydrophilic bile acids, muricholic acids and cholic acids, and thus differs significantly from the more hydrophobic bile

acid pool in humans (Chiang, 2009). Due to these limitations of *in vivo* studies, only little or no details on the intermediate mechanisms of bile acid interactions can be elucidated from their physiological outcomes (Macierzanka et al., 2019). Therefore, *in vitro* studies mimicking the physiological environment in the small intestine are of major interest in order to elucidate the basic mechanisms and identify hypotheses for targeted *in vivo* investigation.

3.2. *In vitro* and *ex vivo* approaches to study bile acid interactions

In vitro bile acid binding capacities have been reported for numerous plant-based foods as recently reviewed by Singh et al. (2019). However, the diversity of *in vitro* model conditions hampers the ability to compare results across different studies. Most results lack in comparability as methods vary regarding the use and parameters of *in vitro* digestion as well as the separation approaches to evaluate bile acid sequestering effects. In the intestine, bile acids form mixed micelles with lipids and cholesterol (Tuncer and Bayramoglu, 2019, Madenci and Egelhaaf, 2010). Nevertheless, many *in vitro* studies apply bile acid concentrations far below critical micelle concentrations, which majorly limits transferability to physiological conditions (Gunness and Gidley, 2010). Centrifugation steps are a common approach to separate unbound bile acids *in vitro* (Dongowski, 2007, Drzikova et al., 2005a, Kahlon and Chow, 2000, Singh et al., 2019). However, centrifugal forces differ significantly from the physiological conditions in the human body. Thus *in vitro* approaches applying centrifugation may not be appropriate to take into account the effects of viscous digestive matrices. Yet, reduction of bile acid diffusion by viscous networks is suggested as a core mechanism of interaction especially for plant-based foods rich in dietary fibre (Gunness et al., 2016b). Centrifugation steps further only separate insoluble undigested material with sufficient density (Minekus et al., 2014). It was thus questioned whether *in vitro* approaches applying centrifugation steps are appropriate to analyse bile acid interactions of soluble plant compounds (Zacherl et al., 2011).

A few studies have examined the course of bile salt micelle passage across membranes in the presence of food digesta containing plant compounds (Cornfine et al., 2010, Gunness et al., 2012, Han et al., 2009, Simonsen et al., 2009). Compared to centrifugation methods, this approach is described as a closer approximation to the reabsorption thought to occur *in vivo*. The dialysis membranes or other semi-permeable barriers thereby allow the passage of bile salt molecules but not of bile salt micelles or polymeric indigested compounds (Gunness et al., 2010, Gunness et al., 2012). When using such dialysis approaches a common behaviour of bile acid permeation versus viscosity was found, indicating that the method covers bile acid interaction effects related to viscosity (Gunness et al., 2012, Zacherl et al., 2011). By evaluation of bile acid retention after reaching equilibrium concentrations, a similar method was also

used to investigate the bile acid adsorbing capacities of oat β-glucans and phenolic derivatives (Simonsen et al., 2009). Dialysis-based methods thus seem appropriate for investigating viscosity-related and molecular interactions of bile acids with food compounds (Cornfine et al., 2010, Gunness et al., 2012, Han et al., 2009).

To further study viscosity effects and understand physiological mechanisms, bile acid diffusion can be investigated using *ex vivo* Ussing chamber experiments. An epithelia membrane separates the Ussing chamber so that each side of the membrane faces a separate chamber half, which is filled with physiological solutions (Westerhout et al., 2015). In a study by Gunness et al. (2016b), tissues from proximal jejunum, mid-jejunum, and terminal ileum derived from pigs were used to study diffusion kinetics. After the addition of oat β-glucan to the mucosal side, a significant decrease in the uptake of a model bile acid across the terminal ileum was reported. Further *ex vivo* studies focus on changes of intestinal mucus after specified diets to elucidate influences on mucosal permeability, which may add to bile acid sequestering effects, especially for soluble fibres (Mackie et al., 2016).

To elucidate the nature of molecular interactions, a variety of structural techniques can be used. These include nuclear magnetic resonance (NMR) methods to study chemical shift changes in bile salt resonances as a function of the concentration of digested plant compounds (Gunness et al., 2010, Ogawa et al., 2016, Pigliacelli et al., 2019). Additionally, small-angle X-ray and/or neutron scattering, microcalorimetry, surface plasmon resonance analysis, and molecular docking experiments can be performed to elucidate binding stoichiometry and energetics of interactions (Kobayashi et al., 2014, Gunness et al., 2016a).

Table 1. ***In vivo*** **and** ***in vitro*** **analysis of interactions between bile acids and plant compounds: approaches, benefits and limitations.**

Approach	Details	Benefits	Limitations	Ref.
Human studies	Bile acid analysis of faecal samples	• Holistic assessment of effects on primary and secondary bile acid compositions in the colon	• Transformation and reabsorption of bile acids in the colon • Compensatory physiological processes	(Ridlon et al., 2014)
Human ileostomy studies	Bile acid analysis of ileal contents	• Shorter and less variable transit time • Minimal bacterial degradation of plant compounds and bile acids • Short term studies on bile acid metabolism	• Availability of human ileostomy subjects • Transferability of long term effects on physiological processes in subjects without ileostomy	(Bosaeus et al., 1986, Ellegard and Andersson, 2007)
Animal models	Bile acid analysis of contents of intestinal sites or faecal samples	• Understanding of bile acid concentrations along the intestinal tract • Holistic assessment of effects on primary and secondary bile acid compositions	• Deviating bile acid profiles in animals • Compensatory physiological processes • Transferability to human physiological processes	(Chiang, 2009, Gunness et al., 2016b)
In vitro models based on centrifugation	Bile acid analysis in supernatant	• Easily applicable • High prevalence in literature	• Variations regarding the use and parameters of *in vitro* digestion (i.e. critical micelle concentrations of bile acids not considered) • Coverage of viscosity-related effects • Applicability for soluble plant compounds	(Kahlon and Smith, 2007, Kim and White, 2010)
In vitro models based on dialysis	Bile acid transport across a dialysis membrane	• Differentiation of viscosity-related and molecular bile acid interactions • Applicability for soluble plant compounds • Bile acid concentrations above critical micelle concentrations	• Simplified model of unstirred water layer • Estimation of physiological concentrations and viscosity	(Gunness et al., 2012)
Structural *in vitro* techniques	Nuclear magnetic resonance, micro-calorimetry, etc.	• Assessment of molecular interactions • Elucidation of molecular mechanisms	• Transferability to physiological processes • Coverage of viscosity-related effects	(Gunness and Gidley, 2010, Li et al., 2019)

4. Bile acid interactions as related to plant tissues

Most studies agree that dietary fibres contribute substantially to bile acid interaction in digests of plant compounds (Gunness and Gidley, 2010, Macierzanka et al., 2019, Singh et al., 2019). To elucidate the cascade of events related to the health attributes of plant-based foods, a considerable number of research activities have thus addressed the interactions between bile acids and dietary fibre (Singh et al., 2019). Dietary fibre may occur in isolated form or as part of complex cell wall architectures. Therefore, the group of dietary fibres comprises a multitude of different structures (Macierzanka et al., 2019). Human ileostomy studies have shown that a diet fortified in dietary fibre from oat induces increased bile excretion within 24 hours after consumption (Macierzanka et al., 2019, Ellegard and Andersson, 2007, Lia et al., 1995). However, studies on dietary fibre retaining intact cell wall structures fail to provide conclusive results regarding the nature and mechanism of the interactions with bile acids (Macierzanka et al., 2019). In particular, high variabilities were reported when comparing results on an equal dietary fibre basis for various fruits (Kahlon and Smith, 2007). These studies suggest that bile acid interactions with other plant compounds, such as proteins and phytochemicals, may add to the bile acid retarding effects of dietary fibres.

4.1. Interactions between bile acids and dietary fibres

In 1967, Cookson et al. (1967) first published on the relationship between dietary fibre and bile acids focusing on the modification or prevention of cholesterol-induced atherosclerosis. Since then, numerous studies have focused on the interaction between soluble and insoluble dietary fibre and bile acids, resulting in several hypotheses about potential interaction mechanisms (Macierzanka et al., 2019, Singh et al., 2019).

The interaction of most soluble dietary fibres, such as pectin, β-glucan, and guar gum, is mostly ascribed to their viscous properties (Gunness and Gidley, 2010). Accordingly, Gunness et al. (2012) investigated *in vitro* bile acid diffusion in the presence of wheat arabinoxylan and barley mixed linkage β-glucan and found that the viscous polymers slowed down the passage of bile acid micelles. A permanent molecular interaction between the two soluble fibres and bile acids was not found. However, non-permanent molecular interactions depended on the source of the fibre, and were revealed by NMR and small-angle X-ray scattering analyses (Gunness et al., 2016a). These interactions were classified in two main classes. β-glucan caused mostly small chemical shift changes in the NMR bile acid resonances, indicating dynamic interactions leading to effective local changes of micellar bile concentrations. On the other hand, arabinoxylan mainly reduced the intensities of NMR bile acid resonances, indicating trapping of bile micelles within polymer aggregates. A recent study further focused on the oxidation and partial hydrolysis of β-glucan and confirmed that the bile acid

sequestering effect of β-glucan can primarily be ascribed to its viscous properties (Marasca et al., 2020). Accordingly, the *in vitro* study revealed that the most viscous native β-glucan extracts exhibited the strongest retardation of bile acid diffusion. In agreement with these findings, reduced reabsorption of bile acids was found after short-term *in vivo* interventions with β-glucan and arabinoxylan (Gunness et al., 2016b, Gunness et al., 2016c). *In vivo* interventions with structurally different guar gums or pectins showed increased faecal bile acid concentrations after 3 weeks exposure (Ghaffarzadegan et al., 2016). Individual bile acid concentrations varied depending on the degree of pectin methoxylation and molecular weight of guar gums and were accompanied with changes in bile acid distribution compared to a fibre-free diet. The correlation between bile acid interaction, and pectin structures and viscosity was further shown *in vitro*, and revealed an increased interaction depending on the bile acid hydrophobicity (Dongowski, 1995, 1997). An early study by Pfeffer et al. (1981) focused on pectin bile acid interactions using NMR and dialysis experiments. The authors could not find permanent molecular interactions for a purified fraction of native high-molecular pectin, but revealed bile acid adsorbing properties for contaminants in commercial pectin preparations. From all these studies focusing on structurally different soluble fibres, a central role of viscosity in the interaction with bile acids can be deduced, while no permanent molecular interaction between soluble fibre structures and bile acids is currently established.

In the last decades, interactions of bile acids with plant compounds rich in insoluble fibre were reported for a number of different feedstocks, including barley, oat, wheat, rice, and soybean (Kahlon and Woodruff, 2003, Zhang et al., 2011). These interactions were mostly independent of viscosity and increased depending on bile acid hydrophobicity. The findings indicated that hydrophobic interactions between bile acids and plant compounds could be related to adsorptive properties of the insoluble fibre structures. However, focusing on different fibre preparations derived from fruits, vegetables, or cereals, Dongowski (2007) was unable to establish a direct correlation between bile acid sequestering effects and dietary fibre compositions (proportion of soluble to insoluble fibre). Bile acid adsorbing capacity was also frequently postulated for lignin (Eastwood and Hamilton, 1968, Gallaher and Schneeman, 1986, Górecka et al., 2002, Kritchevsky and Story, 1976, Sayar et al., 2006, Dziedzic et al., 2015). Lignin has a special position among dietary fibres because it is not a polysaccharide but a phenolic macromolecule (American Association of Cereal Chemists, 2001). However, some studies have also produced results that contradict a possible contribution of lignin to bile acid interactions. In particular, no increase in bile acid adsorption could be achieved by artificial lignification of maize cell walls (Funk et al., 2008). Furthermore, no correlation of bile acid adsorption could be estabilshed to lignin contents of starchy legumes (Elhardallou, 1992). It is thus not entirely understood whether lignin adds to molecular interactions observed for dietary fibre enriched food ingredients.

The beneficial health aspects of dietary fibres highly depend on their physicochemical properties (Blackwood et al., 2000). Since no mechanism for binding bile acids at the molecular level has been established to date, the influence of physiochemical properties on molecular interactions remains unclear. On the other hand, there is a clear dependence of the bile acid interaction of dietary fibres on their viscosity increase in the gastrointestinal tract (Vuksan et al., 2011). Viscous dietary fibres induce thickening when mixed with liquids, which depends on a number of factors such as fibre solubility, molecular weight, surface and hydration properties (Dikeman and Fahey, 2006). The physiochemical properties of dietary fibres are further dependent on the processes applied during ingredient preparation and food formulation. The effects of physiochemical properites of dietary fibres on their rheological properties and influences of food processing will be discussed in 4.1.1 and 4.1.2.

4.1.1. Physiochemical properties affecting the viscous properties of dietary fibres

The sub-division of dietary fibres into soluble and insoluble fractions was driven by the general recognition that soluble fibres exert different physiological effects from insoluble. Soluble dietary fibres are attributed a high water binding capacity, viscosity increasing capacity, and fermentability by intestinal microbiota (Fuller et al., 2016, Gunness and Gidley, 2010, Kumar et al., 2012). On the other hand, insoluble dietary fibres are described as less fermentable and only have little effect on viscosity in the gastrointestinal tract (van Bennekum et al., 2005, Ul Ain et al., 2019). However, some soluble dietay fibre components of plants are not viscous polysaccharides, e.g., oligosaccharides, which are nevertheless classified as soluble dietary fibre (Wood, 2007). Fibre categorisation on basis of solubility thus provides limited links to functionality (Gidley and Yakubov, 2019).

Solubility and viscosity result from the polymers structural features. The relative stability of the ordered and disordered forms determine whether or not a polysaccharide will dissolve in water. Polysaccharide chains may adopt regular, ordered conformations and pack together into insoluble ‘crystalline’ assemblies (e.g. cellulose). These polymers are thus energetically more stable in the solid state than in solution. Otherwise, polysaccharides tend to be soluble if they have some irregularities in their structure, in the backbone or as side chains (e.g. guar gum, cellulosic polymers with attached functional groups). Furthermore, neutral polysaccharides (e.g. cellulose) have a strong tendency to self-association. On the other hand, for polysaacharides with charged groups (e.g. pectin) solubility is promoted by introducing electrostatic repulsion between the chains that inhibits ordered packing (Guillon and Champ, 2000, Morris, 2001).

The nature of the structures adopted by different polysaccharides, depends strongly on the way in which the individual monomers are linked together. If a sufficiently long section of

soluble polysaccharides such as guar gum and cereal β-glucan is considered, the polymer chain conformation is described as 'random coil'. In dependence of the nature of the glycosidic linkage and monosaccharide units, the branching in the molecules varies. That's why at the same molecular weight, more compact structures, which occupy less volume and more extended or less 'dense' structures, may occur (Wood, 2007). Molecular weight also has a large effect on apparent viscosity. At equal concentrations, there is a positive non-linear relationsship between the molecular weight of polysaccharides in solution and viscosity (Dikeman and Fahey, 2006, Rieder et al., 2017). Next to these intrinsic characteristic, the viscosity of polysaccharide solutions depends on the concentration. At low concentrations molecular domains are well separated and behave individually due to free movement. When the concentration increases, polymer domains occupy the solvent volume and molecules are forced to interpenetrate one another. Due to the overlapping coils, entangled networks are formed (Guillon and Champ, 2000, Wood, 2007). While dilute polysaccharide solutions show Newtonian flow behaviour, networks of polysaccharides with concentrations higher than critical concentration of entanglement (C*) show non-Newtonian, shear-thinning flow behaviour, which is is accompanied by a sharp increase in the concentration-dependence of viscosity (Morris, 2001). Furthermore, some polysaccharides can form gels under certain conditions by association of their ordered regions (Guillon and Champ, 2000).

Water binding capacity is associated with certain viscous polysaccharides. The kinetic of water uptake is controlled by structural fibre characteristics and the chemical affinity of fibre components towards water. Consequently, water may be held in capillaries formed by the insoluble fibres due to surface tension strength or water may interact with molecular components of fibre by hydrogen bonding or dipole interactions (Chaplin, 2003, López et al., 1996). Consequently, soluble and insoluble polysaccharides alike have the ability to hold water. The most obvious expression of the water binding capacity of soluble fibres is the phenomenon of gelation. A relatively small amount of polysaccharide, such as 1% agarose, may be sufficient to trap the water in a three-dimensional network of polysaccharide molecules. The water is retained in the polysaccharide matrix and the system has the semi-solid characteristic of a gel (Oakenfull, 1996). Insoluble fibres can absorb water, as they also form a hydrophilic matrix in which water is trapped – but in which the quasi-crystallinity of the polysaccharide is retained and water fills the interstices (Oakenfull, 2001). That's why the insoluble fibre matrix can exhibit high swelling capacity and exhibit high water retention capacity (Guillon et al., 2007). Accordingly, *in vivo* studies focusing on insoluble fibres found that the viscosity of the rat digesta was negatively correlated with its free water content, which was reduced by fibre that held water and swelled (Molist et al., 2009, Takahashi et al., 2009).

4.1.2. Effects of processing on the physiochemical properties of dietary fibres

The nutritional and functional properties of dietary fibres are influenced by process-related changes in the various stages and unit operations of the production process to the final food product (Poutanen, 2001). These processes include chemical, enzymatic, mechanical, thermal or thermo-mechanical treatments. Hereby, the impact of the processing is influenced by the fibre source, its history and the operating conditions (Guillon and Champ, 2000).

Targeted chemical and enzymatic treatments can lead to solubilisation and depolymerisation of dietary fibre, accompanied by an increase of soluble and decrease of insoluble fibre. Conversions of insoluble to soluble dietary fibre are described to improve sensory and textural properties. Studies of different enzyme applications have shown to significantly change the insoluble to soluble ratio. Tailored preparations of *Trichoderma* enzymes with high endohydrolase activity and low exohydrolase activity in durum wheat fibre and barley spent grain lead to a significant increase in soluble dietary fibre (250 %), with constant total dietary fibre contents. Sensory properties improved, as an increase in solubility decreased grittiness and the hard texture of the fibres. However, these enzymatic changes also lead to opened pore structures, a decreased water retention and oil retention capacity (Napolitano et al., 2006). Besides enzymatic treatment, dietary fibre structures can also be partially hydrolysed by acidic treatments. Accordingly, Cornfine et al. (2010) observed the formation of smaller fibre fractions after acidic hydrolysis with varying reaction times (4, 18, 48 h). The authors suggested that the presence of hydrolysis products may have induced higher viscosities, which was further shown by increased bile acid interactions.

Mechanical processing can change the dietary fibre structure by applying shear forces, which can cause depolymerisation of polysaccharides. Most fibre preparations are ground to increase acceptance. Huang et al. (2018b) described increased water retention capacity after ultrafine grinding of glucomannan, β-glucan, guar gum, and inulin preparations. Using a high-pressure micronizer particle size of insoluble carrob fibre were greatly reduced (−94 %), while fibre solubility and water retention capacity were significantly increased (Chau et al., 2007). Raghavendra et al. (2006) studied the correlation between disc mill micronization of dietary fibres from coconuts and the different particle size characteristics. Reduction of particle size from 1127 µm to 550 µm resulted in increased hydration properties, whereas hydration properties of particles smaller than 550 µm decreased with decreasing particle size. Generally, the fat absorption capacity was increased with a decreasing particle size. The effect of grinding on the hydration properties may result from the increase of surface area, total pore volume, and the structural modification, which influence the kinetics of water uptake. However, in certain cases, grinding may cause alteration and collapsing of the fibre matrix, which has

trapped water, and result in a decrease of the water retention and water absorption (Guillon et al., 2007).

By heating or chilling dietary fibres, their physiochemical properties are intentionally altered, which results in different soluble to insoluble fibre ratios. Both increases and decreases in the total dietary fibre content have been reported (Poutanen, 2001). Different thermal treatments, however, have varied impacts on dietary fibre properties. Zia-ur-Rehman et al. (2003) reported that both boiling and pressure-cooking of vegetables reduced the total dietary fibre content, especially cellulose and hemicellulose, while lignin content was not affected. Depending on the duration and intensity of thermal treatments, hemicellulose and cellulose hydrogen bonding's are possibly overcome. As a result, water molecules can hydrolyze the glycosidic bonds and release cellulose and hemicellulose from the plant cell wall materials. As lignin has a far greater molecular mass, the boiling temperature of water possibly is not sufficient to break the strong intramolecular bonding's of the inert carbon-linkages. Solubilisation and depolymerisation of polysaccharides was reported after repeated microwave treatments of green peas (Svanberg, 1999). Air-drying further causes a product collapse and its overall shrinkage, which changes the porosity and hydration properties of the fibres (Guillon and Champ, 2000).

Freeze-thawing can significantly change the physiochemical properties of foods. Alternating crystallisation and melting of water within the cell materials may lead to a reduction of molecular weight and viscosity, e.g., as described for β-glucans in oat bran. During frozen storage of oat bran enriched muffins, crystallisation of water caused a freeze-concentration of β-glucan (Lan-Pidhainy et al., 2007). As intramolecular hydrogen bonding's are furthered in higher concentration, β-glucans self-associate and form aggregates (Tosh et al., 2004). The followed thawing then increased mobility and accelerated the build-up of sections of accumulated intermolecular hydrogen bonds, thus, stabilising the aggregates. As a result, fibre solubility decreased and the viscosity increasing effect of oat β-glucan after *in vitro* digestion of the muffins was decreased (Lan-Pidhainy et al., 2007).

Significant changes of the fibre structures can be caused by combination of thermal and mechanical energy, e.g., due to extrusion processing. Extrusion is a commonly used technique for altering food materials high in insoluble fibre aiming to increase soluble dietary fibre contents. Zhong et al. (2019) researched the effect of extrusion processing on Australian sweet lupin seed coats, which are rich in dietary fibre. Extrusion did not affect the total dietary fibre level. However, significant increases in soluble dietary fibre content were determined (3-fold). The combination of a high screw speed (400 rpm), temperature (140 °C), and a low moisture content in the barrel (35 %) showed the highest soluble dietary fibre increase. As an

increased screw speed shortened the dietay fibre's residence time in the barrel, it could generate higher shear stress and friction upon the fibres. This possibly loosened the cell walls and the fibre matrix, which could have further accelerated the mixing and heat transfer throughout. Therefore, high mechanical energy and temperatures could build up (Duque et al., 2017). The water binding capacity of lupin seeds coats slightly decreased due to extrusion, which contradicts previous findings that show either no significant changes (Arrigoni et al., 1986) or an increase in water binding capacity (Huang and Ma, 2016). While conversion of insoluble to soluble fibre induced by the extrusion process has been extensively studied and reported, existing data on modification of physiological relevant physiochemical properties is contradictory (Ul Ain et al., 2019). Some studies further indicate increased interactions between bile acids and dietary fibres after extrusion processing. However, the intermediate mechanism of interaction was not elucidated (Camire et al., 1993, Huth et al., 2000). As some studies report solubilisation of high molecular weight fractions and gel forming after extrusion processing (Guillon and Champ, 2000), the effect of extrusion on the physiochemical properties and bile acid interactions of dietary fibres should be considered in more detail in future studies.

4.2. Interactions between bile acids and proteins

Little scientific attention has been paid to the interaction between bile acids and plant proteins (Macierzanka et al., 2019). Referring to the bile acid adsorbing capacity of cholestyramine as 100%, bile acid adsorbing capacities were reported for soy protein (14.5%), pinto beans (5.5%), black beans (8.2%), and wheat gluten (8.8%) by Kahlon and Woodruff (2002). High adsorbing capacities were reported for lentil and lupin proteins and hydrolysates, which partly exceeded the values reported for cholestyramine (Yoshie-Stark and Wäsche, 2004, Barbana et al., 2011). All referenced studies assessed the bile acid adsorbing capacity by centrifugation techniques and measured bile acid concentrations by photometric assay kits. Thus, no details on the molecular mechanisms can be elucidated from the results (Macierzanka et al., 2019). Higaki et al. (2006) reported that a soybean protein resistant to digestion captured bile acids and stimulated faecal bile acid excretion applying *in vitro* testing and a rat model. In contrast, Bosaeus et al. (1988) investigated soy bean proteins as alternatives to meat proteins and did not find significant influences on ileostomy bile acid concentrations. A recent study of Wang and Fan (2019) focused on structural characteristics of the interaction between zein, a plant protein isolated from maize, and sodium taurocholate. The study showed sodium taurocholate-dependent changes in secondary and tertiary protein structures and electrostatic binding between the bile salt and the protein. However, the investigations were carried out at acidic pH without the addition of digestive enzymes and it is thus unclear to what extent the results can be transferred to physiological conditions in the

small intestine. From the current state of the literature, interactions of bile acids with plant proteins are not ruled out, but further studies are needed to specify interactions and potential contributions to health benefits of plant-based foods more precisely.

4.3. Interactions between bile acids and phytochemicals

Indications of interactions between bile acids and phytochemicals have been repeatedly reported in recent literature, mostly focusing on polyphenolic compounds. In particular, McDougall et al. (2016) found substantial alterations in bile acid concentrations of ileal fluids of human ileostomy subjects after consumption of raspberries rich in polyphenols. While unconjugated bile acids were reduced, glyco- and tauroconjugated cholic acids and desoxycholic acids were increased by up to 120-fold over pre-supplementation levels. Raspberry pomace was further described to favourably alter bile acid profiles in a high-fat mouse model by Fotschki et al. (2017).

Similar results were also found in a high-fat mouse model for the most abundant green tea polyphenol epigallocatechin-3-gallate (Huang et al., 2018a). Epigallocatechin-3-gallate supplementation decreased intestinal bile acid concentrations and thus alleviated the potentially harmful expansion of the bile acid pool size typically observed in high-fat diets. Furthermore, epigallocatechin-3-gallate was shown to increase faecal bile acid excretion and significantly increase levels of cholesterol 7α-hydroxylase. Modifications of bile acid and steroid excretion were further reported for a number of polyphenol-rich feedstocks, including apples (Sembries et al., 2006, Hosoyamada and Yamada, 2017), grapes (Quifer-Rada et al., 2016, Olivero-David et al., 2018, Sembries et al., 2006), red beets (Sembries et al., 2006), asparagus roots (Visavadiya and Narasimhacharya, 2009), and peanut skins (Tamura et al., 2012). Similar physiological outcomes were also described for extracts from black bean seed coats rich in flavonoids and saponins, indicating synergistic effects of these phytochemicals (Chavez-Santoscoy et al., 2014). Saponins, a group of amphiphilic glycosides, have been repeatedly linked to cholesterol-reducing properties in the last decades. Nevertheless, as recently reviewed by Zhao (2016), the underlying molecular mechanisms are not elucidated yet.

Based on an NMR study, Ogawa et al. (2016) proposed a novel mechanism for the interaction of polyphenols and bile acids. Focusing on tea polyphenols, their results indicate a regiospecific interaction between epigallocatechin-3-gallate or oolongtheanins and bile acids. The authors suggested that these polyphenols form a hydrophobic space that adsorbs the bile acids. Due to this interaction, the micellar solubility of phosphatidylcholine and cholesterol is lowered, thus decreasing absorption and increasing faecal excretion (Ogawa et al., 2016). These results are corroborated by the study of Ikeda et al. (2010), who described that

polyphenols of black tea decrease cholesterols micellar solubility *in vitro* and showed that intestinal cholesterol absorption is decreased in rats. Gallic acid, catechin, and epicatechin (0.2 mg/mL) were shown to reduce the micellar solubility of cholesterol by 27%, 12%, and 19%, respectively (Ngamukote et al., 2011). Furthermore, Raederstorff et al. (2003) reported that the diameter of bile acid micelles was increased when epigallocatechin-3-gallate was added. Applying a model emulsion containing olive oil, phosphatidylcholine, and bile acids, the addition of catechins further markedly increased emulsion droplet size (Shishikura et al., 2006). Li et al. (2019) described the formation of a new complex from condensed tannins and bile acids. Interactions were characterised by turbidity, particle size, microstructure, and physicochemical condition analyses, which indicated that the binding occurred through hydrogen bonding and hydrophobic interactions. Furthermore, the stability and digestion properties of bile acid emulsions were analysed, suggesting that the observed complex formation may inhibit lipid digestion and reduce fat absorption.

Bile acid adsorbing capacities of polyphenols were studied applying different *in vitro* approaches. Yang et al. (2017) investigated the bile acid adsorption capacity of kale using an *in vitro* centrifugation method. The authors found that kale, which is rich in polyphenols and dietary fibre, preferentially bound the hydrophobic bile acids chenodesoxycholic acid and desoxycholic acid. In a second study, the authors compared the adsorbing capacity of raw kale and polyphenol extracts and concluded that certain polyphenolic compounds may have an affinity to adsorb bile acids (Yang et al., 2018). Applying a similar method, Hamauzu and Suwannachot (2019) further linked the bile acid adsorbing capacity of persimmon fruits to its polyphenolic pattern (epigallocatechin-3-gallate, epigallocatechin, epicatechin, and epicatechingallate).

The mentioned studies indicate that plant polyphenols may interact with bile acids on a molecular level, which may be mainly due to hydrophobic interactions. Besides these direct polyphenol-induced alterations of the bile acid pool, polyphenols are described to significantly change microbiota compositions and affect gen expressions linked to bile acid metabolism (Ushiroda et al., 2019, Zhou et al., 2018, Chambers et al., 2019). For instance, resveratrol was reported to decrease ileal bile acid contents, repress farnesoid X receptor, and increase cholesterol 7α-hydroxylase and bile acid synthesis. However, this functionality was lost after treatment with antibiotics (Chen et al., 2016). Therefore, the remodelling of the gut microbiota could represent an alternative physiological mechanism. Future research activities will aim to clarify the role and mechanism of polyphenols in bile acid metabolism.

5. Lupin (*Lupinus* L.) – Relevance, composition and biofunctionality

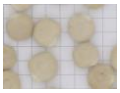
Since ancient times lupins have been cultivated in the Mediterranean and the Andean area. However, due to their high content of anti-nutritional alkaloids, the integration of lupins into the daily diet was limited to the consumption after extensive soaking in water or brine. For this reason lupin was primarily cultivated as preceding crop for grain production taking advantage of its nitrogen-fixing properties. By breeding of sweet lupin varieties at the beginning of the 20st century, alkaloid contents were reduced to levels suitable for human nutrition. Among more than 180 described species, the domesticated lupins include *Lupinus angustifolius* L.*, Lupinus albus* L.*, Lupinus luteus* L., and *Lupinus mutabilis* Sweet (Borek et al., 2009, Gresta et al., 2017).

As a sustainable native European alternative to soybean products, there is a growing interest in the addition of lupins to food due to their elevated and high-quality protein content, potential health benefits, suitability for sustainable production, and consumer acceptance (Lucas et al., 2015). The high proportion of protein and dietary fibre is a characteristic attribute of lupins, which is unique among legumes. Lupin seeds have technological properties suitable for industrial processing (Melini et al., 2017). Lupin proteins have high solubility and show good oil-binding properties, as well as emulsification/stabilisation properties (Bader et al., 2011). In contrast to insoluble cereal fibres, fibre preparations derived after protein isolation of lupin kernels have a smooth texture, a neutral taste, and a white colour. This makes lupin kernel fibres an excellent non-intrusive ingredient for fibre enrichment showing high palatability and sensory acceptability (Hall et al., 2010). Lupins are further described to promote various health benefits in humans including prevention of dyslipidaemia, hyperglycaemia and hypertension (Arnoldi et al., 2015).

5.1. Anatomical features and chemical composition of lupins

The anatomical features of lupin seeds are listed in Table 2. Among the species the seeds differ in shape, size, and colour. While most seeds are oval, the shape of the seeds of *L. albus* L. is rather square and flat. In addition, this seed is characterised by a higher weight and lower proportion of hull. The colour spectrum of the hulls ranges from light beige and slightly pigmented to dark brown.

Table 2: Anatomical features of lupin seeds of selected cultivars of *Lupinus angustifolius* L., *Lupinus albus* L., *Lupinus luteus* L., and *Lupinus mutabilis* Sweet.

Species	Cultivar	dimensions [mm]	weight [g/1000 seeds]	hull [%]	seed	cotyledon
L. albus L.	Typ Top	10.3 x 9.6 x 5.6	439	15.7		
L. angust. L.	Probor	7.2 x 6.1 x 5.4	175	18.7		
L. luteus L.	Bornal	7.1 x 5.8 x 4.3	152	19.1		
L. mutabilis Sweet	Pinta Baer	8.7 x 7.8 x 4.6	210	10.3		

The protein contained in lupin seeds accounts for 28–53% of dry weight and is mainly composed of albumins and globulins in an approximate ratio of 1 to 9 (Martínez-Villaluenga et al., 2006, Musco et al., 2017, Sujak et al., 2006, Duranti et al., 2008, Carvajal-Larenas et al., 2016). The protein classes α-conglutin (legumins) and β-conglutin (vicilins) are most abundant in lupins, while δ- and γ-conglutins are present as minor constituents (Ogura et al., 2014, Sirtori et al., 2010).

Lupin seeds are also an excellent source of dietary fibre containing up to 58.8% fibre, composed of 77–91% insoluble fibre and 9–23% soluble fibre (Musco et al., 2017). Legume seed fibres are derived from two main tissues, the hull (seed coat, outer fibre) and the cotyledons (kernels, inner fibre). Legume hulls are rich in fibre and mainly consist of cellulose. Cell walls of lupin cotyledons contain high amounts of non-cellulose, non-starch polysaccharides (Pfoertner and Fischer, 2001). The main sugar component is galactose, followed by arabinose and uronic acids (Brillouet and Riochet, 1983, Carre et al., 1985, Al-Kaisey and Wilkie, 1992). According to Al-Kaisey and Wilkie (1992) the polysaccharides present are galactans and arabinans as well as hetero-polysaccharides in the form of arabinogalactans, rhamnogalacturonans, and galactoxyloglucans. The principal pectic cotyledon polysaccharide described by Cheetham et al. (1993) contains a rhamnogalacturonan backbone with linear side chains containing 1–4-glycosidic-linked galactans and highly branched 1–5-glycosidic-linked arabinosyl residues. The content of non-structural carbohydrates is lower than that of most legumes and consists mainly of oligosaccharides (6–10%), while starch is absent or scarce (Arnoldi et al., 2015, Bähr et al., 2014).

Lupin seeds vary in fat contents, which ranges from 3–25% depending on the species (Carvajal-Larenas et al., 2016, Musco et al., 2017). Lupin oil shows a favourable content of essential fatty

acids, with linoleic acid (C18:2*n*6) comprising 20–51% and alpha–linolenic acid (C18:3*n*3) comprising 6–10% of total fatty acids (Chiofalo et al., 2012). The unsaponifiable fraction of lupin oil is composed of sterols and triterpene alcohols (Hamama and Bhardwaj, 2004). Lupin seeds further contain 6–13 mg/100 g tocopherols (mainly γ-tocopherol) and 50–230 µg/100 g of carotenoids (mainly zeaxanthin together with lutein, β-carotene and α-carotene) (Boschin and Arnoldi, 2011, Coïsson et al., 2011, Wang et al., 2008).

The main phytochemicals of the oil-free lupin fractions are saponins (57 mg/100 g (Petterson and Fairbrother, 1996)) and polyphenols. The main families of lupin polyphenols are phenolic acids, isoflavones, and flavonoids (Arnoldi et al., 2015). Siger et al. (2012) identified the phenolic acids gallic acid (0.06–0.4 mg/100 g), protocatechuic acid (1.3–7.4 mg/100 g), p-hydroxybenzoic acid (0.1–4.4 mg/100 g), caffeic acid (0.01–0.1 mg/100 g), and p-coumaric acid (0.003–0.07 mg/100 g). The isoflavones genistein and 2'-hydroxygenistein are present in concentrations of 0.3–0.5 mg/100 g and 0.1–0.5 mg/100 g, respectively (Katagiri et al., 2000, Mellenthin and Galensa, 1999). The most abundant polyphenols in lupin seeds are flavonoids represented by two main flavonoids derived from apigenin namely apigenin-6,8-di-C-β-glucopyranoside and apigenin 7-O-β-apiofuranosyl-6,8-di-C-β-glucopyranoside (12–63 mg/100 g and 26–88 mg/100 g DM of lupin seeds, given as vitexin equivalents) (Siger et al., 2012).

5.2. Biofunctional properties of lupin seed flours and isolated fractions

Available *in vitro* and *in vivo* studies focusing on lupin seed flours and its isolated fractions indicate that lupins may provide some useful health benefits in the area of hypercholesterolaemia, diabetes, and hypertension prevention. Hypocholesterolaemic effects have been linked with the dietary fibre and the protein fraction of lupin seeds (Arnoldi et al., 2015). Fechner et al. (2014) evaluated the preventive effects on cardiovascular disease by addition of lupin kernel fibre to the diet of hypercholesterolemic adults. Compared to a control diet, a reduction of total (−9%) and LDL (−12%) cholesterol as well as triacylglycerols (−10%) was observed after a four week period of lupin kernel fibre consumption. The authors proposed that lupin's effect on blood lipids may be mainly related to the short chain fatty acid formation in the colon, which could inhibit the hepatic cholesterol synthesis. Otherwise, Martins et al. (2005) investigated the hypocholesterolaemic effects of lupin seed flour in intact and ileorectal anastomosed pigs and found that ileorectal anastomosis did not modify cholesterol metabolism, which suggests that the caecum and the colon are poorly involved in this metabolism. The substantial decrease in plasma LDL-cholesterol was thus attributed to impaired intestinal cholesterol absorption, involving increased bile acid reabsorption and higher contents of dietary phytosterols, which reduce the micellar solubilisation of

cholesterol. Lupin kernel fibre consumption was further positively associated with improvement of colonic functions and beneficial alterations of risk factors for colorectal cancer. These effects were mainly attributed to an increase of faecal mass, reduction of transit time, and fermentation of the fibre to short chain fatty acids, which reduce faecal pH and formation of secondary bile acids (Fechner et al., 2013, Fechner et al., 2014).

Incorporation of lupin protein isolate in a portfolio of different food items significantly lowered total and LDL cholesterol in humans with enhanced activities found in subjects with severe hypercholesterolemia (Bähr et al., 2015). Hypocholesterolaemic and hypotensive effects of lupin proteins are proposed to be linked to the release of specific peptides encrypted in the protein sequence and released during digestion (Arnoldi et al., 2015, Boschin et al., 2014a, b, Lammi et al., 2014). In addition, the hypoglycaemic activities of lupin protein were linked to γ-conglutin, which is suggested to be absorbed in intact form in the intestine (Capraro et al., 2011). In rats administered to a hyperglycaemic diet, γ-conglutin reduced fasting glucose and insulin blood concentrations by about 20–25% (Lovati et al., 2012).

Little attention has been paid on the health benefits provided by lupin polyphenols (Arnoldi et al., 2015). Some studies reported a positive correlation between total phenolic contents and antioxidant activity (Siger et al., 2012). Globulins of lupin seeds were described to form stable complexes with the main flavonoids of lupin, which were released during *in vitro* digestion (Czubinski et al., 2018). However, further studies are needed to evaluate the bioavailability and bioactivity of lupin polyphenols (Arnoldi et al., 2015).

6. Aims of the study

Numerous health benefits are associated with the consumption of plant-based products rich in dietary fibre and phytochemicals. The lack of these compounds in the current Western diet may therefore contribute to the predisposition of the modern population to chronic diseases including cardiovascular disease, diabetes, and colon cancer. The development of these chronic ailments are related to changes in bile acid metabolism. To elucidate the cascade of events related to the health attributes of plant-based foods, a considerable number of research activities have thus addressed the interactions between bile acids and plant compounds, especially focusing on interactions between bile acids and dietary fibres. However, most of these studies fail to provide conclusive results regarding the nature and mechanism of the interactions with bile acids. Therefore, the main objective of this work was to investigate the mechanisms of interaction between bile acids and plant compounds with special emphasis on lupin kernel fibres, associated plant compounds and changes related to fibre processing.

Most studies explaining the interaction between bile acids and plant compounds allow a classification into two possible mechanisms attributed to the viscous or adsorptive effects of plant compounds. As the term ´bile acid binding´ is frequently and erroneously used regardless of the underlying mechanism, it often remains ambiguous whether the analysed fibres actually have binding properties by adsorption. Since viscous or adsorptive characteristics are difficult to investigate *in vivo*, suitable *in vitro* methodologies can contribute to further understand the mechanistic principles of bile acid retention in the small intestine. Yet, common *in vitro* methods to measure the interaction with bile acids lack in precision and differ significantly from the physiological conditions in the human body. As a consequence, the widespread centrifugal approach was compared with a model based on *in vitro* digestion and dialysis. In order to find an appropriate *in vitro* method to differentiate viscous or adsorptive effects, results derived for lupin kernel fibre were compared with commercially available fibre-enriched preparations and their effects on *in vitro* bile acid retention were assessed using cellulose and cholestyramine as negative and positive controls (CHAPTER 1).

A number of disease phenotypes are linked to changes in the size and composition of the bile acid pool. In particular, procarcinogenic and proinflammatory secondary bile acids, derived from microbial transformation of primary bile acids in the colon, are described to accumulate in the bile acid pool during a 'Western diet'. In a second study, the method established in CHAPTER 1 has thus been extended to the analysis of the main components abundant in human bile. To improve the understanding of changes in bile acid profiles associated with plant constituents, the interactions of dietary fibre enriched food ingredients with primary bile acids (cholic acids and chenodesoxycholic acids) as well as with desoxycholic acids as important representatives of secondary bile acids were investigated. Furthermore, the dependence of bile conjugation and the degree of bile acid hydroxylation on bile acid interactions was examined to elucidate mechanistic principles of interactions (CHAPTER 2).

It is frequently hypothesised that the bile acid retention effect of plant compounds is a synergistic effect of the viscous fibre constituents and the surface properties of insoluble dietary fibres. To evaluate whether the mechanisms of interactions investigated in CHAPTER 2 can be linked to the fibre constituents of lupin seeds, bile acid interactions were compared after isolation and fractionation of fibres from lupin hull and cotyledon. Isolation of cell wall components was achieved by enzymatic hydrolysis of proteins followed by alcoholic extraction procedures. The purified fibre fraction was then sequentially extracted to separate pectin-like, hemicellulosic, and lignocellulosic structures. Cellulose and lignin were used as references for bile acid interaction studies. The assessment of *in vitro* bile acid interactions was combined with rheological and dietary fibre characterisations to obtain a profound

knowledge about the fractions of the fibres responsible for interactions with specific bile acids (CHAPTER 3).

In addition to the fibres, proteins are abundant in lupin seeds. The protein and fibre fractions further contain associated secondary plant metabolites, such as polyphenols. Therefore, possible contributions of these compounds to bile acid interactions needed to be evaluated. For this purpose, a pilot-scale procedure was applied to generate protein isolates containing α-, β-, and δ-conglutin (precipitated fraction, LPI-E) and others containing γ-conglutin (ultrafiltrated fraction, LPI-F). Bile acid interactions of protein isolates were then investigated and compared with interactions observed for lupin cotyledon flour. Finally, protein and fibre fractions were purified using repeated alcohol extraction procedures, and the resulting polyphenol extracts were assessed for their composition and bile acid interactions (CHAPTER 4).

Apart from the composition, metabolic and physiological benefits of dietary fibre enriched ingredients further depend on the process applied during food preparation. As most plant-based fibres are predominantly insoluble, efforts have been made to convert insoluble into soluble fibre in order to enhance their physiological effectiveness. Extrusion processing is a widely used process to change physiochemical properties of food components, which was reported to affect bile acid interactions. However, the intermediate mechanism of interaction was not elucidated. Thus, the impact of extrusion processing on the physiochemical (dietary fibre composition, colour, water binding, and oil binding capacity) and functional properties (viscosity and bile acid interactions *in vitro* under simulated gastrointestinal conditions) of lupin kernel fibre was evaluated. These investigations aimed to contribute to the understanding of whether extrusion processing can improve the health benefits of lupin dietary fibre (CHAPTER 5).

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CHAPTER 1: Differentiation of Adsorptive and Viscous Effects of Dietary Fibres on Bile Acid Release by Means of *In Vitro* Digestion and Dialysis [1]

Abstract

To explain the cholesterol-reducing effects of dietary fibres, one of the major mechanisms proposed is the reduced reabsorption of bile acids in the ileum. The interaction of dietary fibres with bile acids is associated with their viscous or adsorptive effects. Since these fibre characteristics are difficult to investigate *in vivo*, suitable *in vitro* methodologies can contribute to understanding the mechanistic principles. We compared the commonly used centrifugal approach with a modified dialysis method using dietary fibre-rich materials from different sources (i.e., barley, citrus, lupin, and potato). Digestion was simulated *in vitro* with oral, gastric, and small intestinal digestion environments. The chyme was dialysed and released bile acids were analysed by high-performance liquid chromatography. The centrifugation method showed adsorptive effects only for cholestyramine (reference material) and a high-fibre barley product (1.4 µmol taurocholic acid/100 mg dry matter). Alternatively, the dialysis approach showed higher values of bile acid adsorption (2.3 µmol taurocholic acid/100 mg dry matter) for the high-fibre barley product. This indicated an underestimated adsorption when using the centrifugation method. The results also confirmed that the dialysis method can be used to understand the influence of viscosity on bile acid release. This may be due to entrapment of bile acids in the viscous chyme matrix. Further studies on fibre structure and mechanisms responsible for viscous effects are required to understand the formation of entangled networks responsible for the entrapment of the bile acids.

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1 Naumann, S., Schweiggert-Weisz, U., Bader-Mittermaier, S., Haller, D., Eisner, P. (2018). Differentiation of adsorptive and viscous effects of dietary fibres on bile acid release by means of *in vitro* digestion and dialysis. *The International Journal of Molecular Sciences*, 19, 2193, doi: 10.3390/ijms19082193.

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Keywords: cholesterol; bile acid binding; bile acid excretion; centrifugation; dialysis; diffusion kinetics; barley fibre; β-glucan; citrus fibre; lupin kernel fibre; potato fibre

1. Introduction

Hypercholesterolemia is one of the major risk factors for coronary heart disease—the most frequent cardiovascular disease in developed and developing countries (Stamler et al., 2000). With regard to the protective effect of a healthy and balanced nutrition, the adequate intake of dietary fibres is of particular importance. Threapleton et al. (2013) have demonstrated the inverse correlation between coronary risk factors and an increased dietary fibre intake.

While a correlation of cholesterol-lowering effects and the structural and physiochemical properties of dietary fibres has already been reported, the underlying mechanisms are not yet fully understood (Gunness and Gidley, 2010). One hypothesis is the impairment of fat and cholesterol absorption and their increased secretion induced by the interaction with dietary fibres or the inhibition of relevant enzymes (Lairon, 2001). Besides the decreased intake of cholesterol, various substances may impact its endogenous synthesis. Due to viscous fibres, the glucose absorption rate decelerates, and therefore the postprandial insulin secretion decreases (Würsch and Pi-Sunyer, 1997). As insulin is an activator of a rate-limiting enzyme in cholesterol synthesis, this mechanism could contribute to the reduction of cholesterol (Erkkilä and Lichtenstein, 2006). Another hypothesis is related to the bacterial fermentation of dietary fibres to produce short chain fatty acids, which could inhibit the hepatic cholesterol synthesis.

Evidence for the latter mechanism is still inconsistent; there is broad agreement on the decrease in cholesterol level by a reduced reabsorption of bile acids (Gunness and Gidley, 2010, Theuwissen and Mensink, 2008). Bile acids are synthesised in the liver from cholesterol and secreted into the small intestine via the gallbladder. Therein, they contribute to the enzymatic degradation and absorption of fats. In the ileum, about 95% of the bile acids are recycled by reabsorption – a process called enterohepatic circulation. Dietary fibres are reported to interact with the bile acids preventing their reabsorption (Gunness and Gidley, 2010). Therefore, the bile acids are actively excreted and need to be resynthesised in the liver from cholesterol, which presumably reduces high cholesterol levels (Dawson et al., 2009). Evidence of this process has already been reported in a variety of *in vivo* studies, which have shown an excess of faecal bile acid excretion after the consumption of specific dietary fibres (Lia et al., 1995, Borum et al., 1992, Garcia-Diez and Garcia-Mediavilla, 1996).

Until now, the properties and the nature of the interaction between the dietary fibres and bile acids leading to low bile absorption is not fully elucidated. Moreover, these aspects are difficult to investigate *in vivo* due to the limited access to small intestinal samples and the fermentative alteration of bile acids in the colon. Most studies focussing on possible mechanisms to explain the interaction of dietary fibres with bile acids can be concluded in two possibilities. Either bile acids are directly adsorbed to dietary fibres and/or a viscous network is formed by the dietary fibre polymers thereby restricting the bile acid release (Gunness and Gidley, 2010). As the term bile acid binding is frequently and erroneously used regardless of the underlying mechanism, it often remains indistinct whether the analysed fibres actually have binding properties by adsorption.

The comparison of results and mechanisms between *in vivo* and *in vitro* are limited by the use of different methods. Most studies on bile acid reabsorption have used centrifugation steps to separate unbound bile acids in the supernatant (Dongowski, 2007, Drzikova et al., 2005, Kahlon and Chow, 2000). However, centrifugal forces differ significantly from the physiological conditions in the human body. Therefore, bile acid binding measured by centrifugation methods may not be appropriate to take into account the viscosity and matrix effects. In addition, methods differ in fibre concentration, parameters of *in vitro* digestion, and centrifugation conditions. Other approaches include pressure filtration and dialysis to separate unbound bile acids. Some *in vitro* studies have already demonstrated the suitability of dialysis to determine bile acid release from simulated chymes (Adiotomre et al., 1990, Gunness et al., 2012, Zacherl et al., 2011, Cornfine et al., 2010). Little account has been taken of whether this approach can reflect adsorption as well as viscous properties of dietary fibres. Moreover, it remains to be shown if these properties can be linked to the water soluble fraction of dietary fibres.

As dialysis is a diffusion-based approach, the concentration gradient between the *in vitro* digested chyme and the dialysate at initial conditions of dialysis represents the driving factor of bile acid release. Correspondingly, first-order kinetics can be applied to the analysis of bile acid release across a dialysis membrane. This could enable the evaluation of viscous and adsorptive effects of dietary fibres based on the parameters of diffusion kinetics (Gunness et al., 2012, Macheras et al., 1986). In the present study, we evaluated this approach by dialysis of *in vitro* digested chyme containing dietary fibre from different sources (i.e., barley, citrus, lupin, and potato) and studied adsorptive as well as viscous effects under simulating conditions of the small intestine.

2. Materials and methods

2.1. Chemicals and enzyme preparations

Cholestyramine resin (C4650), taurocholic acid sodium salt hydrate (T4009), L-α-lecithin (egg yolk, highly purified, CAS 8002-43-5), α-amylase from human saliva Type IX-A (A0521), pancreatin from porcine pancreas (P7545), and pepsin from porcine gastric mucosa (P6887) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other reagents and chemicals were of analytical grade and supplied by VWR (Darmstadt, Germany).

2.2. Dietary fibre-rich materials

A high-fibre barely product SANACEL® betaG containing 19 ± 2% β-glucan (ICC 166) was obtained from CCF GmbH & Co. KG (Gehren, Germany). Citrus fibre Herbacel AQ Citrus-N was kindly provided by Herbafood Ingredients GmbH (Werder, Germany). Lupin kernel fibre (*Lupinus angustifolius* L. Boregine) obtained after de-oiling, extraction, and separation of protein fractions were lyophilised and ground using a Retsch ZM-200 mill (Düsseldorf, Germany) to pass through a 500 µm screen. Lupin seeds were processed on a pilot scale plant based on the method described by D'Agostina, et al. (2006). Cellulose VITACEL® (L 600-30) and potato fibre VITACEL® (KF 200) were supplied by J. Rettenmaier & Soehne GmbH & Co. KG (Rosenberg, Germany).

2.3. Fibre composition

The soluble and insoluble dietary fibre content was determined on an enzymatic–gravimetric basis described in the AOAC Official Method 991.43 (Association of Official Analytical Chemists, 2016).

2.4. Viscosity

Viscosity of the pre-digested samples was analysed using a rotational rheometer (Physica MCR 301, Anton Paar, Graz, Austria) equipped with RheoPlus software version 3.40 (Anton Paar, Graz, Austria). A parallel plate geometry was selected (diameter: 50 mm, shear gap: 1 mm) and the temperature was kept constant at 37 °C. The samples were pre-sheared at a shear rate of 5 s^{-1} for 20 s and allowed to rest for 20 s before starting the measurement. The viscosity was monitored as a function of the shear rate, which was increased linearly (0–1000 s^{-1}).

2.5. *In vitro* determination of bile acid reabsorption

The *in vitro* determination of bile acid reabsorption of chymes containing different fibres was conducted based on a digestion method described by Minekus et al. (2014), followed by simulation of bile acid release from the simulated chyme by dialysis or bile acid binding by centrifugation.

2.5.1. *In vitro* digestion

The static *in vitro* digestion was determined with slight modifications to the methods as described by Minekus et al. (2014). Electrolyte stock solutions (simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)) were exactly prepared as published in Minekus et al. (2014). To avoid microbial growth, 0.04% of sodium azide was added to all electrolyte fluids to reach a concentration of 0.02% in the final digestion mixture. The dietary fibre-rich materials and references were weighed in Erlenmeyer flasks and diluted with demineralised water to obtain a final dry matter concentration of 50% (*w/w*). For the preparation of substrate blank, the sample volume was substituted by demineralised water. All steps were performed at 37 °C under constant shaking using the water bath G76D (New Brunswick Scientific Co., Edison, NJ, USA) at 150 rpm.

During the oral phase, samples were mixed with SSF (ratio 50:50 (*w/w*)) containing α-amylase to achieve 75 $IU \cdot mL^{-1}$ in the final mixture and incubated for 2 min. To simulate the acidic conditions in the stomach, the pH was adjusted to 3 with 1 M HCl. The gastric phase included the addition of SGF (ratio 50:50 (*w/w*)) containing pepsin and lecithin to reach a final concentration of 2000 $IU \cdot mL^{-1}$ and 0.17 mM in the digestion mixture and mixing for 2 h. Finally, to simulate the digestion in the small intestine, the pH was neutralised (pH 7) with 1 M NaOH and SIF (ratio 50:50 *w/w*) was added, which contained pancreatin to reach 100 $IU \cdot mL^{-1}$ of trypsin activity in the digestion mixture. Taurocholic acid was selected as representative bile acid and used at a concentration of 10 mM (Minekus et al., 2014, Zacherl et al., 2011). The resulting chyme was mixed for 2 h for simulating intestinal digestion. Due to the dilution during the digestion phases, the final chyme resulted in a fibre content of 6.25% dry matter for all samples.

2.5.2. Bile acid binding with centrifugation method

The centrifugation of the pre-digested samples (prepared as described in Section 2.5.1) was based on the method described by Kahlon and Chow (2000). A graphic scheme of the method set-up is presented in Figure 1a. Ten mL of *in vitro* digested sample material was centrifuged using a Sigma 3 K 30 ultracentrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz,

Germany) at 30,000 *g* and 25 °C for 18 min. The supernatants were decanted and 5 mL of phosphate buffer (0.1 M, pH 6.3) was added, mixed, and centrifuged as before. Supernatants were pooled, diluted by a factor of 10 with demineralised water, filtrated through a 45 µm syringe filter, and analysed for unbound bile acids using high-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA, USA), as described in Section 2.7. Bile acid concentration was calculated and corrected for the mean recovery of bile acids in the substrate blank.

2.5.3. Bile acid release from simulated chyme using dialysis

4 g of the pre-digested samples (prepared as described in Section 2.5.1), containing 10 mM of taurocholic acid, was transferred into membranes prepared from 16 mm Servapor® 12–14 kDa cut-off dialysis tubings (SERVA Electrophoresis GmbH, Heidelberg, Germany). Dialysis was carried out against 36 mL of 50 mM phosphate buffer (pH 7) containing 0.02% sodium azide using a shaking water bath at 150 rpm and 37 °C. A graphic scheme of the method set-up is presented in Figure 1b. At regular time intervals (2, 4, 8, 12, 24, and 48 h) a 100 µL-aliquot of dialysate was collected and analysed for permeated bile acids by HPLC (Agilent, Santa Clara, CA, USA) as described in Section 4.7.

2.6. Inverse dialysis model for the determination of adsorptive effects on bile acids

The inverse model for determining adsorptive effects on bile acids based on the methods described in Sections 2.5.1 and 2.5.3. Analogous 4 g of the pre-digested sample (Section 2.5.1) was filled in the Servapor® tubings and transferred into a flask containing 36 mL of 50 mM phosphate buffer (pH 7) and 0.02% sodium azide. In addition, 10 mM of taurocholic acid was added to the outside buffer (same initial concentration of taurocholic acid on both sides of the dialysis membrane) and the dialysis was conducted in a shaking water bath at 150 rpm and 37 °C. A graphic scheme of the method set-up is presented in Figure 1c. At regular time intervals (2, 4, 8, 12, 24, and 48 h), a 100 µL-aliquot of dialysate was collected and the decrease of taurocholic acid due to potential adsorptive effects were analysed by means of HPLC (Agilent, Santa Clara, CA, USA), as described in Section 2.7.

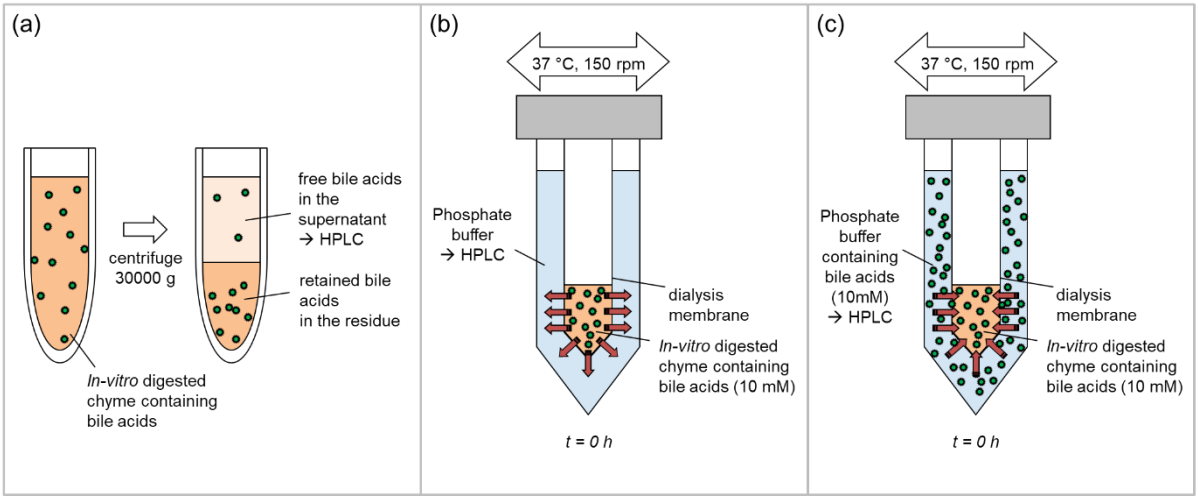

Figure 1. ***In vitro*** **model approaches for simulation of bile acid reabsorption: (a) centrifugation method (Section 2.5.2), (b) bile acid release from simulated chyme using dialysis (Section 2.5.3), and (c) inverse dialysis model for the determination of adsorptive effects (Section 2.6).**

2.7. Bile acid quantification using HPLC

Bile acids were quantified using an Agilent HPLC series 1200 system (Agilent, Santa Clara, CA, USA) equipped with ChemStation software version B.04.02, a G1379 degasser, a G1312B binary gradient pump, a G1367D thermo autosampler, a G1316B column oven, and a G1315C diode-array detector. The column used was a NUCLEOSIL® 120-5 C18 (125 × 3.0 mm i.d.; 5 µm particle size) from Macherey–Nagel (Düren, Germany), operated at 40 °C. The mobile phase consisted of 15 mM phosphate buffer (pH 6.5) in water (eluent A) and 15 mM phosphate buffer (pH 6.5) in water and methanol (30:70, *v/v*, eluent B). A gradient program was applied as follows: 70% B in the first minute, linearly gradient 70–100% B at 1–3 min, 100% B at 4–7 min, linearly gradient 100–70% B at 8–10 min and hold for 5 min. The injection volume was 10 µL, the flow rate was 0.5 mL/min, and the total run time was 15 min. Taurocholic acid was detected at 200 nm and quantified using a calibration curve.

2.8. Statistical analysis

Data obtained by triplicate measurements are presented as mean ± standard deviation. The results were evaluated statistically using R version 3.2.4 (R Development Core Team, 2014). In all analyses $p \leq 0.05$ was considered significant. After testing for homogeneity of variance (Bartlett test) and normal distribution (Shapiro–Wilk test), one-way ANOVA with post-hoc Tukey test were performed to separate significant means. Regressions were calculated using SigmaPlot®, version 12.5 (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Fibre composition

Table 1 represents the soluble dietary fibre (SDF), insoluble dietary fibre (IDF), total dietary fibre (TDF), and a ratio of SDF to TDF. The high-fibre barley product showed a TDF value of 29.2 g/100 g dry matter (DM) while the SDF and IDF were 19.8 g/100 g DM and 9.5 g/100 g DM, respectively. In comparison to the high-fibre barley product, the citrus fibre had differing results for TDF (91.2 g/100 g DM) and an IDF value of 79.8 g/100 g DM. Significant ($p \leq 0.05$) differences were observed between all the samples as analysed by the Tukey test. The cellulose mainly consisted of IDF (99.1 g/100 g DM), which is consistent with the values reported in the literature (Kahlon and Chow, 2000). Lupin showed the second highest TDF (83.4 g/100 g DM) with a significantly ($p \leq 0.05$) lower value of SDF (4.8 g/100 g DM) in comparison with other investigated fibres (except cellulose).

Table 1. Dietary fibre composition of dietary fibre-rich materials and the reference cellulose (soluble dietary fibre (SDF), insoluble dietary fibre (IDF), total dietary fibre (TDF), *n* = 3).

Sample	TDF [g/100 g DM]	IDF [g/100 g DM]	SDF [g/100 g DM]	SDF/TDF [%]
High-fibre barley product	29.2 ± 1.5 [a]	9.5 ± 0.2 [a]	19.8 ± 1.5 [e]	67.3 ± 1.0 [e]
Citrus fibre	91.2 ± 0.0 [d]	79.8 ± 1.2 [d]	14.9 ± 0.3 [d]	15.7 ± 0.3 [c]
Lupin kernel fibre	83.4 ± 0.7 [c]	78.6 ± 0.4 [c]	4.8 ± 0.6 [b]	5.8 ± 0.7 [b]
Potato fibre	67.8 ± 1.9 [b]	56.5 ± 0.8 [b]	11.2 ± 1.7 [c]	16.5 ± 2.2 [d]
Cellulose	100.0 ± 0.5 [e]	99.1 ± 0.2 [e]	0.8 ± 0.4 [a]	0.8 ± 0.4 [a]

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

3.2. Viscosity

The chyme containing citrus fibre was highly viscous in comparison to other selected samples (Figure 2). Interestingly, the viscosity values were surprising due to the smaller amount of SDF in comparison to the high-fibre barley product. The viscosity of the high-fibre barley product was significantly ($p \leq 0.05$) lower than citrus fibre at all measured shear rates. The viscosity of lupin and potato samples were lower than the above samples, but showed a similar pattern of shear thinning at high shear rates. No definite pattern was observed for the other samples due to low values of viscosity.

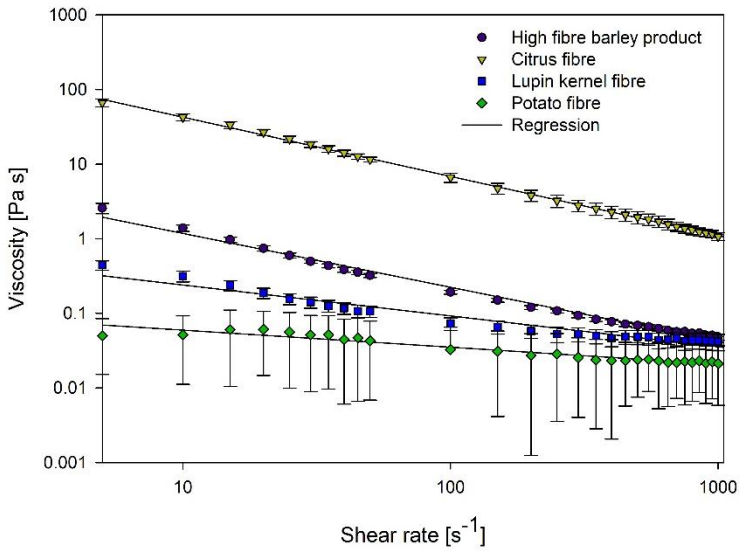

Figure 2. Viscosity of *in vitro* digested chymes containing different dietary fibre-rich materials as a function of the shear rate (*n* = 3).

3.3. *In vitro* determinations of bile acid reabsorption

Centrifugation method was used as a reference method for determination of bile acid reabsorption (Dongowski, 2007, Drzikova et al., 2005, Kahlon and Chow, 2000). Among the selected dietary fibre-rich materials and references, only the high-fibre barley product and cholestyramine showed bile acid binding properties. The proportion of taurocholic acid bound by the high-fibre barley product was 1.4 ± 0.1 µmol/100 mg DM. Cholestyramine showed bile acid binding of 10.8 ± 0.1 µmol taurocholic acid/100 mg DM. The amount of bile acids bound by cholestyramine was similar to the findings of Kahlon et al. (2007) (10.1 ± 0.1 µmol/100 mg DM) and Kim and White (2011) (10.7 ± 0.1 µmol/100 mg DM). Our results for the high-fibre barley product are comparable to the amounts of glycoconjugated bile acids bound by barley flour as reported by Dongowski (2007) (1.18–1.55 µmol/100 mg DM). Kahlon and Woodruff (2003) reported lower values of bile acid binding for β-glucan enriched barley (0.67 ± 0.02 µmol/100 mg DM), but values (5.3 ± 0.1 µmol/100 mg protein) were similar to our findings (6.0 ± 0.5 µmol/100 mg protein) when compared on equal protein basis.

To determine the kinetic of bile acid release using dialysis, the amount of free permeating taurocholic acid was quantified using HPLC. Figure 3 shows the bile acid release curves obtained after *in vitro* digestion and dialysis of simulated chymes containing different dietary fibre-rich materials, the references (i.e., cellulose and cholestyramine), and the substrate blank.

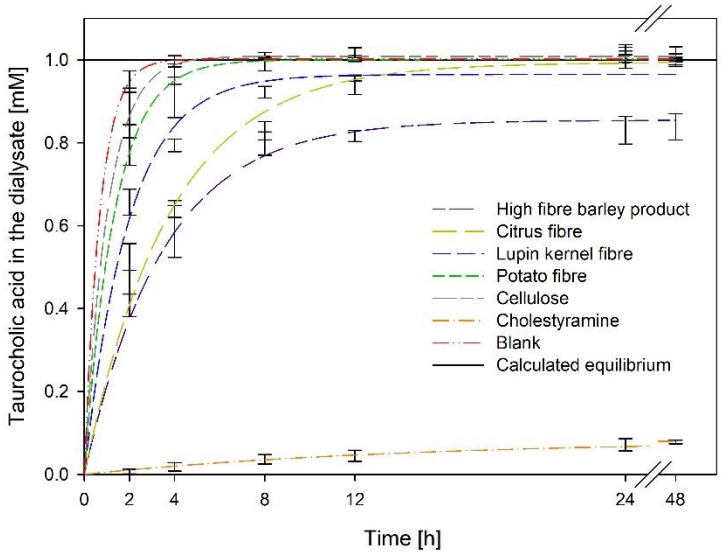

Figure 3. Diffusion kinetics of taurocholic acid release across a dialysis membrane without (blank) and with different dietary fibre-rich materials and references (*n* = 3).

The data obtained by triplicate measurements at six dialysis times were fitted using first-order kinetics (Equation 1).

$$C_t = C_f \times (1 - e^{-k \times t}) \quad (1)$$

This describes the simplest model for diffusion under dialysis (Gunness et al., 2012, Macheras et al., 1986). According to Equation 1, the concentration of taurocholic acid after reaching equilibrium (C_f) and the apparent permeability rate constant (k) were calculated by non-linear regression (Table 2). As can be seen by the correlation coefficients (R^2) in Table 2, this model is appropriate to describe the release of bile acids as a function of time.

Table 2. Correlation coefficients (R^2), concentration of taurocholic acid after reaching equilibrium (C_f), and apparent permeability rate constant (k) determined by first-order kinetic fitting of bile acid release from simulated chymes (*n* = 3).

Sample	R^2	C_f [mM]	k [h^{-1}]
High-fibre barley product	0.96	0.86 ± 0.02 ^{b}	0.29 ± 0.03 ^{a,b}
Citrus fibre	0.99	0.99 ± 0.01 ^{c}	0.27 ± 0.01 ^{a,b}
Lupin kernel fibre	0.99	0.96 ± 0.01 ^{c}	0.51 ± 0.03 ^{b,c}
Potato fibre	0.99	1.00 ± 0.01 ^{c}	0.75 ± 0.04 ^{c,d}
Cellulose	1.00	1.01 ± 0.01 ^{c}	0.98 ± 0.05 ^{d}
Cholestyramine	0.92	0.09 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}
Blank	1.00	1.00 ± 0.00 ^{c}	1.45 ± 0.07 ^{e}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

Without adsorption, the equilibrium concentration (C_f) of 1 mM can be calculated based on the dilution factor of 10 (*in vitro* digested chyme diluted with buffer used for dialysis).

Adsorptive effects can be recognised at lower C_f values, as in the case of reference cholestyramine. The equilibrium concentration in this case was 0.09 mM, which means that 91% of the bile acids were adsorbed. This corresponds to the expectations since cholestyramine is a strong ion exchange resin and forms insoluble complexes with bile acids (Oakenfull and Fenwick, 1978). The high-fibre barley product adsorbed about 14% of the bile acids. Lupin kernel fibre may have bound about 4% of the bile acid present, though the variability of the results were not within the confidence interval ($p = 0.25$). All other dietary fibre-rich materials did not significantly ($p > 0.05$) deviate from the calculated equilibrium concentration and the negative reference cellulose.

Although significant ($p \leq 0.05$) adsorptive effects were only detected for the high-fibre barley product, all other dietary fibres showed a retarded bile acid release from the simulated chymes, as their apparent permeability rate constant k differed significantly ($p \leq 0.05$) from the blank sample (Figure 3 and Table 2). Cellulose showed a higher rate of bile acid release which was consistent with previous results (Dongowski, 2007). Citrus fibre and the high-fibre barley product showed the highest impact on bile acid release, which was more than five times slower than the blank sample.

3.4. Determination of adsorptive effects on bile acids

To confirm the observations of adsorptive effects, an inverse dialysis model for *in vitro* adsorption was applied (Figure 4). The amount of bile acids adsorbed by the high-fibre barley product (15%) was in accordance with the previous dialysis release kinetics (Table 2). This experiment also clearly displayed the adsorptive potential of cholestyramine, which gradually depleted the bile acid concentration in the dialysate ($C_f = 1.2$ mM, 88% of adsorbed bile acids). These results correspond to the samples that have shown bile acid binding properties using the centrifugation method (high-fibre barley product and cholestyramine). This suggests that only adsorptive effects can be measured with the centrifugation method.

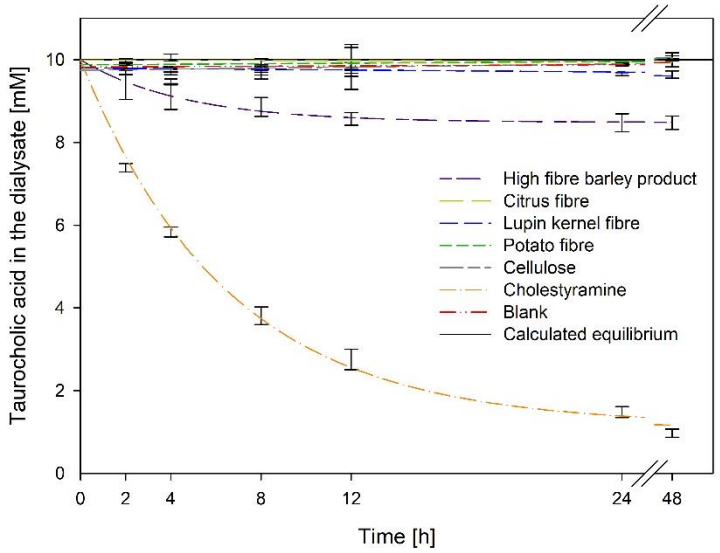

Figure 4. Adsorption of taurocholic acid measured by inverse dialysis model: bile acid concentration as a function of time without (blank) and with different dietary fibre-rich materials and references (*n* = 3).

In addition to the concentration gradient between the chyme and the dialysate, the diffusion of bile acids was mainly influenced by the viscosity of the *in vitro* digested chyme. The impact of adsorptive and viscous effects can be displayed by correlating the apparent permeability rate (k) and the viscosity (Figure 5). Since only small shearing forces occur in the gastrointestinal tract, the correlation was based on the shear viscosity at a shear rate of 15 s^{-1} (Gunness et al., 2012).

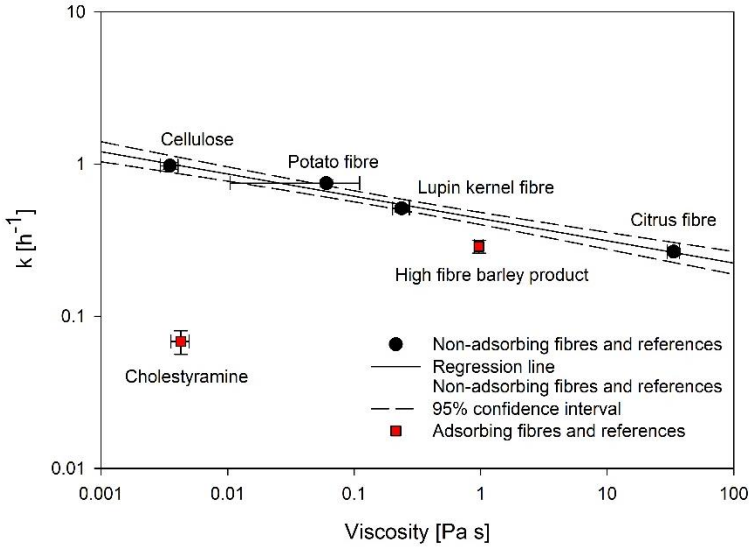

Figure 5. Correlation of apparent permeability rate (k) and the viscosity (shear rate 15 s^{-1}) of *in vitro* digested chymes.

Without adsorption, the apparent permeability rate (k) correlates with the viscosity of the fibres. For fibres showing adsorptive properties, the impact of adsorption on the release rate was evaluated by the distance to the regression line. Correspondingly, the influence of viscosity on bile acid release in the presence of cholestyramine was minimal. On the other hand, the bile release rate of the sample containing the high-fibre barley product was mainly influenced by viscosity.

4. Discussion

Our results support the hypothesis that the influence of dietary fibre-rich materials on the reabsorption of bile acids can be explained as a combination of adsorptive and viscous effects. The impact of both effects has already been discussed based on a variety of *in vitro* and *in vivo* findings (Gunness and Gidley, 2010). *In vitro* digestion combined with dialysis and kinetic analysis enables the differentiation of both effects, which could be underlined by additional measurements of adsorption and viscosity.

4.1. *In vitro* methodologies

Our results substantiate the suitability of dialysis method for *in vitro* determination of bile acid release from the simulated chyme. The parameters of first-order kinetics can be linked to the viscosity and bile acid adsorption of fibres. As the concentration gradient decreases with increasing adsorptive effects of dietary fibres, the equilibrium concentration (C_f) decreases correspondingly. The evaluation of this parameter revealed adsorptive properties of the high-fibre barley product and the reference cholestyramine, which were confirmed by an additional measurement of adsorptive effects via an inverse adsorption experiment (Figure 4). By reducing the concentration of free bile acids present in the *in vitro* digested chyme, adsorptive effects of dietary fibres also decrease the concentration gradient and thereby influence the apparent release rate (k) (Gunness et al., 2012, Macheras et al., 1986). This parameter is inversely controlled by the viscosity of the chyme. As the viscosity of the chyme increases, the diffusion and the release rate decreases. Additional viscosity measurements of fibres (e.g., the high-fibre barley product) were helpful to understand the viscous and adsorption effects individually (Figure 5).

Using the common centrifugation method, bile acid binding was detected in chymes containing the high-fibre barley product and cholestyramine. These samples corresponded to the results of adsorptive effects during dialysis experiments. This suggests that by using methods based on centrifugation exclusively adsorptive effects could be determined. The comparison of results also indicated that the adsorptive effects were underestimated by

centrifugation: the amount of bile acids bound by the anion exchange resin cholestyramine was 10.8 ± 0.1 µmol taurocholic acid/100 mg DM for centrifugation method; using the dialysis method we detected an adsorption of 13.1 ± 0.1 µmol taurocholic acid/100 mg DM. An even higher deviation between the two methods could be demonstrated for the high-fibre barley product (centrifugation method: 1.4 ± 0.1 µmol taurocholic acid/100 mg DM, dialysis method: 2.3 ± 0.3 µmol taurocholic acid/100 mg DM).

To ensure the accuracy of the centrifugation method, a complete precipitation of all possibly binding components and constant ratios of supernatants to residues are required. Precipitation is affected by the water solubility of the components, the matrix composition, the dry matter concentration, and the centrifugation conditions (Minekus et al., 2014, Zacherl et al., 2011). Therefore, it remains unclear to what extent soluble fibres and other soluble matrix components are precipitated using centrifugal approaches. Analogously, we observed different residue volumes depending on the sample composition (e.g., residue volume was higher for samples showing high values of IDF (citrus fibre and lupin fibre)). This could explain the underestimation of adsorptive effects using centrifugation in comparison to the dialysis method. In addition, the inconstant deviations of the methods (centrifugation and dialysis) for cholestyramine and the high-fibre barley product could be attributed to different sample compositions. If samples differ in their composition or solubility, conditions for precise measurements by centrifugation method cannot be achieved. The suitability of this method should thus be considered critically, especially for the analysis of soluble fibre.

4.2. Evaluation of dietary fibre-rich materials

Differences between dietary fibre-rich materials from different sources (i.e., barley, citrus, lupin, potato) were assessed using cellulose and cholestyramine. Cellulose is known to have a low bile acid binding capacity (van Bennekum et al., 2005), which could be confirmed by centrifugation and the *in vitro* release experiments. Cholestyramine was used as reference for direct binding forces, as it is a strong ion exchange resin adsorbing bile acids (Cornfine et al., 2010).

The retardation of bile acid release induced by citrus fibre and potato fibre could be correlated to viscous effects. Soluble fibre content is often described as an indicator for viscous effects caused by soluble fibre components (Dikeman and Fahey, 2006, Levrat-Verny et al., 2000, Dongowski et al., 2003). However, based on the current data, a correlation between the soluble fibre content and the viscosity of *in vitro* digested chymes could not be established. Accordingly, despite similar contents of soluble fibre in citrus fibre and potato fibre, the viscosity of the chymes and the bile acid release differed significantly ($p \leq 0.05$).

Different soluble fibre sources could be associated with different molecular weights and branching patterns. Our results suggest that the soluble fraction of citrus fibre could consist of longer molecules, which are able to form more entanglements, contributing to a higher viscosity of the solution. Depending on the surface area and porosity, insoluble fibres act as filler particles, which could also contribute to the viscosity of the chymes. The particularly high viscous effects of citrus fibre could thus partially be explained by its high insoluble fibre content. This hypothesis is corroborated by the study of Chen et al. (1984) which described a high, specific volume of citrus fibre in comparison to other fibre sources.

Our findings for citrus fibre and potato fibre correspond to previous *in vivo* studies. The influence of the tested potato fibre on the *in vitro* bile acid release was comparatively low. Accordingly, there is no indication for cholesterol-lowering of potato fibre by increased bile acid excretion *in vivo* (Lærke et al., 2007). The cholesterol-lowering effects of the soluble (Brouns et al., 2012, Kay and Truswell, 1977, Bosaeus et al., 1986) and the insoluble fraction (Chau et al., 2004) of citrus fibre were reported to be associated with an increased faecal bile acid excretion. Our results suggest this effect to be linked with the viscous effects of this fibre. Prospective clinical studies should thus be combined with rheological studies and structure elucidation of this fibre to further elucidate the mechanism of cholesterol reduction.

Our findings suggest that the cholesterol-reduction of the high-fibre barley product could be attributed to the reduced reabsorption of bile acids. Both adsorptive and viscous effects were tested and reported. By correlating the apparent permeability rates and the viscosity of all samples, it could be shown that the release of bile acids in the presence of the high-fibre barley product was mainly influenced by viscosity. Using a similar *in vitro* dialysis method, Gunness et al. (2012) already described the viscous effects of purified barley β-glucan. The viscous effects found in our study may thus be subscribed to the high content of β-glucan in the barley product. The cholesterol-lowering effect of barley β-glucan is well established based on a variety of *in vivo* studies (EFSA NDA Panel, 2011). Yet, the mechanisms underlying this effect remain to be fully elucidated. Our results substantiate the influence of viscosity on the reduced reabsorption of bile acids by β-glucan-rich fractions. This is in accordance with the *in vivo* findings of Wolever et al. (2010) that demonstrated higher cholesterol-lowering effects of oat β-glucan dependent on its molecular weight, and thus its contribution to viscosity. In contrast, Kim and White (2010) reported the highest values of bile acid binding for oat fractions containing low molecular weight β-glucan. The authors concluded that other components of the oat fraction might also have contributed to the bile acid binding. This is supported by the study of Sayar et al. (2005) which described that partial lichenase hydrolysis did not affect bile acid binding of β-glucan-rich oat fractions using an *in vitro* centrifugation method. These results support our hypothesis that centrifugation-based methods do not

reflect the impact of viscosity on the reabsorption of bile acids. The comparability on *in vitro* centrifugation method with *in vivo* results is thus greatly impaired.

Referring to the results of previous studies, we assume that the adsorptive effects observed for the high-fibre barley product are not related to its β-glucan content. This is substantiated by the *in vitro* study of Gunness et al. (2012) who reported exclusively viscous effects for purified barley β-glucan on porcine bile acids using an *in vitro* dialysis method. These results confirmed former nuclear magnetic resonance studies indicating that barley β-glucan does not bind tauroconjugated (Gunness et al., 2010) or glycoconjugated (Bowles et al., 1996) bile acids. Therefore, the contribution of other components must be considered. The adsorptive effects of the barley fraction could be related to insoluble dietary fibre components, which was described for β-glucan enriched barley by Kahlon and Woodruff (2003) applying an *in vitro* centrifugation method. Besides fibre, starch and protein represent the main fractions of barley (Oscarsson et al., 1996). Kahlon and Woodruff (2002) already described bile acid binding of protein-rich plant materials and also observed the highest relative values of bile acid binding for β-glucan-enriched barley on equal protein basis (Kahlon and Woodruff, 2003). A study by Araki et al. (2001) described high-binding values for unconjugated bile acids and germinated barley foodstuff. These references indicate a potential role of barley protein in bile acid binding, which should be addressed in further studies.

Zacherl et al. (2011) have already demonstrated a potential influence of lupin kernel fibre on the reabsorption of bile acids *in vitro.* The bile concentration was assessed in comparison to industrial fibre references after three hours of dialysis. Turnbull et al. (2005) similarly concluded that dietary fibre derived from lupin kernels could be suitable as a functional ingredient due to the high-water binding capacity and high induced viscosity during digestion in comparison to other legume sources and negative controls. These results are consistent with the findings of the current study. An *in vivo* study conducted by Fechner et al. (2014) demonstrated that the consumption of lupin kernel fibre contributes to the decrease of blood cholesterol levels in hypercholesterolemic adults. Even though the incorporation of lupin kernel fibre resulted in a slight increase in primary bile acid excretion, the authors hypothesised the formation of short chain fatty acids to be the main contributor to the reduced cholesterol levels. In the current study, the increase in viscosity caused by lupin kernel fibre was low in comparison to the other analysed fibres. Consequently, it could be assumed that the cholesterol reduction found *in vivo* is caused by several interacting effects.

5. Conclusions

The *in vitro* model presented in this study describes the measurement of adsorptive and viscous effects on the reduced reabsorption of bile acids. This was confirmed by the correlation of release kinetics with viscosity and adsorption properties. Correspondingly, in accordance to *in vivo* findings, the release rate in presence of a high-fibre barley product depended mostly on viscous effects, while the adsorption was about 15%. Citrus and lupin kernel fibre preparations, on the other hand, retarded bile acid release mainly by increasing the viscosity of the chyme. Despite its comparably high-soluble fibre content, the apparent release rate was decelerated only slightly in presence of potato fibre. Further experiments confirmed that the soluble dietary fibre content is not suitable as an indicator for bile acid retardation. Using a method based on centrifugation, bile acid binding could primarily be attributed to adsorptive effects. Viscous effects, especially from soluble fibre components, were not represented by this centrifugation method.

Our findings showed that the reduced reabsorption of bile acids should be assessed holistically to account for interactive effects like the interaction of soluble and insoluble compounds, the impact of porosity, and adsorptive properties. The established method could be further helpful to investigate the mechanisms responsible for cholesterol-reducing effects of dietary fibres. For this purpose, the effects on different bile acids should be studied.

To link fibre properties and physiological outcomes, clinical studies should be supplemented with structure elucidation and rheological characterisation. By differentiating adsorptive and viscous effects the *in vitro* dialysis method helps to define relevant parameters for prospective clinical studies on cholesterol-lowering effects of dietary fibres. Furthermore, it could act as an initial indicator on components and structures responsible for the reduced reabsorption of bile acids.

To elucidate the mechanisms responsible for viscous effects, further research has to focus on the formation of entangled networks. Further investigations on the structure and composition of dietary fibre-rich materials need to be studied in detail to gain deeper insight into the molecular interactions of dietary fibres and other indigestible food components with bile acids.

Abbreviations

DM	dry matter
HPLC	high-performance liquid chromatography
IDF	insoluble dietary fibre
SDF	soluble dietary fibre
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SSF	simulated salivary fluid
TDF	total dietary fibre

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 2: *In Vitro* Interactions of Dietary Fibre Enriched Food Ingredients with Primary and Secondary Bile Acids [2]

Abstract

Dietary fibres are reported to interact with bile acids, preventing their reabsorption and promoting their excretion into the colon. We used a method based on *in vitro* digestion, dialysis, and kinetic analysis to investigate how dietary fibre enriched food ingredients affect the release of primary and secondary bile acids as related to viscosity and adsorption. As the main bile acids abundant in humans interactions with glyco- and tauroconjugated cholic acid, chenodesoxycholic acid, and desoxycholic acid were analysed. Viscous interactions were detected for apple, barley, citrus, lupin, pea, and potato derived ingredients, which slowed the bile acid release rate by up to 80%. Adsorptive interactions of up to 4.7 µmol/100 mg dry matter were significant in barley, oat, lupin, and maize preparations. As adsorption directly correlated to the hydrophobicity of the bile acids the hypothesis of a hydrophobic linkage between bile acids and dietary fibre is supported. Delayed diffusion in viscous fibre matrices was further associated with the micellar properties of the bile acids. As our results indicate changes in the bile acid pool size and composition due to interactions with dietary fibre rich ingredients, the presented method and results could add to recent fields of bile acid research.

Keywords: bile acid binding; bile acid excretion; cholesterol; colorectal cancer; *in vitro* digestion; critical micelle concentration

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2 Naumann, S.; Schweiggert-Weisz, U.; Eglmeier, J.; Haller, D.; Eisner, P. (2019). *In vitro* interactions of dietary fibre enriched food ingredients with primary and secondary bile acids. *Nutrients*, 11, 1424, doi: 10.3390/nu11061424.

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1. Introduction

Primary bile acids are synthesised in the liver from cholesterol and stored in the gall bladder. The most common types of primary bile acids are cholic acid (CA) and chenodesoxycholic acid (CDCA), which are conjugated either with taurine or glycine to form water-soluble bile salts (Russell and Setchell, 1992). After a meal gall bladder contraction is stimulated and bile salts are secreted. In the intestinal tract, they act as detergents facilitating the absorption of dietary lipids and fat-soluble vitamins (Chiang, 2013).

Bile acids are reabsorbed by active transport and passive diffusion and transported back to the liver by the hepatic portal vein. The bile acid pool, which contains about 2.5 to 5 g of bile acids, is thereby recycled 4 to 12 times a day. This process, called the enterohepatic circulation, is highly efficient and recycles about 95% of the bile acid pool. Daily, 400 to 800 mg of bile acids escape this circulation and becomes substrate for microbial transformation to secondary bile acids (Vlahcevic and Heuman, 1996).

The most common secondary bile acids are desoxycholic acid (DCA) and lithocholic acid. These result from deconjugation and dehydroxylation of primary bile acids by the human gut microbiota (Ridlon et al., 2006). As lithocholic acid is the most hydrophobic bile acid, reabsorption rates back into the enterohepatic circulation are small (Hanafi et al., 2018). DCA, on the other hand, is less hydrophobic and is reabsorbed in the colon, transported back to the liver and is recycled with CA and CDCA. Therefore, the bile acid pool consists of about 40% CA, 40% CDCA, 20% DCA, and trace amounts of lithocholic acid (Chiang, 2013).

Besides lipid metabolism bile acids are involved in several physiological processes. The hepatic production of primary bile acids represents the main pathway to remove an excess of cholesterol from the body (Lairon, 2001). Recent studies indicate that bile acid pool size and bile acid composition contribute to the regulation of gut microbial community structures (Ridlon et al., 2014). Additionally, bile acids support glucose regulation and energy homeostasis, are involved in several cellular signalling pathways and are ligands for numerous nuclear hormone receptors (Hanafi et al., 2018).

In contrast, bile acids are also associated with a number of disease phenotypes. High concentrations of secondary bile acids promote carcinogenesis in the colon (Nguyen et al., 2018). Changes in bile constituents are linked to the development of biliary stones (Dosch et al., 2019). Cytotoxicity due to microbial changes of bile acid structures may cause inflammation, apoptosis, and cell death. Changes in the size or composition of the bile acid pool are further related to cardiac dysfunctions, liver diseases, and diabetes (Chiang, 2013, Hanafi et al., 2018).

Dietary fibres are reported to interact with bile acids preventing their reabsorption and promoting their transit to the colon. Reducing the reabsorption rate of bile acids affects the bile acid pool size, increases the hepatic synthesis of primary bile acids from cholesterol, and thus, changes the composition of the bile acid pool (Gunness and Gidley, 2010). Using a pig model Gunness et al. (2016) demonstrated that a decrease in blood cholesterol in the presence of oat β-glucan was associated with restricted bile acid diffusion, reduction of circulating bile acids and a change in bile acid profile. Furthermore, a change in the bile acid profile towards a higher concentration of 12α-hydroxylated bile acids was associated with insulin resistance in humans (Haeusler et al., 2013). Interestingly, various types of dietary fibres or fibres with different physio-chemical characteristics were related to changes in bile acid profiles of rats (Ghaffarzadegan et al., 2016). Dietary fibre intake is thus directly related to bile acid metabolism, its various physiological functions and the development of bile acid related diseases. A further understanding of dietary fibre–bile acid interactions is thus needed to understand fibre related changes of bile acid profiles as a measure of physiological homeostasis (Li and Chiang, 2014).

Food processing following the industrial and agricultural revolutions entailed the development of a 'Western diet' high in calories, sugars, and animal fats and low in dietary fibre. Therefore, daily consumption is far below recommendations for fibre intakes, which range from 25 to 35 g/day depending on country specific guidelines (Lunn and Buttriss, 2007). Dietary fibres are characterised by their resistance to enzymatic degradation within the upper gastrointestinal tract. However, they differ greatly in their structural and nutritional properties (American Association of Cereal Chemists, 2001, Lunn and Buttriss, 2007). Elucidation of the structures and properties of dietary fibres responsible for their health promoting effects is thus needed to make specific recommendations and promote the fibre fortification of food.

Hypotheses to explain the interaction of dietary fibres with bile acids can be ascribed to two main effects: Dietary fibres directly adsorb bile acids (e.g. by hydrophobic interactions) and a viscous matrix formed by the dietary fibres reduces bile acid release rates, respectively (Gunness and Gidley, 2010).

Due to bacterial alterations of bile acids and dietary fibres in the colon and the structural diversity of bile acids in animals, *in vivo* studies to directly measure interactions between dietary fibres and human bile acids are very limited. Yet, common *in vitro* methods to measure the interaction of dietary fibres with bile acids lacked in precision and differed significantly from the physiological conditions in the human body. A recent comparison of *in vitro* methods in our laboratory revealed that the combination of *in vitro* digestion, dialysis, and kinetic analysis enables the differentiation of adsorptive and viscous interactions (Naumann et al.,

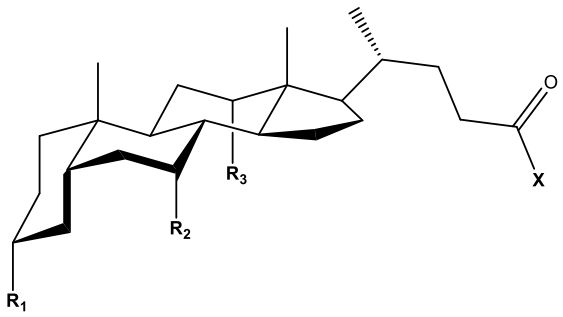
2018). In the present study, this method has been extended to the analysis of the main components abundant in human bile.

The aim of this study is to reveal viscous and adsorptive interactions of dietary fibre enriched food ingredients on conjugated primary bile acids (CA and CDCA) and DCA as an important representative of the secondary ones. Furthermore, the dependence of conjugation and the degree of bile acid hydroxylation should be examined. By this means, we want to contribute to a further understanding of the health promoting effects of dietary fibres as related to bile acid metabolism.

2. Materials and methods

2.1. Bile acids

Taurocholic acid sodium salt hydrate (CAS 345909-26-4), sodium taurochenodeoxycholate (CAS 6009-98-9), sodium glycocholate hydrate (CAS 338950-81-5), sodium glycochenodeoxycholate (CAS 16564-43-5), sodium taurodeoxycholate hydrate (CAS 207737-97-1), and sodium glycodeoxycholate (CAS 16409-34-0) were purchased from Merck KGaA (Darmstadt, Germany). Structural differences in hydroxylation and conjugation of the investigated primary and secondary bile acids are given in Figure 1.



R: Hydroxylation

Bile acid	R_1	R_2	R_3
Cholic acid (CA)	OH	OH	OH
Chenodesoxycholic acid (CDCA)	OH	OH	H
Desoxycholic acid (DCA)	OH	H	OH

X: Conjugation

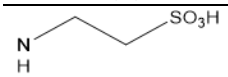
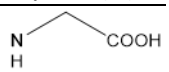
Taurine (T)	Glycine (G)
	

Figure 1. Chemical structure of primary and secondary bile acids mainly abundant in human bile.

2.2. Chemicals and enzyme preparations

L-α-lecithin (egg yolk, highly purified, CAS 8002-43-5), α-amylase from human saliva (Type IX-A, 1,000–3,000 units/mg protein), pancreatin from porcine pancreas (8 × USP specifications) and pepsin from porcine gastric mucosa (3,200–4,500 units/mg protein) were purchased from Merck KGaA (Darmstadt, Germany). All other reagents and chemicals were of analytical grade and supplied by VWR (Darmstadt, Germany).

2.3. Dietary fibre enriched food ingredients

A resistant starch preparation NUTRIOSE® FM 06 was provided by Roquette Frères (Lesrem, France). A maize bran derived preparation SOFABRAN 184-80 was purchased from Limagrain Céréales Ingrédients (Saint-Ignat, France). An oat preparation VITACEL HF 401-30 (extracted from oat spelt bran), a potato preparation VITACEL KF 200, and a wheat preparation VITACEL WF 600 were supplied by J. Rettenmaier & Soehne GmbH & Co. KG (Rosenberg, Germany). An apple preparation Herbacel AQ Plus Apple - A 09 and a citrus preparation Herbacel AQ Plus Citrus - N (derived from apple or citrus fruit pomace) were provided by Herbafood Ingredients GmbH (Werder, Germany). A barley preparation SANACEL® betaG was obtained from CCF GmbH & Co. KG (Gehren, Germany). A pea preparation Emfibre EF 200 (derived from yellow pea cotyledons) was supplied by Emsland-Stärke GmbH (Emlichheim, Germany). A lupin preparation was obtained from lupin cotyledons processed on a pilot scale plant based on the method described by D'Agostina et al. (2006).

2.4. Dietary fibre composition

Soluble and insoluble dietary fibre contents were determined by enzymatic–gravimetric analysis according to AOAC 991.43 (Association of Official Analytical Chemists, 2016).

2.5. Viscosity

Viscosity measurements were conducted using a rotational rheometer (Physica MCR 301, Anton Paar, Graz, Austria). Triple determinations were performed using a parallel plate geometry (diameter: 25 mm, shear gap: 1 mm) (PP25-SN23060, Anton Paar, Graz, Austria) at constant temperature of 37 ± 0.1 °C. An aliquot of 2 g of the *in vitro* digested fibre preparation was positioned in the centre of the tempered plate geometry, the opposite plate was lowered and the sample was trimmed prior to measurement. The samples were pre-sheared at a shear rate of 5 s^{-1} for 20 s and allowed to rest for 20 s before starting the measurement. The viscosity η was monitored as a function of the shear rate, which was increased in a logarithmic scale ranging from 0.01–1000 s^{-1}.

2.6. *In vitro* interactions with bile acids

2.6.1. *In vitro* digestion

The *in vitro* digestion was performed according to the harmonised protocol, developed by the COST INFOGEST network, with slight modifications (Minekus et al., 2014). The digestion procedure included an oral phase, a gastric phase, and an intestinal phase. The dietary fibre enriched food ingredients were weighed in Erlenmeyer flasks and diluted with demineralised water to obtain a final dry matter concentration of 50% (*w/w*). Simulated digestion fluids (simulated salivary fluid, simulated gastric fluid, and simulated intestinal fluid) were exactly prepared as published in Minekus et al. (2014). To avoid microbial growth, 0.04% of sodium azide was added to all electrolyte fluids to reach a concentration of 0.02% in the final digestion mixture. The diluted samples were mixed with pre-warmed (37 °C) simulated salivary fluid (ratio 50:50 (*w/w*)) containing α-amylase to achieve 75 IU·mL^{-1} in the final mixture to start the oral phase. The mixture was incubated at 37 °C for 2 min and then subjected to gastric phase digestion by adjusting the pH of the mixture to 3.0 with 1 M HCl and mixing with simulated gastric fluid (ratio 50:50 (*w/w*)) containing pepsin and lecithin to reach a final concentration of 2000 IU·mL^{-1} and 0.17 mM in the digestion mixture. After incubation for 2 h, the intestinal digestion phase was started by raising the pH to 7.0 with 1 M NaOH and adding simulated intestinal fluid (ratio 50:50 *w/w*), which contained pancreatin and bile acids to reach 100 IU mL^{-1} of trypsin activity and 10 mM in the digestion mixture. Due to overlapping retention times in high-performance liquid chromatography with diode-array detection (HPLC-DAD) (2.6.3.), the interactions of dietary fibre enriched ingredients were examined separately for primary and secondary bile acids. To study interactions with primary bile acids, a mixture (1:1:1:1) containing taurocholic acid (TCA), glycocholic acid (GCA), taurochenodesoxycholic acid (TCDCA), and glycochenodesoxycholic acid (GCDCA) was used. To investigate interactions with secondary bile acids, taurodesoxycholic acid (TDCA) and glycodesoxycholic acid (GDCA) were selected as representatives and were used in equal proportions. The mixture was incubated for 2 h. The digestion was performed in a shaking water bath at 37 °C. A blank digestion was performed by using a mixture of simulated digestion fluids and the same concentration of lecithin, pepsin, pancreatin, and bile but the sample was replaced with demineralised water.

2.6.2. Dialysis and kinetic analysis

In total, 4 g of the *in vitro* digested samples, with 10 mM primary or secondary bile acid mixtures, was transferred into membranes prepared from 16 mm Servapor® 12–14 kDa cut-off dialysis tubings (SERVA Electrophoresis GmbH, Heidelberg, Germany). Dialysis was carried out against 36 mL of 50 mM phosphate buffer (pH 7) containing 0.02% sodium azide using a

shaking water bath at 150 rpm and 37 °C. 100 µL-aliquots of dialysate were taken at 1, 2, 4, 8, 12, 24, and 48 h dialysis time and analysed for permeated bile acids by HPLC (Agilent, Santa Clara, CA, USA).

Bile acid release kinetics were assessed using SigmaPlot®, version 12.5 (Systat Software Inc., San Jose, CA, USA). To describe the diffusion under dialysis the triplicate measurements at seven dialysis times were fitted using non-linear regression. According to the first-order kinetic described in Equation 1, the concentration of bile acids after reaching equilibrium (C_f) and the apparent permeability rate constant (k) were calculated (Gunness et al., 2012, Macheras et al., 1986).

$$C_t = C_f \times (1 - e^{-kt}) \quad (1)$$

Bile acid adsorption was calculated with respect to the dry matter concentration in the *in vitro* digesta (µmol/100 mg DM).

2.6.3. Bile acid quantification using HPLC

Bile acids were analysed using an Agilent HPLC series 1200 system (Agilent, Santa Clara, CA, USA) equipped with Open Lab CDS software version 2.3, a G1379 degasser, a G1312B binary gradient pump, a G1367D thermo autosampler, a G1316B column oven, and a G1315C diode-array detector. The column used was an Ascentis® Express C18 (150 × 4.6 mm i.d.; 2.7 µm particle size) from Supelco® Analytical (Merck KGaA, Darmstadt, Germany) operated at 40 °C. The binary gradient consisted of a water solution containing 0.012% formic acid and 5 mM ammonium acetate (solvent A) and methanol containing 0.012% formic acid and 5 mM ammonium acetate (solvent B) (Glicksman et al., 2010, Tagliacozzi et al., 2003). A binary gradient program was applied as follows: Linearly gradient 70% to 100% B in 10 min; held at 100% B for 5 min followed by re-equilibration at 70% B for 5 min. The injection volume was 10 µL and column flow was 0.6 mL/min. Bile acids were detected at 200 nm and quantified using peak area analysis.

2.7. Statistical analysis

Results are reported as mean ± standard deviation. Significance is shown by a $p \leq 0.05$. The results were assessed using R version 3.2.4 (R Development Core Team, 2014). Homogeneity of variance and normal distribution were tested by Bartlett and Shapiro–Wilk test. Two-way ANOVA and one-way ANOVA with post-hoc Tukey test were performed to separate significant means.

3. Results

3.1. Dietary fibre composition of food ingredients

Dietary fibre compositions of fibre enriched food ingredients derived from different sources are given in Table 1.

Table 1. Dietary fibre composition (soluble dietary fibre (SDF), insoluble fibre (IDF), and total dietary fibre (TDF), *n* = 3) of dietary fibre enriched food ingredients derived from different sources.

	SDF	IDF	TDF	SDF/TDF
Source	[g/100 g DM]	[g/100 g DM]	[g/100 g DM]	[%]
Apple	15.7 ± 1.6	73.0 ± 1.9	88.7 ± 2.5	17.6 ± 1.3
Barley [1]	19.8 ± 1.5	9.5 ± 0.2	29.2 ± 1.5	67.3 ± 1.0
Citrus [1]	14.9 ± 0.3	79.8 ± 1.2	94.7 ± 1.2	15.7 ± 0.3
Lupin [1]	4.8 ± 0.6	78.6 ± 0.4	83.4 ± 0.7	5.8 ± 0.7
Maize	1.3 ± 0.8	78.9 ± 0.8	80.2 ± 1.1	1.6 ± 1.0
Oat	1.4 ± 1.1	92.6 ± 0.4	94.0 ± 1.2	1.5 ± 1.1
Pea	2.0 ± 0.4	52.8 ± 0.1	54.8 ± 0.5	3.6 ± 0.8
Potato [1]	11.2 ± 1.7	56.5 ± 0.8	67.8 ± 1.9	16.5 ± 2.2
Res. starch [2]	84.7 ± 0.7	-	-	-
Wheat	2.5 ± 0.2	96.0 ± 0.1	98.5 ± 0.2	2.5 ± 0.2

[1] as published by Naumann et al. (2018) determined by AOAC 991.43;
[2] as published by Lefranc-Millot et al. (2010) determined by AOAC 2001.03;
- not detected.

The ingredients used in this study differed in the total dietary fibre contents (TDF) and in the ratio of water-soluble and insoluble fibre components. While TDF was highest for wheat (98.5 g/100 mg DM), lowest TDF was detected in the barley derived ingredient (29.2 g/100 mg DM). However, despite fully soluble resistant starch preparation, the barley derived ingredient showed the highest proportion of soluble fibre (67.3%). High soluble proportions were also found in apple (17.6%), potato (16.5%), and citrus (15.7%) derived ingredients (Naumann et al., 2018), whereas the fibre enriched food ingredients derived from oat, maize, wheat, and pea were mainly composed of insoluble fibre.

3.2. Viscosity of *in vitro* digested dietary fibre enriched food ingredients

Since only small shearing forces occur in the gastrointestinal tract, the viscosity of *in vitro* digested dietary fibre enriched food ingredients were compared at a shear rate of 15 s^{-1} (Figure 2a) (Gunness et al., 2012, Naumann et al., 2018). The oat, wheat, and resistant starch preparations did not significantly increase the viscosity of the digesta, as the viscosity was comparable to the blank digestion. The chymes containing apple and citrus fibre preparations

were highly viscous in comparison to all other analysed samples. Viscous networks were also formed by barley, lupin, pea, and potato derived preparations, which significantly increased the viscosity in comparison to the blank digestion.

Due to low values of viscosity, no definite shear rate dependency was observed for *in vitro* digesta of oat, wheat, and resistant starch preparations. All other samples showed a similar pattern of shear thinning at high shear rates (Figure 2b). This flow behaviour is typical of entangled polymer solutions. By increasing the shear rate the polymers align with the direction of the shear as well as with the direction of the shear gradient. Thus, they partially disentangle, which decreases the flow resistance.

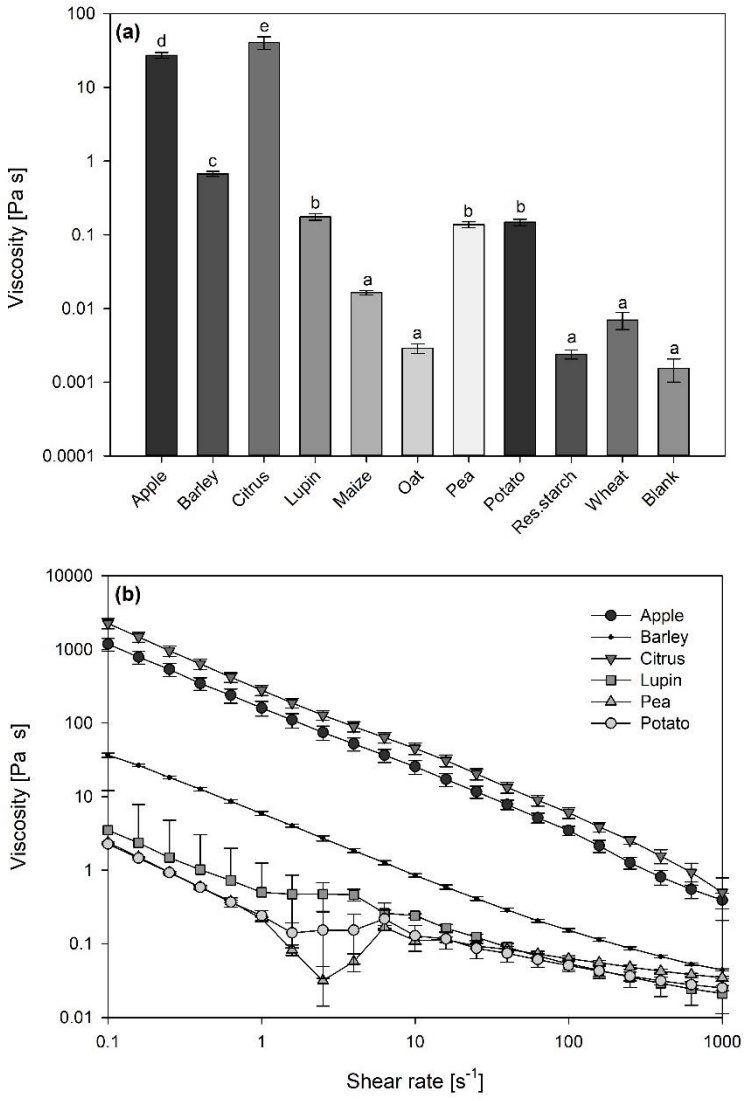
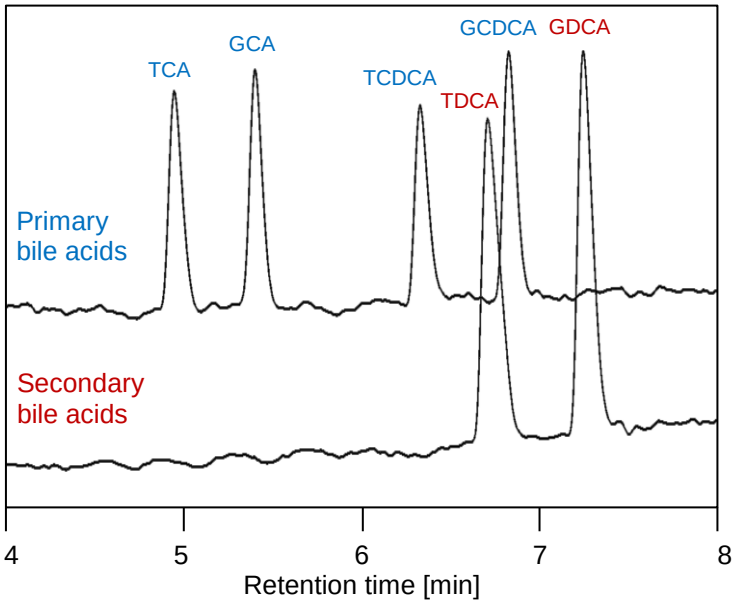


Figure 2. Comparison of viscosity at shear rate 15 s^{-1} (a) and viscosity as a function of the shear rate (b) of *in vitro* digested dietary fibre enriched food ingredients derived from different sources. Different letters indicate significant differences on a $p \leq 0.05$ level basis ($n = 3$).

3.3. Interactions with bile acids

Complete separation was achieved for separate analysis of primary and secondary bile acids by HPLC-DAD (Figure 3). Bile acid concentrations and bile acid release kinetics were calculated. Correlation coefficients for non-linear regression of bile acid release were in the range of 0.963 to 0.999, which shows the high agreement of the kinetic fitting with the experimental data.

Figure 3. Separation of primary and secondary bile acids using HPLC-DAD at 200 nm (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDCA), taurochenodesoxycholic acid (TCDCA), glycodesoxycholic acid (GDCA), taurodesoxycholic acid (TDCA)).



In Figure 4, the diffusion kinetics of bile acid release are exemplified by maize fibre preparation (a) showing adsorptive effects and by apple fibre preparation (b) showing viscous effects in comparison to blank digestion (c).

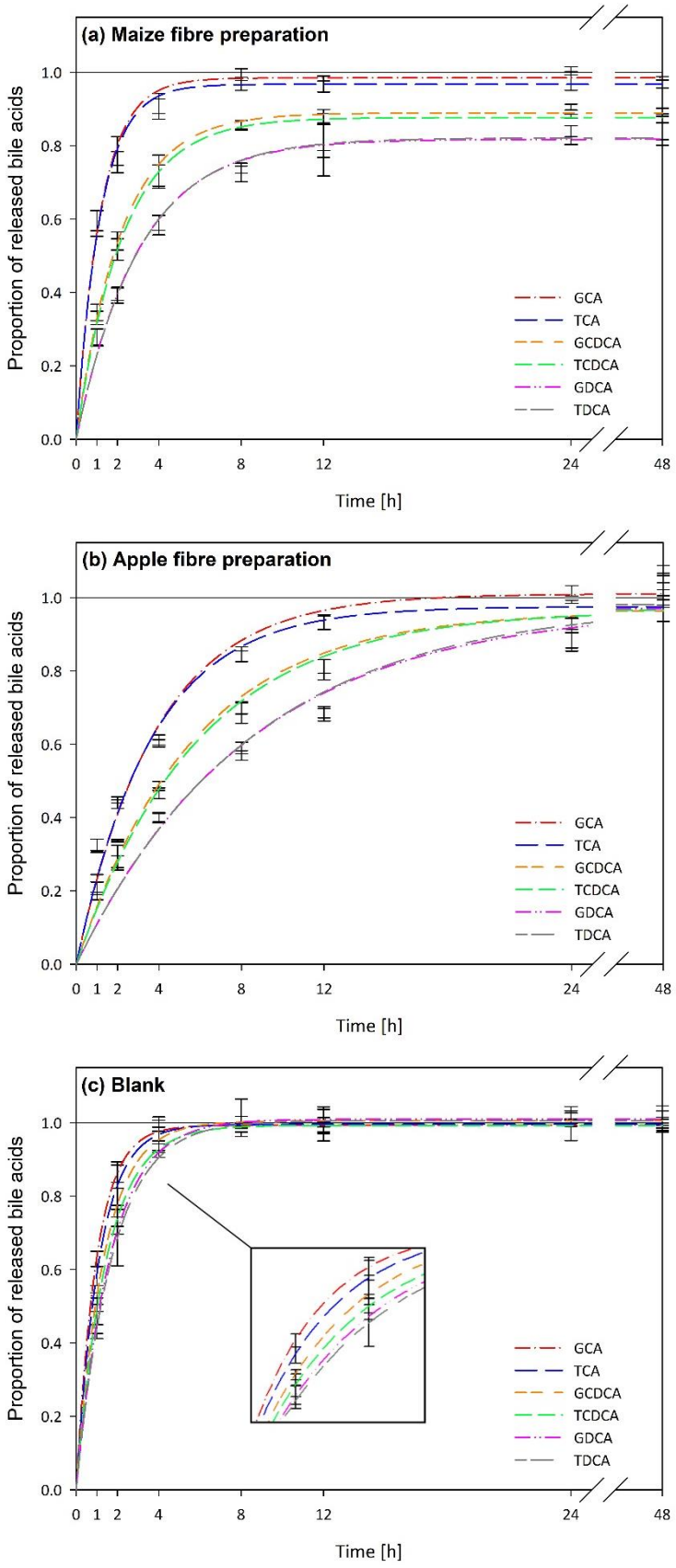


Figure 4. Diffusion kinetics of bile acid release (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDCA), taurochenodesoxycholic acid (TCDCA), glycodesoxycholic acid (GDCA), taurodesoxycholic acid (TDCA)) of *in vitro* digested (a) maize fibre preparation, (b) apple fibre preparation, and (c) blank digestion.

3.3.1. Adsorptive effects as related to bile acid structures

Significant adsorption in comparison to the blank digestion was detected for barley, oat, lupin, and maize fibre enriched food ingredients (Table 2). Adsorptive effects depended strongly on the structure of the bile acids as shown for the maize preparation in Figure 4a. Dihydroxy bile acids (CDCA and DCA) were adsorbed to a higher degree than trihydroxy bile acids (CA), with DCA showing highest adsorption (two-way ANOVA, $p < 0.001$). Conjugation had no significant effect on the adsorption (two-way ANOVA, $p = 0.6$) of bile acids. Therefore, the data for glyco- and tauroconjugated CA, CDCA, and DCA are summarised in Table 2.

Table 2. Bile acid adsorption of dietary fibre enriched food ingredients (given as sum of glyco- and tauroconjugated cholic acids (CA), chenodesoxycholic acids (CDCA), desoxycholic acids (DCA)).

Source	Bile acid adsorption [µmol/100 mg DM]		
	CA	CDCA	DCA
Apple	0.11 ± 0.05 [a]	0.56 ± 0.18 [a]	0.29 ± 0.97 [a]
Barley	1.19 ± 0.13 [b]	3.20 ± 0.32 [c]	4.65 ± 0.17 [d]
Citrus	0.38 ± 0.48 [a]	0.37 ± 0.42 [a]	0.25 ± 0.14 [a]
Lupin	0.44 ± 0.15 [a]	1.74 ± 0.26 [b]	2.18 ± 0.11 [c]
Maize	0.38 ± 0.13 [a]	1.87 ± 0.10 [b]	2.88 ± 0.24 [c]
Oat	1.07 ± 0.11 [b]	2.57 ± 0.06 [c]	2.01 ± 0.57 [c]
Pea	−0.04 ± 0.21 [a]	0.65 ± 0.28 [a]	0.87 ± 0.16 [b]
Potato	−0.10 ± 0.29 [a]	0.29 ± 0.29 [a]	0.72 ± 0.28 [a]
Res. starch	0.03 ± 0.05 [a]	0.21 ± 0.08 [a]	0.19 ± 0.07 [a]
Wheat	0.09 ± 0.11 [a]	0.18 ± 0.12 [a]	−0.03 ± 0.28 [a]
Blank	0.09 ± 0.01 [a]	0.02 ± 0.11 [a]	0.01 ± 0.19 [a]

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

3.3.2. Adsorptive effects as related to dietary fibre sources

CA were adsorbed by barley (1.19 ± 0.13 µmol/100 mg DM) and oat preparations (1.07 ± 0.11 µmol/100 mg DM). Adsorption of dihydroxy bile acids (CDCA and DCA) was higher and significant (in comparison to the blank digestion) in barley, oat, lupin, and maize preparations. In maize (Figure 4a) and barley derived ingredients, the adsorption of DCA was significantly higher than for CDCA. Highest adsorption rates for all analysed bile acids were found for the ingredients derived from barley and oat. Adsorption of DCA was significantly higher in barley derived preparation than in all other analysed samples.

3.3.3. Viscous effects as related to bile acid structures

Blank digestion revealed that bile acid release in aqueous media varied depending on bile acid structures (Figure 4c). This was more evident in samples, which increased the viscosity of the digesta e.g., apple preparation (Figure 4b). Release rates differed depending on the

conjugation and the degree of hydroxylation of the bile acids. Slightly reduced release rates were observed for tauroconjugated bile acids in comparison to glycoconjugated bile acids in the blank digestion and in the samples (Figure 4b and 4c, Table 3). Two-way ANOVA revealed significant differences in apparent permeability rates for glyco- and tauroconjugated bile acids with $p = 0.003$. Hydroxylation affected the release rates to a greater extent: Apparent permeability rates were higher for trihydroxycholic acids (CA) than dihydroxycholic acids (CDCA and DCA) with DCA showing the lowest release rates (two-way ANOVA, $p < 0.001$).

Table 3. Apparent permeability rate constants (k) of kinetic bile acids release analysis (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDCA), taurochenodesoxycholic acid (TCDCA), glycodesoxycholic acid (GDCA), taurodesoxycholic acid (TDCA)) of dietary fibre enriched food ingredients derived from different sources.

	Apparent Permeability Rate k [h^{-1}]					
Source	GCA	TCA	GCDCA	TCDCA	GDCA	TDCA
Apple	0.26 ± 0.01 ^{a}	0.27 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	0.17 ± 0.01 ^{a}	0.12 ± 0.02 ^{a}	0.12 ± 0.02 ^{a}
Barley	0.35 ± 0.04 ^{a,b}	0.31 ± 0.04 ^{a}	0.25 ± 0.01 ^{a}	0.24 ± 0.01 ^{a,b}	0.30 ± 0.05 ^{b,c}	0.28 ± 0.02 ^{b,c}
Citrus	0.26 ± 0.06 ^{a}	0.27 ± 0.04 ^{a}	0.17 ± 0.04 ^{a,b}	0.17 ± 0.04 ^{a}	0.15 ± 0.01 ^{a,b}	0.15 ± 0.01 ^{a,b}
Lupin	0.26 ± 0.06 ^{a}	0.46 ± 0.04 ^{a}	0.39 ± 0.01 ^{b,c}	0.34 ± 0.02 ^{b,c}	0.36 ± 0.07 ^{c,d}	0.34 ± 0.04 ^{c,d}
Maize	0.54 ± 0.05 ^{b}	0.86 ± 0.10 ^{b}	0.46 ± 0.05 ^{c,d}	0.45 ± 0.05 ^{c,d}	0.33 ± 0.04 ^{c,d}	0.33 ± 0.03 ^{c}
Oat	0.85 ± 0.12 ^{c}	0.74 ± 0.12 ^{b}	0.55 ± 0.11 ^{d,e,f}	0.52 ± 0.11 ^{d,e,f}	0.54 ± 0.04 ^{e,f}	0.53 ± 0.04 ^{f,g}
Pea	0.79 ± 0.17 ^{c}	0.77 ± 0.03 ^{b}	0.51 ± 0.04 ^{c,d,e}	0.55 ± 0.08 ^{d,e,f}	0.39 ± 0.07 ^{c,d,e}	0.37 ± 0.05 ^{c,d,e}
Potato	0.88 ± 0.06 ^{c}	0.89 ± 0.07 ^{b}	0.55 ± 0.04 ^{d,e,f}	0.52 ± 0.07 ^{d,e}	0.39 ± 0.11 ^{c,d,e}	0.38 ± 0.10 ^{c,d,e,f}
Res. starch	0.92 ± 0.07 ^{c}	0.85 ± 0.08 ^{b}	0.63 ± 0.04 ^{e,f,g}	0.60 ± 0.04 ^{d,e,f}	0.50 ± 0.05 ^{d,e,,f}	0.48 ± 0.04 ^{d,e,f}
Wheat	0.92 ± 0.10 ^{c}	0.80 ± 0.09 ^{b}	0.68 ± 0.08 ^{f,g}	0.63 ± 0.07 ^{e,f}	0.53 ± 0.03 ^{e,f}	0.51 ± 0.04 ^{e,f,g}
Blank	1.02 ± 0.07 ^{c}	0.90 ± 0.15 ^{b}	0.74 ± 0.05 ^{g}	0.68 ± 0.04 ^{f}	0.61 ± 0.09 ^{f}	0.58 ± 0.08 ^{g}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

3.3.4. Viscous effects as related to dietary fibre sources

For samples showing no adsorption, the apparent permeability rate (k) correlates with the viscosity of the fibres (Naumann et al., 2018). Accordingly, a wheat fibre and a resistant starch preparation did not interact with the bile acids. Kinetic analysis revealed interactions for all other analysed samples by means of retarded bile acid permeability rates (Table 3). The highest retardation of release of all bile acids was found in highly viscous chymes containing apple (Figure 4b) and citrus fibre preparations. Pea and oat preparations showed slight reductions of CDCA and DCA release rates, while the apparent release rate constant for CA was not significantly different from the blank digestion.

As only minor adsorption rates of CA were detected, viscous interactions of adsorbing ingredients (barley, oat, maize, lupin) were compared based on the apparent permeability rate constants of CA: While oat and maize preparations did not show significant viscous interactions, apparent permeability rate was slowed down by 65% by barley and by 48% by lupin derived ingredients in comparison to the blank digestion.

4. Discussion

Our results indicate that both viscous and adsorptive interactions of dietary fibres with bile acids are influenced by the properties of the fibre ingredients as well as by bile acid structure.

4.1. Adsorptive interactions as related to bile acid structures

The degree of bile acid hydroxylation strongly influenced adsorptive interactions with dietary fibre enriched food ingredients: Dihydroxycholic acids were adsorbed more strongly (DCA > CDCA) than trihydroxycholic acids (CA). CA were adsorbed only to a minor extent by barley and oat preparations. CDCA and DCA showed larger adsorption rates of up to 26%. These were significant in barley, oat, lupin, and maize preparations. The results of our study support the hypothesis of a hydrophobic linkage between bile acids and dietary fibre components. This was already proposed by several authors mostly focusing on differences between dihydroxy-bile acids and trihydroxy-bile acids (Camire et al., 1993, Drzikova et al., 2005, Dziedzic et al., 2015, Trautwein et al., 1999). Cornfine et al. (2010) further described bile acid binding to be positively correlated with the degree of fibre acetylation. However, the exact mechanism remains to be fully elucidated. As soluble dietary fibres (like pectin, arabinoxylans, and mixed linkage β-glucans) are hydrophilic, our results suggest that soluble fibre components do not contribute to the adsorptive capacity of the dietary fibre enriched ingredients. Accordingly, no direct molecular interaction with bile acids could be established for β-glucan, the soluble fraction of oat, by Gunness et al. (2010). Adsorption could thus be related to insoluble dietary fibre structures (Kahlon and Woodruff, 2003). Furthermore, contributions of components associated to the dietary fibres could contribute to the adsorption, which was already proposed for lignin, proteins and phytochemical structures (Dongowski, 2007, Kahlon and Smith, 2007, Sayar et al., 2006).

Hydrophobicity of bile salts decreases in the order of DCA > CDCA > CA, which correlates with the results of our study describing a significant decrease in adsorption following this order (Hanafi et al., 2018). However, although glycine-conjugates are more hydrophobic than tauro-conjugates, no significant influence resulting from conjugation was detectable. This is in line with the recent finding of Parker et al. (2014) focusing on the adsorption–desorption behaviour of bile salts to hydrophobic surfaces. The structure function relationships of bile salts were studied using dual polarisation interferometry and atomic force microscopy. The authors described that under physiological conditions simulating the small intestinal milieu (pH 7) glycine as well as taurine are fully charged increasing the solubility of the bile acids in a similar way. The minor role of the conjugation is further substantiated by flexible attachment by a four carbon chain separating the conjugating group from the main amphiphilic group. This explains why minor structural changes in bile acid hydroxylation have a much greater

impact on adsorption than the major structural differences resulting from changes in the conjugated group (Parker et al., 2014). The concordance of our results with this study focusing on adsorption to hydrophobic surfaces further corroborates the hypothesis of a hydrophobic linkage.

4.2. Viscous interactions as related to bile acid structures

The conjugation of bile acids slightly affected the apparent release rate with tauroconjugated bile acids being released more slowly than glycoconjugated bile acids. Hydroxylation had a greater impact on apparent release rate; effects were significant in the blank digestion (Figure 4c) and enhanced in increasingly viscous matrices e.g., formed by apple fibre (Figure 4b). Release rates decreased with increasing hydrophobicity (DCA < CDCA < CA) (Hanafi et al., 2018). We propose these findings to be mainly associated with the micellar properties of the bile acids, which are driven by hydrophobic effects and hydrogen bonding (Madenci and Egelhaaf, 2010). Referring to the Equation of Stokes and Einstein (2), the diffusion (D) at constant temperature (T) and viscosity (η) is influenced exclusively by the radius (r) of the diffusing particles (k_B: Boltzmann's constant) (Miller and Walker, 1924).

$$\mathrm{D} = \frac{k_B\, T}{6\, \pi\, \eta\, r} \tag{2}$$

In the case of bile acids, this radius depends on the proportion of micelles in the solution and the number of molecules within a micelle (aggregation number (N_{agg})). In solution, the concentration of monomeric bile acids increases with increasing concentration until the critical micellar concentration (CMC) is reached and the monomer concentration saturates (Madenci and Egelhaaf, 2010). As recommended by Minekus et al. (2014), a total bile acid concentration of 10 mM was used in our study. During dialysis, this concentration dilutes to 1 mM. Since critical micelle formation concentrations are between 0.9 and 18 mM, this results in different micellar proportions for the different bile acids. Differences in the aggregation number represent an additional parameter influencing the micelle diameters (Table 4).

Table 4. Critical micelle concentration (CMC) and aggregation number (N_{agg}) of main bile acids as summarised by Parker et al. (2014) taken from Madenci and Egelhaaf (2010).

Bile acid	Abbreviation	CMC [mM]	N_{agg}
Taurocholic acid	TCA	3–18	3–7
Glycocholic acid	GCA	4	9
Taurochenodesoxycholic acid	TCDCA	0.9–7	5–26
Glycochenodesoxycholic acid	GCDCA	1–2	15
Taurodesoxycholic acid	TDCA	2–3	12–19
Glycodesoxycholic acid	GDCA	1–2	13–16

According to Table 4 highest CMC and lowest aggregation numbers are described for CA, which therefore form less and smaller micelles causing faster diffusion compared to CDCA and DCA. Analogously, a decrease of diffusion rates of CDCA > DCA is expected by the combination of CMC and the aggregation number (Table 4).

The data displayed in Table 4 refers to aqueous solutions, in which bile salts self-assembly to primary and secondary micelles due to hydrophobic effects and hydrogen bonding (Madenci and Egelhaaf, 2010). Under physiological conditions bile salts form mixed micelles with phospholipids. This increases the capacity of the bile salts to solubilise cholesterol and lipids. These mixed micelles are greatly expanded, show higher aggregation numbers and lower CMC in comparison to micelles formed in aqueous solutions (Dietschy, 1968). A recent study has shown that the internal structure of mixed micelles is independent of the lipid concentration (Tuncer and Bayramoglu, 2019). During the *in vitro* digestion applied in our study bile salts were mixed with phospholipids. We therefore suggest that the micelles, formed under these *in vitro* conditions, resemble the mixed micelles in the intestine under physiological conditions. Reabsorption of mixed micelles due to micellar diffusion is greatly retarded (Dietschy, 1968). Therefore, it is assumed that the *in vivo* reabsorption of bile salts can be explained by the uptake of monomeric constituents that are in equilibrium with the micellar structures (Wilson, 1981). These reabsorption conditions are represented by the *in vitro* system, as the dialysis membrane (cut-off: 12–14 kDa) prevents micelles from diffusion. In addition to a slower diffusion of the micelles to the membrane interface, the decrease of the apparent permeability rates CA > CDCA > DCA, can be explained by the higher concentration of monomeric bile acids as controlled by the CMC of the individual bile acids (Table 4). Micellisation is further controlled by the conjugation of the bile acids: Tauroconjugated bile acids show slightly lower CMC values than glycoconjugated bile acids (Matsuoka et al., 2006), which explains the slightly reduced apparent permeability rates observed for tauroconjugated bile acids in comparison to glycoconjugated bile acids.

4.3. Bile acid interactions as related to dietary fibre sources

The source and the structural composition of the tested dietary fibre enriched food ingredients highly affected the degree of interactions with bile acids. While some ingredients showed no significant or only minor interaction (wheat, resistant starch, pea, and potato), others showed predominantly viscous (apple, citrus) or adsorptive interactions (oat). A combination of viscous and adsorptive interactions was observed for barley, lupin, and maize derived ingredients.

In comparison to the blank digestion, no consistent change of bile acid release was observed for the wheat fibre preparation. This contradicts the *in vitro* results obtained by centrifugation

and described by Kahlon and Chow (2000), who reported bile acid binding of 30.4% on equal TDF basis considering cholestyramine as 100% bound. However, our results correspond to an *in vivo* study conducted by Bosaeus et al. (1986), who did not find an effect of wheat bran supplementation on bile acid excretion in ileostomy patients.

Consistent with our results, dextrin based resistant starches (RS4), like NUTRIOSE®FM, are known as a non-viscous fibre (Johnston et al., 2010, Le Thanh-Blicharz et al., 2014). Increased faecal excretion of bile acids was described for native granular starch (RS2), retrograded, crystalline, nongranular starch (RS3), and chemically modified or re-polymerised starch (RS4) (Dongowski et al., 2005, Shang et al., 2017, Simsek and El, 2012, Ebihara et al., 1998). Contradictory data is described by Chezem et al. (1997), who did not observe a significant increase of faecal bile acid excretion after RS2 in rats. However, they reported highest bile acid excretion after a starch-rich diet, which is in line with the *in vitro* data supplied by Simsek and El (2012). In addition to a possible influence of digestible starch on bile acid excretion, RS-induced changes in microbiota metabolites may alter bile acid absorption and thus influence faecal bile acid excretion (Bindels et al., 2015, Shang et al., 2017). It therefore remains to be clarified to what extent bile acid excretion in faeces can be attributed to adsorptive interactions between RS and bile acids. In our study dextrin-based RS did not show adsorptive interactions with bile acids. As RS encompass a wide variety of structures, further studies are needed to test the transferability of our results to other types of RS.

We found only minimal interactions of pea fibre with CDCA and DCA. Although pea has shown to lower serum cholesterol levels *in vivo*, further studies do not indicate that cholesterol lowering is due to interactions with bile acids (Liu et al., 2014, Martins et al., 2004, Nyman et al., 1990). The cholesterol lowering effect, however, is assumed to be attributed to pea protein or inhibition of cholesterol synthesis by short chain fatty acids because of colonic fermentation of the pea fibre (Nyman et al., 1990, Parolini et al., 2013). Our results support these hypotheses as they do not indicate any direct influence of pea fibre on bile acid metabolism.

Potato fibre also interacted minimally with bile acids, which was shown by a slight but significant retardation of the bile acid release for CDCA and DCA. Therefore, we hypothesise that potential health effects of potato fibre are not directly related to interactions with bile acids. This is supported by the findings of Lærke et al. (2007), who reported no significant reduction of plasma cholesterol or glycaemic response after potato fibre intervention in rats.

Apple fibre preparation formed highly viscous chymes, which significantly slowed down bile acid release by up to 80% while no adsorptive interactions were detected. The viscous properties may be attributed to its high soluble fibre content of 15.7 g/100 mg DM. Accordingly, Parolini et al. (2013) described activation of cholesterol 7α-hydroxylase, the rate

limiting enzyme of bile acid synthesis after intervention with highly viscous apple pectin. This was ascribed to an increase in faecal loss of bile acids, which was also described in two studies conducted by Sembries et al. (2004, 2006). Our results suggested that this influence on bile acid metabolism may be attributed to the formation of a viscous chyme matrix reducing bile acid diffusion and absorption. A similar flow behaviour and bile acid interaction as in apple was observed in citrus fibre enriched ingredient. This is in line with the study conducted by Bosaeus et al. (1986), who found a significant increase in ileostomy bile acid excretion after citrus pectin. Besides pectin, insoluble fibre components may interact with bile acids by formation of entangled fibre networks (Chau et al., 2004, Naumann et al., 2018).

The cholesterol-lowering effect of some oat and barley-based food products is already described and mostly attributed to the viscosity increase of the digesta induced by β-glucan. Accordingly, EFSA allows health claims for foods enriched in β-glucan derived from both barley and oat although the exact mechanism responsible for cholesterol reduction is still not elucidated (EFSA NDA Panel, 2010, 2011). However, it is already known that the cholesterol-lowering effect is strongly influenced by the dose and processing as related to the refinement and molecular weight of the β-glucan enriched products (Grundy et al., 2018). Recent studies also indicate that, in addition to viscosity effects, β-glucan may decrease the permeability of the intestinal mucus (Mackie et al., 2016). Since the oat fibre used in our study showed only a minor proportion of water-soluble dietary fibre (1.5%), including β-glucan, the influence of β-glucan on the investigated bile acid interactions is probably negligible. This is also confirmed by the measurement of shear viscosity, which resulted in a viscosity of 0.003 Pa s (shear rate 15 s^{-1}, Figure 2a), which did not differ significantly from the value observed after blank digestion. Adsorptive interactions must therefore be caused by insoluble dietary fibre or other components of the food matrix. This is in line with the study performed by Zacherl et al. (2011), who investigated the bile acid binding capacity of heat damaged oat fibre. Although the viscosity of the fibre was almost completely lost due to the thermal treatment, a dose-dependent bile acid binding of up to 26% was observed. This binding capacity was suggested to result from hydrophobic interactions. In our former study we already hypothesised that the adsorption of the barley product was independent of its β-glucan content (19 ± 2%). However, the described interactions with TCA could nonetheless mostly be ascribed to the viscosity-increasing properties of the water-soluble β-glucan-rich fraction (Naumann et al., 2018). The current study has been extended to the analysis of six different bile acids, showing DCA to be adsorbed almost four times more than the previously investigated CA. The adsorption shown in both oat and barley derived ingredients could help to explain the cholesterol lowering effects already found in extracts and fractions low in β-glucan as well as in low molecular weight β-glucan hydrolysates (Araki et al., 2001, Bae et al., 2010, Grundy et al., 2018, Kahlon

and Woodruff, 2003, Sun et al., 2006). Further studies are needed to identify the responsible structures and to characterise the adsorptive mechanism.

Although cholesterol lowering effects are described for both lupin and maize derived dietary fibre enriched ingredients, there is a lack of *in vivo* data that directly correlates this effect with bile acid interactions (Fechner et al., 2014, Kreuzer et al., 2002). On the one hand, this could be explained by the low overall number of studies regarding these specific fibres. On the other hand, it could be concluded that interaction with bile acids are not the main mechanism of cholesterol reduction. While the maize fibre preparation did not significantly increase the viscosity of the digesta, the viscosity of chyme containing lupin fibre was comparable with the fibre preparations derived from pea and potato. Concomitantly, there are no *in vivo* studies on these fibres indicating positive nutritional effects due to increased viscosities. Therefore, potential viscosity effects of this magnitude are either insufficient to cause significant outcomes or are superimposed on other effects during *in vivo* digestion. To the best of our knowledge there are no former studies describing the adsorptive effects of lupin and maize fibre preparations on CDCA and DCA. The enhanced binding of DCA might be a possible explanation for the increased excretion and accumulation of primary bile acids in the bile acid pool already described in *in vivo* studies using blue lupin products (Fechner et al., 2014, Martins et al., 2005).

Responsible structures and mechanisms of bile acid retention remain to be fully elucidated. To understand structure function relationships detailed knowledge of the plant tissue, processing of fibre preparations and their physiochemical properties is required. Future *in vitro* studies focusing on bile acid retention should be combined with dietary fibre characterisations. Furthermore, to understand the relationship of dietary fibre structures and health, characterisation and reporting of dietary fibre sources is crucially needed in *in vivo* studies to identify responsible compounds.

5. Conclusions

The comparability of our results for fibre enriched food ingredients showing different structural and functional properties with *in vivo* studies, especially with ileostomy studies, highlights the advantages of the applied *in vitro* method to study interactions with bile acids. The influence of micelle formation on the bile acid release demonstrated further illustrates the limitation of many studies using concentrations below the CMC (Gunness and Gidley, 2010). We were able to link viscosity and adsorption as influenced by fibre sources with the structural properties of the main bile acids abundant in human bile. Adsorption directly correlated to the hydrophobicity of the bile acids, which supports the hypothesis of a

hydrophobic linkage between bile acids and dietary fibre. Delayed diffusion in viscous fibre matrices was further associated with the micellar properties of the bile acids. Secondary bile acids, especially DCA, are associated with a number of disease phenotypes (Ridlon et al., 2014). DCA is known to accumulate in the bile acid pool, if a 'Western diet' low in fibre is consumed. Our results suggest that the extent of the interaction of DCA with bile acids is increased by its hydrophobicity and micellar properties. The elucidation of this increased interaction in comparison to primary bile acids is especially important as the human liver is unable to undertake 7α-hydroxylation of secondary bile acids (Ridlon et al., 2006). Our results could therefore contribute to the elucidation of mechanisms responsible for the health promoting effects of dietary fibres. The applied *in vitro* methodology could act as an initial indicator to identify principal mechanisms and structures responsible for bile acid retention. However, dialysis is a simplified model to simulate absorption. The influence of the mucous layer, small intestinal peristaltic, and active bile acid transporters are not covered by this model. To ensure the transferability to physiological processes, *in vivo* studies need to be conducted to corroborate the presented *in vitro* findings. As dietary fibre intake and changes in bile acid profiles are directly related to microbial shifts and the activity of the gut microbiota (Ghaffarzadegan et al., 2019, Ghaffarzadegan et al., 2018), future studies need to focus on the interplay between dietary fibre, bile acids, and the microbiome.

Abbreviations

CA	cholic acid
CDCA	chenodesoxycholic acid
CMC	critical micelle concentration
DCA	desoxycholic acid
DM	dry matter
GCA	glycocholic acid
GCDCA	glycochenodesoxycholic acid
GDCA	glycodesoxycholic acid
HPLC-DAD	high-performance liquid chromatography with diode-array detection
RS	resistant starch
TCA	taurocholic acid
TCDCA	taurochenodesoxycholic acid
TDCA	taurodesoxycholic acid

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 3: Retention of Primary Bile Acids by Lupin Cell Wall Polysaccharides under *In Vitro* Digestion Conditions [3]

Abstract

Interference of dietary fibres with the enterohepatic circulation of bile acids is proposed as a mechanism for lowering cholesterol. We investigated how lupin hull and cotyledon dietary fibres interact with primary bile acids using an *in vitro* model under simulated upper gastrointestinal conditions. Cell wall polysaccharides were isolated and extracted to separate pectin-like, hemicellulosic, and lignocellulosic structures. Lupin hull consisted mainly of structural components rich in cellulose. The viscosity of the *in vitro* digesta of lupin hull was low, showing predominantly liquid-like viscoelastic properties. On the other hand, lupin cotyledon fibre retarded bile acid release due to increased viscosity of the *in vitro* digesta, which was linked with high contents of pectic polymers forming an entangled network. Molecular interactions with bile acids were not measured for the hull but for the cotyledon, as follows: A total of 1.29 µmol/100 mg dry matter of chenodesoxycholic acids were adsorbed. Molecular interactions of cholic and chenodesoxycholic acids were evident for lignin reference material but did not account for the adsorption of the lupin cotyledon. Furthermore, none of the isolated and fractionated cell wall materials showed a significant adsorptive capacity, thus disproving a major role of lupin cell wall polysaccharides in bile acid adsorption.

Keywords: cholesterol; dietary fibre; bile acid binding; bile acid excretion; viscosity; viscoelastic properties; lignin; cellulose

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1. Introduction

As a sustainable alternative to soybean products, interest in the inclusion of sweet lupins in food is growing (Lucas et al., 2015). Next to their large proportions of plant protein, lupin seeds are a fibre source which has received little attention up to now (Kouris-Blazos and Belski, 2016). Dietary fibres are mainly plant cell wall polysaccharides, such as celluloses, hemicelluloses, and pectin. They are characterised by their resistance to enzymatic degradation in the upper gastrointestinal tract, varying in their structural and nutritional properties (American Association of Cereal Chemists, 2001, Lunn and Buttriss, 2007). Lupin kernel fibres—in contrast to insoluble cereal fibres—have a smooth texture, a neutral taste, and a white colour. This makes lupin kernel fibres an excellent non-intrusive ingredient for fibre enrichment, which shows high palatability and sensory acceptability in dietary interventions (Hall et al., 2010).

Legume seed fibres are derived from two main tissues, as follows: The hull (seed coat) and the cotyledons (kernels), which are therefore often referred to as outer and inner fibre. Legume hulls are rich in fibre, contain high amounts of cellulose and insoluble hemicelluloses and vary in lignin contents. Cotyledons contain less dietary fibre and are poorer in structural components (cellulose and lignin). A special feature of lupin cotyledons is the storage of high amounts of non-cellulose non-starch polysaccharides in their cell walls (Pfoertner and Fischer, 2001). Dietary fibre contents of lupin cotyledons range considerably, between 11.4 and 40.1 g/100 g dry matter (DM), depending on genetic and environmental effects (Bähr et al., 2014, Brillouet and Riochet, 1983).

There is clear evidence that a diet rich in dietary fibre provides protection against diet-related disorders such as obesity, type 2 diabetes, cardiovascular disease, and colon cancer (Bingham et al., 2003, Blackwood et al., 2000). Accordingly, health benefits of lupin kernel fibre have been demonstrated in several *in vivo* studies. The consumption of lupin kernel fibre was associated with a decrease of blood cholesterol levels, lowering of blood pressure, improvement of insulin sensitivity, and favourable alteration of the gut microbiome (Fechner et al., 2013, Fechner et al., 2014, Hall et al., 2005, Johnson et al., 2007).

Our recent *in vitro* findings suggest that the interaction of lupin kernel fibres with primary bile acids may contribute to the cholesterol-lowering properties of lupins (Naumann et al., 2019). Primary bile acids, tauro- and glycoconjugated cholic, and chenodesoxycholic acids, are synthesised in the liver from cholesterol and act as emulsifiers in the small intestine, enabling fat digestion and absorption. Bile acid reabsorption is mainly achieved by active transport in the terminal ileum (Dawson et al., 2009). Dietary fibres may interact with bile acids, withdrawing them from the enterohepatic circulation. By this means endogenous bile acid

synthesis is stimulated, which could explain the cholesterol-lowering effect of dietary fibres (Gunness and Gidley, 2010). Responsible components for decreased re-uptake of bile acids in the ileum and mechanisms of bile acid retention remain to be fully elucidated.

Interactions of dietary fibre with bile acids can be differentiated in adsorptive effects and the entrapment of bile acids in viscous chyme matrices using an *in vitro* model based on *in vitro* digestion, dialysis, and kinetic analysis (Naumann et al., 2018). For a lupin kernel fibre preparation (with a dietary fibre content of 83.4 g/100 g DM), a retardation of bile acid release of about 50% due to increased viscosity of the digesta was observed for all primary bile acids. While cholic acids were not significantly adsorbed, an adsorption of 1.74 µmol/100 mg DM was reported for chenodesoxycholic acids (Naumann et al., 2019, Naumann et al., 2018). However, it is unclear which lupin fibre fractions contribute to these interactions. Increasing the viscosity during digestion is mainly attributed to water-soluble fibres, which impede the diffusion and absorption of bile acids (Guillon and Champ, 2000). Although lupin kernel fibre is described as primarily insoluble, the increased viscosity under simulated digestion may be due to pectic substances with rheological properties similar to pectin (Turnbull et al., 2005).

While there is a broad agreement on the contribution of dietary fibre to viscosity formation, data regarding adsorptive capacities of individual dietary fibre structures are contradictory. As recently reviewed by Singh et al. (2019), bile acid binding capacities were reported for numerous dietary fibre sources during the last decades. However, the term bile acid binding is regularly chosen by authors without considering the underlying mechanism, which impedes the differentiation of adsorptive and viscous effects (Naumann et al., 2018). On the other hand, some studies reported bindings, which were distinctly independent of viscosity due to thermal or enzymatic treatment of dietary fibres, e.g., as described for oat (Sayar et al., 2005, Zacherl et al., 2011). Since no direct molecular interaction with bile acids could be determined for β-glucan (Gunness et al., 2010), the soluble fraction of oat, adsorption could be related to insoluble dietary fibre structures (Kahlon and Woodruff, 2003). Analogously, adsorption by insoluble dietary fibres could account for the bile acid adsorption revealed for lupin fibre (Naumann et al., 2019). However, further studies are needed to identify the relevant structures and to characterise the adsorptive mechanism.

In our previous study, we positively correlated bile acid adsorption with bile acid hydrophobicity, which indicates an underlying mechanism based on hydrophobic interactions (Naumann et al., 2019). The main hydrophobic component of fibre is lignin, which occurs predominantly in structural plant components. Lignin could be introduced to lupin kernel fibre preparations through remaining hulls after the technical de-hulling processes (Cornfine et al., 2010). Lignin has long been considered as the responsible component for bile acid adsorption

(Eastwood and Hamilton, 1968, Gallaher and Schneeman, 1986, Górecka et al., 2002, Kritchevsky and Story, 1976, Sayar et al., 2006). However, the major role of lignin in bile acid adsorption was questioned by other studies (Elhardallou, 1992, Funk et al., 2008, Górecka et al., 2005, Nixon et al., 1986). It is thus not entirely understood whether lignin adds to molecular interactions of lupin fibre with bile acids.

We hypothesise that bile acid retardation by dietary fibres is a synergic effect of viscosity formation and hydrophobic interactions of dietary fibres with bile acids (Guillon and Champ, 2000, Gunness et al., 2012, Gunness and Gidley, 2010, Naumann et al., 2018, Naumann et al., 2019). It is essential to gain detailed knowledge of the plant tissue, processing of fibre preparations, and their physiochemical properties to understand the underlying relationships between cell wall polymer structures and their functional effects on bile acid metabolism.

The aim of this study was to evaluate whether and to what extent lupin dietary fibres contribute to the viscous and adsorptive interactions with primary bile acids. Cell wall components were isolated from lupin cotyledon and hull and sequentially extracted to separate pectin-like, hemicellulosic, and lignocellulosic structures. Cellulose and lignin were used as references for bile acid interaction studies. The assessment of *in vitro* bile acid interactions was combined with rheological and dietary fibre characterisations to obtain a profound knowledge about the fractions of the fibre responsible for interactions with specific bile acids.

2. Materials and methods

2.1. Chemicals and enzyme preparations

Cellulose VITACEL® (L 600-30) was supplied by J. Rettenmaier & Soehne GmbH & Co. KG (Rosenberg, Germany). Taurocholic acid sodium salt hydrate (CAS 345909-26-4), sodium taurochenodeoxycholate (CAS 6009-98-9), sodium glycocholate hydrate (CAS 338950-81-5), sodium glycochenodeoxycholate (CAS 16564-43-5), PRONASE® (53702, Protease, *Streptomyces griseus*, CAS 9036-06-0), lignin alkali (CAS 8068-05-1), L-α-lecithin (egg yolk, highly purified, CAS 8002-43-5), pancreatin from porcine pancreas (8 × USP specifications), and pepsin from porcine gastric mucosa (3200–4500 units/mg protein) were purchased from Merck KGaA (Darmstadt, Germany). All other reagents and chemicals used were of analytical grade and supplied by VWR (Darmstadt, Germany).

2.2. Raw material and pretreatment of lupin seeds

Lupinus angustifolius L. Boregine (harvest 2016) were provided by Saatzucht Steinach GmbH & Co. KG (Steinach, Germany). The cleaned lupin seeds were de-hulled using an underrunner disc sheller (Streckel & Schrader KG, Hamburg, Germany). The cotyledons were separated from the hulls using a zigzag air-classifier (Hosokawa Alpine AG, Augsburg, Germany). Remaining hull material was removed manually and the germ was discarded. Subsequently, the kernels were flaked using a flaking mill (Streckel & Schrader KG, Hamburg, Germany). The obtained yellow flakes were de-oiled by Soxhlet extraction using n-hexane ('white flakes'), which was evaporated in a cold air stream after oil removal. Hulls were sieved to remove adherent residues of the cotyledons. Finally, the hulls and de-oiled white flakes were milled using a Retsch ZM-200 mill (Duesseldorf, Germany) to fit through a 500 µm screen.

2.3. Chemical composition

Dry-matter and ash contents were analysed on a thermogravimetric basis at 105 °C and 550 °C using a TGA 601 analyser (Leco, St. Joseph, MI, USA) following the official methods, L 18.00-12 and L 15.00-7, respectively (Deutsches Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2005). Nitrogen (AOAC Official Method 968.06) was determined according to the Dumas combustion method using a Nitrogen Analyzer TruMac N (LECO Instrumente GmbH, Mönchengladbach, Germany) and protein was calculated as N × 5.8 (Association of Official Analytical Chemists, 2016). Fat content was analysed following the method of Caviezel (Pendl et al., 1998). Determination of starch was carried out by glucose determination applying the hexokinase method described by Beutler (1978). Carbohydrates were calculated as 100 − (ash + protein + fat + total dietary fibre).

2.4. Dietary fibre analyses

The soluble and insoluble dietary fibre content was determined on an enzymatic–gravimetric basis (AOAC Official Method 991.43, Association of Official Analytical Chemists (2016)). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed following the method described by van Soest et al. (1991).

2.5. Monosaccharide composition

Cell walls were hydrolysed by Saeman procedure (Sulphuric acid hydrolysis: 1 h 12 M at 30 °C, followed by 3 h 1 M at 100 °C) and monosaccharides (arabinose, rhamnose, galactose, glucose, xylose, mannose) were determined by high-performance anion exchange chromatography with pulsed amperometric detection. The total uronic acid was determined by spectrophotometric quantification (Blumenkrantz and Asboe-Hansen, 1973).

2.6. Fractionation of lupin cotyledons and hulls

Hull flour and de-oiled cotyledon flour were obtained as described in Section 2.2 and fractionated as illustrated in Figure 1. Protein in lupin cotyledons was removed by Pronase hydrolysis (Section 2.6.1). Subsequently, the cell wall material (CWM) was isolated from the residue of cotyledon hydrolysis and the hulls (Section 2.6.2). Sequential extraction of the CWM yielded three fractions, as follows: NaOH–EDTA-soluble pectin-like fraction (OHEP), hemicellulose fraction (HC), and cellulose-lignin fraction (CL) (Section 2.6.3) (Schieber et al., 2005, Fügel et al., 2004, Mohamed and Rayas-Duarte, 1995).

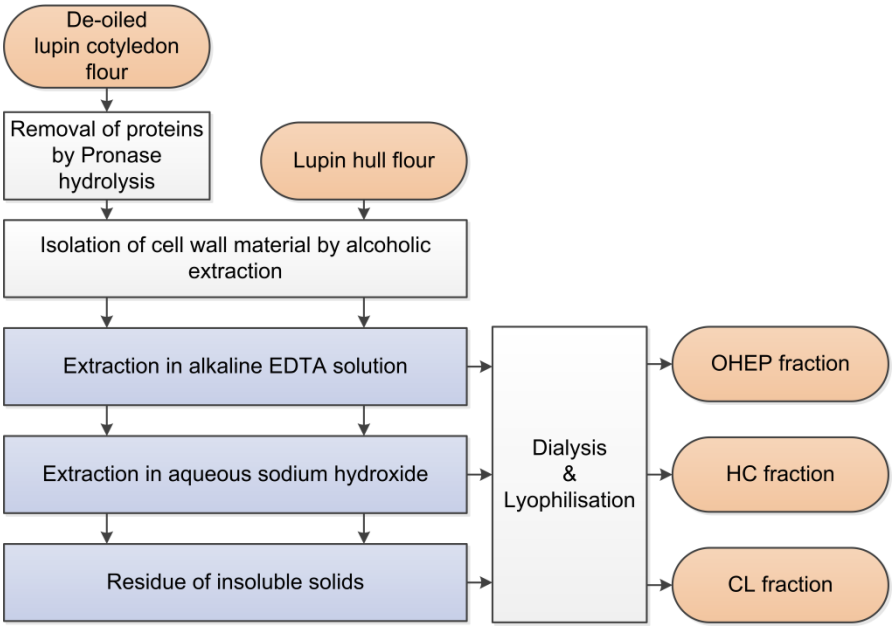

Figure 1. Sequential fractionation of cell wall material obtained from the cotyledon and the hull of *Lupinus angustifolius* L. Boregine (pectin-like fraction (OHEP), hemicellulose fraction (HC), and cellulose-lignin fraction (CL)) (Mohamed and Rayas-Duarte, 1995, Schieber et al., 2005, Fügel et al., 2004).

2.6.1. Removal of protein from lupin cotyledons by Pronase hydrolysis

De-oiled lupin cotyledon flour (5% (*w/v*)) was homogenised in phosphate buffer (0.1 M, pH 7.5) using an Ultra-Turrax blender (IKA, Staufen, Germany). Pronase (5000 IU/100 g lupin flour) was added and incubated at 30 °C for 5 h, followed by centrifugation at 5,000 *g* for 15 min. The residue was washed five times with excess water and recovered by centrifugation (Mohamed and Rayas-Duarte, 1995).

2.6.2. Isolation of cell wall material

The residue of cotyledon Pronase hydrolysis (Section 2.6.1) and the hull flour (Section 2.2) were suspended (10% (*w/v*)) and extracted in boiling ethanol (80% (*v/v*)) for 1 h under

permanent mixing. The solid fraction was isolated from the alcohol phase by filtration using a Büchner funnel and blue ribbon filter paper. The extraction was repeated until the alcohol phase was clear, which was achieved after three repetitions for cotyledon residue and six repetitions for hull flour. The residue was stirred overnight in acetone and then filtered using a G3 glass sinter filter. The solids were transferred into a tared glass vessel and dried at 40 °C for 24 h. The yield of CWM was determined gravimetrically (*n* = 3) (Fügel et al., 2004).

2.6.3. Sequential extraction of cell wall material

The sequential extraction was adapted from Schieber et al. (2005). Extraction conditions were attained using a water bath equipped with a magnetic stirrer. Centrifugation (15,000 *g*, 25 min) with subsequent filtration of the supernatant using a Büchner funnel and black ribbon filtration paper was used to separate the liquid phase from the residue, which was further extracted afterwards. Filtrates were pooled, concentrated using a rotary evaporator, dialysed (Servapor® dialysis tubings 29 mm, MWCO 12–14 kDa, SERVA Electrophoresis GmbH, Heidelberg, Germany) for three days against demineralised water and lyophilised.

2.7. *In vitro* digestion

Reference materials (cellulose and lignin) and lupin fractions were diluted with demineralised water to obtain a constant dietary fibre concentration of 5% in the final digestion mixture. The *in vitro* digestion was performed according to the harmonised INFOGEST protocol (Egger et al., 2016, Minekus et al., 2014) with slight modifications: Oral phase was performed without added α-amylase due to the low content of starch in all samples and 0.04% of sodium azide was added to all electrolyte fluids to reach a concentration of 0.02% in the final digestion mixture to avoid microbial growth. To study interactions with primary bile acids, a mixture (1:1:1:1) containing taurocholic acid (TCA), glycocholic acid (GCA), taurochenodesoxycholic acid (TCDC), and glycochenodesoxycholic acid (GCDC) was used. A blank digestion was performed by using the same concentration of simulated digestion fluids, lecithin, pepsin, pancreatin, and bile acids but the sample was replaced with demineralised water.

2.8. *In vitro* interactions with bile acids

A total of 4 g of *in vitro* digesta (Section 2.7), with 10 mM of primary bile acid mixture, was dialysed (16 mm Servapor® 12–14 kDa cut-off dialysis tubings (SERVA Electrophoresis GmbH, Heidelberg, Germany)) against 36 mL of phosphate buffer (50 mM, pH 7, 0.02% sodium azide) using a shaking water bath at 150 rpm and 37 °C. At 1, 2, 4, 8, 12, 24, and 48 h dialysis time, 100 µL-aliquots of dialysate were analysed for permeated bile acids by high-performance liquid chromatography as described in detail in Naumann et al. (2019). Bile acid release kinetics were exploited applying non-linear regression using SigmaPlot®, version 12.5 (Systat Software Inc., San Jose, CA, USA).

2.9. Rheological measurements

Viscoelastic and viscosity measurements were conducted using a rotational rheometer (Physica MCR 301, Anton Paar, Graz, Austria). Triple determinations were performed using a parallel plate geometry (diameter: 25 mm, shear gap: 1 mm) (PP25-SN23060, Anton Paar, Graz, Austria) at constant temperature of 37 ± 0.1 °C.

2.9.1. Viscosity

Before starting the measurement, samples were sheared at a shear rate of 5 s^{-1} for 20 s and allowed to rest for 20 s. The viscosity η was monitored as a function of the shear rate, which was analysed in a logarithmic scale ranging from 1–1000 s^{-1}.

2.9.2. Dynamic oscillatory rheology

The linear viscoelastic range was determined using strain sweep tests prior to the analysis of the viscoelastic behaviour by frequency sweep tests. Strain sweep tests were performed at an angular frequency of 10 s^{-1} and a strain ramp in a logarithmic scale ranging from 0.01% to 100% deformation of the sample. Based on the results for linear viscoelastic ranges, a constant strain of 0.5% sample deformation was set for the frequency sweep tests. Frequency sweep tests were performed with a logarithmic decreasing angular frequency ranging from 100 to 0.001 s^{-1}. Storage (G') and loss modulus (G") were calculated and used to evaluate the viscoelastic properties of the samples.

2.10. Statistical analysis

The results were evaluated statistically using R version 3.2.4 (www.r-project.org). In all analyses, $p \leq 0.05$ was considered significant. After testing for homogeneity of variance (Bartlett test) and normal distribution (Shapiro–Wilk test), one-way ANOVA with a post-hoc Tukey test was applied to separate significant means.

3. Results and discussion

3.1. Chemical and dietary fibre composition

Lupin seeds of *Lupinus angustifolius* L. Boregine (Figure 2) had an average weight of 189.7 ± 40.5 g/1000 seeds and dimensions of 7.3 mm x 6.2 mm x 5.3 mm and the hulls comprised 18.9 ± 2.4% of the total seeds (*n* = 10).

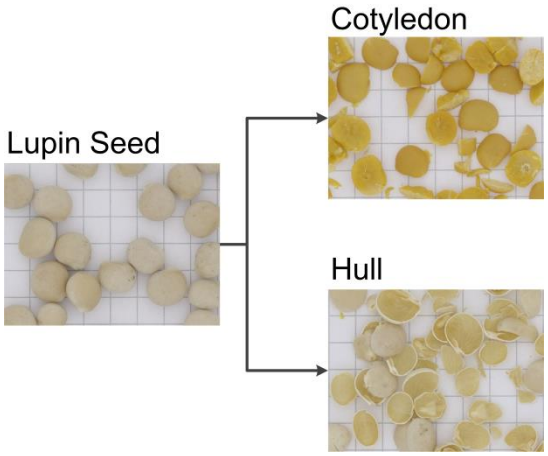

Figure 2. Seeds of *Lupinus angustifolius* L. Boregine.

Chemical and dietary fibre compositions of the cotyledon and the hull of *Lupinus angustifolius* L. Boregine are given in Table 1. Protein (40.13 g/100 g DM), dietary fibre (37.77 g/100 g DM), and fat (9.47 g/100 g DM) represented the main components of lupin cotyledon; the compositional data was within the range reported in the literature (Calabrò et al., 2015, Musco et al., 2017, Muranyi et al., 2013). Starch content of cotyledon was low (0.07 g/100 g DM), which was expected as starch is replaced by galactans as the reserve polysaccharide in lupin (Al-Kaisey and Wilkie, 1992).

Dietary fibre composition detected for cotyledon and hull of *Lupinus angustifolius* L. Boregine was comparable with values reported by Evans et al. (1993) for three cultivars of the same lupin species. The cotyledon of *Lupinus angustifolius* L. Boregine contained high amounts of dietary fibre (37.77 g/100 g DM), which was predominantly water-insoluble (34.59 g/100 g DM). Water-insoluble fibre determined by detergent-extraction (NDF) was 11.34 g/100 g DM. This underestimation in comparison to the enzymatic–gravimetric analysis of insoluble dietary fibre is probably due to a high content of water-insoluble pectin-like substances, which are lost during the NDF procedure (Belo and De Lumen, 1981). Detergent extraction further revealed low levels of cellulose (ADF−ADL: 4.87 g/100 g DM) and lignin (ADL: 0.37 g/100 g DM). The low content of these structural components in the cotyledon was also reported by Evans et al. (1993) and may be explained by the accumulation of non-

structural material within the cell wall, which is presumed as a major polysaccharide reserve (Crawshaw and Reid, 1984).

Table 1. Chemical (dry matter (DM), ash, protein, fat, carbohydrates) and dietary fibre composition (total dietary fibre (TDF), soluble dietary fibre (SDF), insoluble dietary fibre (IDF), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL)) of the cotyledon and the hull of *Lupinus angustifolius* L. Boregine (*n* = 3).

		Cotyledon	Hull
DM	[g/100g]	88.13 ± 0.11	91.53 ± 0.02
Ash	[g/100g DM]	4.26 ± 0.14	2.60 ± 0.05
Protein	[g/100g DM]	40.13 ± 0.13	1.98 ± 0.04
Fat	[g/100g DM]	9.47 ± 0.07	< 0.5
Carbohydrates	[g/100g DM]	8.38 ± 2.15	4.08 ± 0.76
Starch	[g/100g DM]	0.07 ± 0.02	0.03 ± 0.00
TDF	[g/100g DM]	37.77 ± 2.14	91.31 ± 0.76
IDF	[g/100g DM]	34.59 ± 0.96	90.10 ± 0.56
SDF	[g/100g DM]	3.18 ± 1.91	1.21 ± 0.52
NDF	[g/100g DM]	11.34 ± 1.52	82.59 ± 0.40
ADF	[g/100g DM]	5.25 ± 0.30	74.81 ± 0.50
ADL	[g/100g DM]	0.37 ± 0.09	1.95 ± 0.46

As expected, lupin hull mainly consisted of dietary fibre (91.31 g/100 g DM), which was predominantly insoluble (90.10 g/100 g DM) (Pfoertner and Fischer, 2001). Hull fibre contained high amounts of cellulose (ADF−ADL: 72.87 g/100 g DM) while minor lignification was observed (1.95 g/100 g DM).

3.2. Fractionation and monosaccharide composition of cell wall polysaccharides

CWM constituted of 32.6 ± 0.3 g/100 g DM for lupin cotyledon and 93.1 ± 0.7 g/100 g DM for lupin hull. Recovery of the CWM after sequential extraction was 97.2 ± 1.4 % and 89.7 ± 0.5 % for cotyledon and hull, respectively. In a first step solubilisation of pectin-like substances from the isolated CWM was enhanced by EDTA in dilute alkaline solution (OHEP). Hemicelluloses were extracted with increasing alkali concentrations (HC), whereas the remaining alkali-insoluble residue predominantly consisted of cellulose and low amounts of lignin (CL) (Schieber et al., 2005). Sequential extraction yields for OHEP, HC, and CL fractions are given in Figure 3.

HC represented the main fraction of lupin cotyledon (21.4 g/100g DM), followed by OHEP (5.2 g/100g DM) and small amounts of CL (2.1 g/100g DM). CL was predominant in lupin hull (80.8 g/100g DM), while HC (8.4 g/100g DM) and OHEP (2.0 g/100g DM) was low.

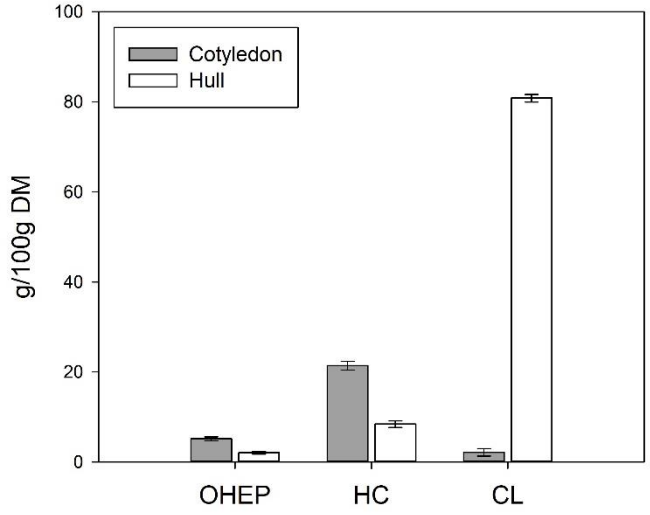

Figure 3. Yields of pectin-like fraction (OHEP), hemicellulose fraction (HC), and cellulose-lignin fraction (CL) isolated from the cotyledon and hull cell wall material of *Lupinus angustifolius* L. Boregine (*n* = 3).

The monosaccharide composition of the cotyledon, hull and isolated pectin-like fraction (OHEP), hemicellulose fraction (HC), and cellulose-lignin fraction (CL) of *Lupinus angustifolius* L. Boregine is given in Table 2. The cell wall composition of the cotyledon (Table 2) is in agreement with previously reported results for *Lupinus angustifolius* L. (Al-Kaisey and Wilkie, 1992, Brillouet and Riochet, 1983, Evans et al., 1993). The main components were galactose (67.6%), arabinose (11.5%), and uronic acids (8.1%), with glucose (7.6%) and xylose (2.6%) being minor constituents.

Table 2. Monosaccharide composition of cotyledon, hull and isolated pectin-like fraction (OHEP), hemicellulose fraction (HC), and cellulose-lignin fraction (CL) of *Lupinus angustifolius* L. Boregine, expressed as a percentage of total polysaccharide sugars.

Sample	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose	Uronic acids
Cotyledon	11.5	1.8	67.6	7.6	2.6	0.8	8.1
C_OHEP	11.3	1.6	73.2	1.1	2.0	0.3	10.5
C_HC	14.0	1.7	71.8	2.0	2.7	0.1	7.8
C_CL	10.6	1.2	46.9	24.8	3.5	0.7	12.3
Hull	8.3	0.4	1.8	59.1	15.7	2.6	12.1
H_OHEP	20.7	1.4	13.6	1.2	7.5	25.9	29.7
H_HC	2.9	0.4	4.5	9.9	71.0	5.3	6.1
H_CL	7.0	0.4	0.4	77.3	3.7	0.8	10.4

The glucose content is in agreement with the cellulose content obtained by detergent extraction (ADF−ADL: 4.87 g/100 g DM) and sequential extraction (Figure 3). The glucose and xylose contents further indicate the presence of xyloglucans or galacto-xyloglucans. The monosaccharide composition coincides with the study of Al-Kaisey and Wilkie (1992), who

described lupin cotyledon polysaccharides to comprise galactans, arabinogalactans, arabinans, rhamnogalacturonans, and galactoxyloglucans. For these galactans, Cheetham et al. (1993) proposed a possible molecular structure. Thereafter, 1–4-linked long-chain galactans and highly branched 1–5-linked arabinans are linked to the rhamnosyl residues of a rhamnogalacturonan backbone. The galactose (71.8%) and rhamnose (1.7%) found in the HC fraction of the cotyledon indicated that this fraction partially consisted of pectic polymers that were not removed by the EDTA extraction. Higher yields for pectic fraction (71.0% and 61.4%) were described by Carre et al. (1985) and Evans et al. (1993) applying higher temperatures and lower pH during extraction with EDTA. Thus, although the yield was highest for HC, pectic polymers represented the main component of lupin cotyledon. The main components of the CL fraction were galactose (46.9%), glucose (24.8%), and arabinose (10.6%). While the glucose content is expected and accounts for cellulosic structures, the compositional data further indicates residual hemicellulose and pectic substances. This indicates that these components are strongly associated with the structural constituents of the cell wall.

As expected for legume seed hulls (Pfoertner and Fischer, 2001), the lupin hulls under study essentially contained cellulose, which accounts for the glucose present in the hull (59.1%) and the CL fraction (77.3%). The other main sugars were xylose (15.7%), uronic acids (12.1%), and arabinose (8.3%), which agrees with the data reported by previous authors for *Lupinus angustifolius* L. and other lupin species (Bailey et al., 1974, Evans et al., 1993). This indicated that the hull, next to cellulose, mainly contained arabinoxylan hemicelluloses and pectic polysaccharides rich in uronic acids (Evans et al., 1993). OHEP, which was the smallest hull fraction (2.0 g/100g DM), showed a strikingly different composition, as follows: Uronic acids were predominant (29.7%), followed by mannose (25.9%), arabinose (20.7%), and galactose (13.6%). Besides pectic substances and arabinoxylan, this composition indicated the presence of galactomannans, which is considered a minor hull constituent (Evans et al., 1993). The dominant sugar in HC was xylose (71.0%), which might originate from the secondary cell wall (Bailey et al., 1974).

3.3. Rheological measurements

3.3.1. Viscosity

The viscosity of *in vitro* digested samples was tested for logarithmically increasing shear rates and which are reported in Figure 4. The digested HC fractions of hull revealed nearly true viscous flow behaviour (viscosity independent of changes in shear rate). All other samples showed a similar pattern of shear thinning at high shear rates, which is typical for insoluble fibre suspensions.

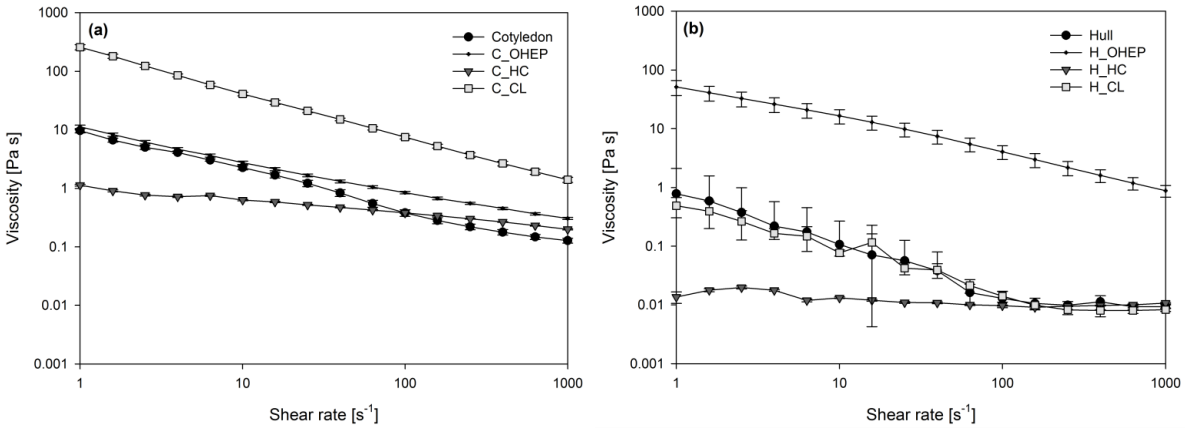

Figure 4. Viscosity of *in vitro* digesta containing fractionated dietary fibre (pectin-like fraction (OHEP), hemicellulose fraction (HC), cellulose-lignin fraction (CL)) derived from lupin cotyledon (a) and hull (b) of *Lupinus angustifolius* L. Boregine (*n* = 3).

The flow curve of the digesta containing lupin hull and its CL fraction tended to a plateau value at high shear rates. This newton-like flow behaviour at high shear rates is typical of polymers without crosslinks or entanglements. As only low shear forces occur in the gastrointestinal tract, viscosity values at low shear rates (approximately 15 s^{-1}) are most relevant with regard to bile acid release (Gunness et al., 2012). At low shear rates, flow curves of digesta containing lupin cotyledon and its OHEP fraction were almost identical (Figure 4a). The highest viscosity was observed for the CL fraction of the cotyledon, while HC showed the lowest viscosity at low shear rates. For digesta containing lupin hull and its dietary fibre fractions (Figure 4b), viscosity of OHEP was almost 2 decades higher than for hull and CL, which showed similar flow behaviour. As observed for cotyledon digesta, the HC fraction of hull showed the lowest viscosity. The viscosities of the *in vitro* digested reference materials cellulose and lignin were low (< 0.02 Pa s at a shear rate of 15 s^{-1}) and showed no significant deviation from blank digestion (p = 0.3).

3.3.2. Dynamic oscillatory rheology

The frequency spectra of storage (G') and loss (G") moduli of *in vitro* digesta containing fractionated dietary fibre derived from lupin cotyledon (a) and hull (b) are shown in Figure 5.

For the whole cotyledon as well as its OHEP and CL fraction (Figure 5a), both moduli were largely independent of frequency and a prevalence of G' over G" was observed, suggesting a highly crosslinked polymer solution or dispersion with high degree of structural integrity. For the HC fraction of the cotyledon, however, G" was higher than G' and the values of both G' and G" continuously decreased with decreasing frequencies towards a plateau at low frequencies. This indicates a weakly crosslinked polymer solution or dispersion with low structural strength. Similar viscoelastic properties were observed for the hull and its HC and

CL fraction (Figure 5b). The OHEP fraction of the hull showed a small decrease of both moduli with continuous G' > G", signalling predominantly elastic properties.

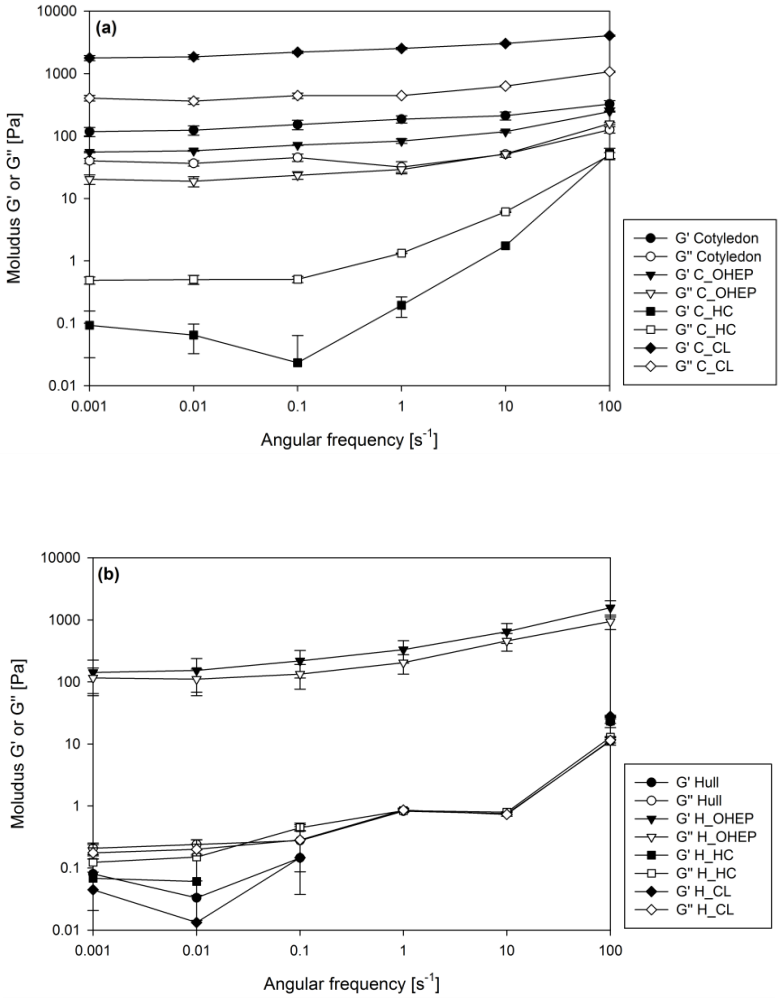

Figure 5. Frequency spectra of storage (G') and loss (G'') moduli of *in vitro* digesta containing fractionated dietary fibre (pectin-like fraction (OHEP), hemicellulose fraction (HC), cellulose-lignin fraction (CL)) derived from lupin cotyledon (a) and hull (b) of *Lupinus angustifolius* L. Boregine (*n* = 3).

3.3.3. Evaluation of rheological properties

Although HC was the largest fibre fraction of lupin cotyledon (Figure 3), the rheological properties of the cotyledon digesta deviated strongly from this fraction. HC showed lower flow viscosity and predominantly liquid-like behaviour (G" > G'). However, the viscosity and viscoelastic properties (predominantly solid-like behaviour (G' > G")) observed for the OHEP fraction was similar to the cotyledon. Therefore, we assume the rheology of the cotyledon to be associated with the polysaccharides extracted in the OHEP fraction. The rheological properties of the HC fraction indicate a low molecular weight and low crosslinking of the

contained polymers. The rheological properties of the OHEP fraction, on the other hand, suggest a high molecular weight and high entanglement of the polymers. This is corroborated by the study of Carre et al. (1985), described a small EDTA soluble fraction of high molecular weight, which mainly consisted of galactose, as we also observed in this study. Due to its high cellulose content, the high viscosity and high structural integrity for the CL cotyledon fraction was not expected. The low viscosity of digesta containing cellulose was already described in previous studies (Naumann et al., 2018). We assume that the rheological properties of CL digesta can thus be ascribed to residual pectic polymers (Table 2). The higher structural strength, in comparison to the OHEP fraction, further suggests synergistic interactions between the pectic polymers and the structural lignocellulosic components.

As expected, the hull digesta showed rheological properties similar to the CL fraction, which was by far the largest hull fraction (Figure 3). In agreement to previous studies, the viscosity of the mostly cellulosic polymers was low (Naumann et al., 2018). Liquid-like behaviour was dominant in the frequency test, which is in line with the low water binding capacity of cellulose (Chen et al., 1984). The OHEP fraction showed a high viscosity and high structural strength, indicating a high molecular weight and entanglement of the polymers. The properties of the hull HC were comparable to the HC fraction extracted from cotyledon. Due to the small proportions of OHEP and HC in the hull (Figure 3), no contribution of these fractions to the rheological behaviour of the hull was expected.

3.4. Interactions with primary bile acids

Bile acid release kinetics were investigated for the primary bile acids GCA, TCA, GCDC, and TCDC. The concentration of primary bile acids after reaching equilibrium (C_f) and the apparent permeability rate constant (k) were calculated following the first-order kinetic described in Equation 1 (Gunness et al., 2012, Macheras et al., 1986).

$$C_t = C_f \times (1 - e^{-k \times t}) \quad (1)$$

Correlation coefficients for non-linear regression of bile acid release ranged between 0.977 and 0.999, which showed the high agreement of the kinetic fitting with the experimental data.

If bile acid release studies are combined with rheological investigations after *in vitro* digestion, viscous and adsorptive interactions of samples with specific bile acid species can be differentiated by the parameters of the kinetic analysis (Naumann et al., 2018). The effects of the samples on the viscosity after *in vitro* digestion can be understood using rheological measurements (Section 3.3). Subsequently, the influence of the viscosity increase on specific

bile acid species can be evaluated by comparing their apparent permeability rates, k (Equation 1), which is further addressed in Section 3.4.1.

Figure 6 shows the bile acid release kinetics for *in vitro* digesta containing lupin cotyledon (a) and hull (b) compared to the reference materials cellulose (c) and lignin (d).

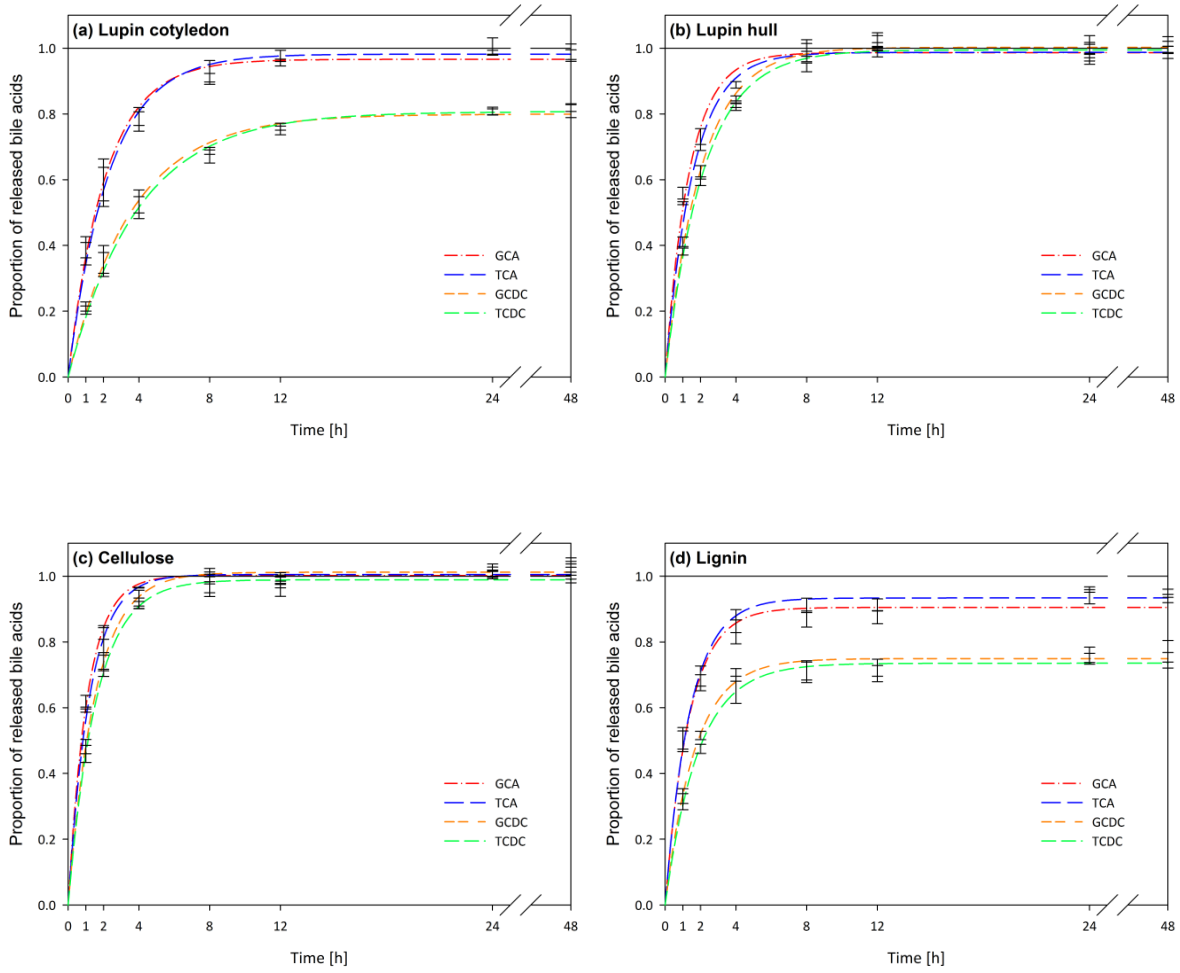

Figure 6. Diffusion kinetics of bile acid release (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDC), and taurochenodesoxycholic acid (TCDC)) of *in vitro* digested cotyledon (a) and hull (b) of *Lupinus angustifolius* L. Boregine, cellulose (c) and lignin (d) (*n* = 3).

In case that bile acids are adsorbed to sample components, less bile acids are available for diffusion, resulting in a decrease of the equilibrium concentration, C_f (Equation 1), in comparison to the blank digestion. In Figure 6 this incomplete release of bile acids is evident for lupin cotyledon (a) and lignin (d), which is examined and discussed in more detail in Section 3.4.2.

3.4.1. Viscous interactions

Apparent permeability rates (k) of fractionated CWM derived from lupin cotyledon and hull and the references, cellulose and lignin, are given in Table 3.

Table 3. Apparent permeability rate constants (k) of kinetic bile acids release analysis (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDC), and taurochenodesoxycholic acid (TCDC)) of *in vitro* digesta containing fractionated dietary fibre (pectin-like fraction (OHEP), hemicellulose fraction (HC), cellulose-lignin fraction (CL)) derived from lupin cotyledon and hull of *Lupinus angustifolius* L. Boregine, cellulose and lignin (*n* = 3).

Sample	k [h^{-1}]			
	GCA	TCA	GCDC	TCDC
Cotyledon	0.48 ± 0.08 ^{a,b}	0.44 ± 0.07 ^{a,b,c}	0.28 ± 0.03 ^{a}	0.26 ± 0.03 ^{a}
C_OHEP	0.36 ± 0.03 ^{a}	0.38 ± 0.04 ^{a,b}	0.25 ± 0.02 ^{a}	0.26 ± 0.03 ^{a}
C_HC	0.58 ± 0.10 ^{b,c}	0.57 ± 0.08 ^{b,c,d}	0.47 ± 0.14 ^{b}	0.45 ± 0.06 ^{b}
C_CL	0.36 ± 0.06 ^{a}	0.31 ± 0.05 ^{a}	0.26 ± 0.04 ^{a}	0.23 ± 0.04 ^{a}
Hull	0.74 ± 0.04 ^{c,d}	0.64 ± 0.04 ^{c,d,e}	0.49 ± 0.03 ^{b}	0.46 ± 0.02 ^{b}
H_OHEP	0.39 ± 0.03 ^{a}	0.34 ± 0.02 ^{a}	0.28 ± 0.01 ^{a}	0.26 ± 0.01 ^{a}
H_HC	0.69 ± 0.05 ^{c,d}	0.65 ± 0.06 ^{d,e}	0.51 ± 0.02 ^{b,c}	0.48 ± 0.02 ^{b}
H_CL	0.85 ± 0.04 ^{d,e}	0.74 ± 0.04 ^{d,e,f}	0.62 ± 0.03 ^{b,c,d}	0.59 ± 0.01 ^{c,d}
Cellulose	0.92 ± 0.07 ^{e,f}	0.82 ± 0.06 ^{e,f}	0.65 ± 0.06 ^{c,d}	0.63 ± 0.06 ^{c,d}
Lignin	0.74 ± 0.04 ^{c,d}	0.71 ± 0.05 ^{d,e,f}	0.59 ± 0.03 ^{b,c,d}	0.54 ± 0.03 ^{b,c}
Blank	0.96 ± 0.04 ^{f}	0.93 ± 0.02 ^{f}	0.72 ± 0.03 ^{d}	0.65 ± 0.01 ^{d}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

The apparent permeability rate constants described in Table 3 differed depending on the conjugation and hydroxylation of the primary bile acids (two-way ANOVA, $p < 0.001$). Release rates decreased following the order GCA > TCA > GCDC > TCDC, which can be explained by the micellar properties (critical micelle concentrations) of the different bile acids, as described in detail in Naumann et al. (2019).

In vitro digested cotyledon and its dietary fibre fractions showed a retarded release of primary bile acids in comparison to the blank digestion (Table 3). The extent of the retardation was comparable to lupin fibre preparation (Naumann et al., 2019), which had an equal TDF content in the final *in vitro* digesta. The other components of the cotyledons have thus not caused any further decrease in bile acid release. Therefore, the decrease in the apparent permeability rate constants can be primarily attributed to the dietary fibre contained in the cotyledon. In agreement with the rheological data (Section 3.3), the OHEP and CL fractions showed the highest decrease of the release rate, of about 65%, compared to the blank digestion. Therefore, increased bile acid excretion reported *in vivo* may be ascribed to impeded bile acid

diffusion (Fechner et al., 2013, Fechner et al., 2014). We assume that this is mainly due to the pectic polymers contained in OHEP and CL fractions.

Lupin hulls also caused a retarded release of all primary bile acids, but it was significantly lower than that observed for the cotyledon. In agreement with its different composition and rheological behaviour, OHEP showed an extended retardation. The decrease in release rate was similar for hull, as well as for the HC and CL fractions. The retarded release for lupin hull can thus mainly be ascribed to these fractions. Since lignin did not show a significant increase in viscosity after *in vitro* digestion (Section 3.3.1), the reduced release rate can be explained by its adsorptive properties (Section 3.4.2). Cellulose is known to have a low bile acid binding capacity (van Bennekum et al., 2005). Accordingly, no significant impact on bile acid release was measurable. Although lupin hull is predominantly composed of cellulose, the hull had a stronger impact on bile acid release, which could be explained by the additional fibre components described in Section 3.2.

3.4.2. Adsorptive interactions

Based on the equilibrium concentrations (C_f, Equation 1), bile acid adsorption of fractionated CWM derived from lupin cotyledon and hull, as well as from the references, cellulose and lignin, were calculated with respect to dry matter concentration in the *in vitro* digesta. Conjugation of bile acids had no significant effect on the adsorption (two-way ANOVA, $p = 0.5$). Therefore, the data for glyco- and tauroconjugated cholic acids and chenodesoxycholic acids are given and summarised in Table 4.

Lignin showed the highest adsorption capacity, which was significant for cholic acids (1.29 µmol/100 mg DM) and chenodesoxycholic acids (4.13 µmol/100 mg DM, Table 4). Lupin cotyledon showed significant adsorption of chenodesoxycholic acids of 1.29 µmol/100 mg DM (Table 4). The higher adsorption of chenodesoxycholic acids compared to cholic acids observed for lupin cotyledon and lignin (Figure 6 and Table 4) is in line with the results of our previous study and supports the hypothesis of a hydrophobic linkage between bile acids and dietary fibres (Naumann et al., 2019). Due to the low lignin content of the cotyledons (0.37 g/100 g DM, Table 1), the adsorption observed for the cotyledon cannot be attributed to lignin. For lupin hull, fractionated hull CWM, and cellulose (Figure 6 and Table 4) no significant adsorption of primary bile acids was observed. Thus, the low proportion of lignin in lupin hull did not affect the adsorptive capacity of the hull.

Table 4. Bile acid adsorption (given as sum of glyco- and tauroconjugated cholic acids and chenodesoxycholic acids) of *in vitro* digesta containing fractionated dietary fibre (pectin-like fraction (OHEP), hemicellulose fraction (HC), cellulose-lignin fraction (CL)) derived from lupin cotyledon and hull of *Lupinus angustifolius* L. Boregine, cellulose and lignin (*n* = 3).

Sample	Bile acid adsorption [µmol/100 mg DM]	
	Cholic acids	Chenodesoxycholic acids
Cotyledon	0.16 ± 0.26 ^{a,b}	1.29 ± 0.09 ^{b}
C_OHEP	−0.02 ± 0.50 ^{b}	0.12 ± 0.70 ^{a}
C_HC	−0.03 ± 0.26 ^{b}	−0.08 ± 0.19 ^{a}
C_CL	0.19 ± 0.23 ^{b}	0.50 ± 0.31 ^{a,b}
Hull	0.22 ± 0.09 ^{b}	0.02 ± 0.20 ^{a}
H_OHEP	0.10 ± 0.19 ^{b}	0.01 ± 0.26 ^{a}
H_HC	0.27 ± 0.36 ^{b}	0.29 ± 0.17 ^{a,b}
H_CL	0.19 ± 0.68 ^{b}	0.26 ± 0.79 ^{a,b}
Cellulose	−0.05 ± 0.12 ^{b}	−0.01 ± 0.25 ^{a}
Lignin	1.29 ± 0.26 ^{a}	4.13 ± 0.42 ^{c}
Blank	0.00 ± 0.06 ^{b}	−0.15 ± 0.04 ^{a}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

In Figure 7, the kinetics of chenodesoxycholic acid release of the whole cotyledon is compared to its cell wall polysaccharide fractions.

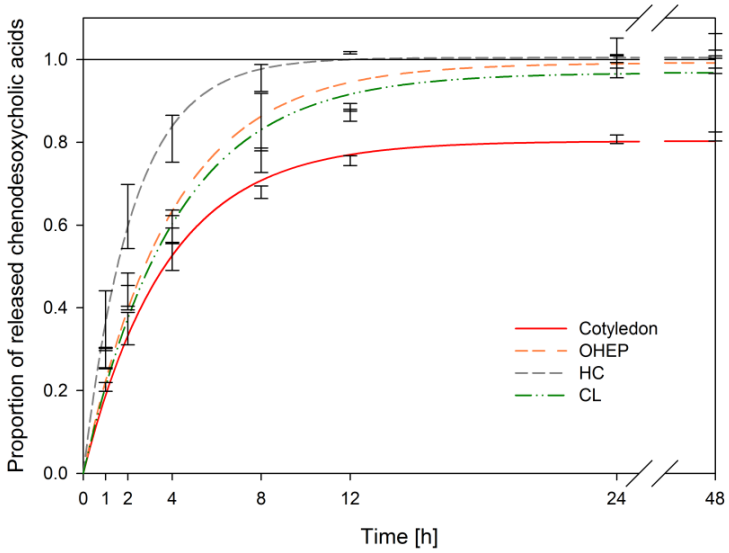

Figure 7. Release kinetics of chenodesoxycholic acids of *in vitro* digested fractionated dietary fibre (pectin-like fraction (OHEP), hemicellulose fraction (HC), cellulose-lignin fraction (CL)) derived from lupin cotyledon of *L. angustifolius* L. Boregine (*n* = 3).

Bile acid release curves of *in vitro* digested cotyledon fractions (OHEP, HC, CL) deviated strongly from the release curve of the whole cotyledon (Figure 7). No significant adsorptive effects were measurable in dietary fibre fractions (Table 4). This contradicts our hypothesis that bile acids are adsorbed by insoluble dietary fibre polymers. As the results obtained after

isolation and fractionation of cell walls disapproved a major role of lupin cell wall polysaccharides in bile acid adsorption, the adsorption observed for lupin cotyledon must be associated with other lupin cotyledon components.

The adsorptive capacity observed for lupin fibre preparation (1.74 µmol chenodesoxycholic acids/100 mg DM, Naumann et al. (2019)) indicates that either the adsorption of lupin is caused by components associated with the cell wall polysaccharides or that the extraction process leads to an accumulation of components in the fibre fraction being able to adsorb bile acids. Besides the fibres (dietary fibre content 83.4 g/100 g DM), residual proteins (11.0 g/100 g DM) are the second main components of the fibre preparation derived after protein isolation. It has been described in several *in vivo* studies that lupin protein acts hypocholesterolaemic (Bähr et al., 2015, Bettzieche et al., 2009, Fontanari et al., 2012), but the underlying mechanism is still not fully elucidated. An *in vitro* study performed by Yoshie-Stark and Wäsche (2004) indicates a high bile acid binding capacity of protein isolates derived from white lupins. The bile acid adsorption observed for the cotyledon in our study may thus result from the adsorption of the lupin protein. Further studies are needed to investigate the adsorptive capacity of the lupin protein and to clarify the role of bile acid adsorption in cholesterol reduction.

Adsorptive capacities have also been suggested for dietary fibre sources with a low protein contents, such as described for a number of fruits by Kahlon and Smith (2007). The authors hypothesised that adsorption could be attributed to polyphenols. This hypothesis is supported by the adsorption we observed for lignin in our study. Unlike most dietary fibres, lignin is not a dietary fibre polysaccharide but a phenolic macromolecule (American Association of Cereal Chemists, 2001). It remains to be investigated to what extent polyphenols contribute to the adsorption of bile acids observed for dietary fibre preparations.

4. Conclusions

In our study, we investigated how bile acid interactions were influenced by the cell wall polysaccharides of lupin hull and cotyledon. Lupin hull mostly consisted of cellulosic polymers, which is in line with low bile acid retardation induced by viscosity. Bile acid adsorption observed for a lignin reference material was not evident in hull or cotyledon due to low lignification. Sequential extraction revealed that pectin-like substances are mainly responsible for viscosity in lupin cotyledon digesta. We suggest that the formation of entangled networks, causing predominantly elastic properties, majorly contributes to the increase of viscosity. Due to the characterisation of components accountable for the viscous effects on bile acid reabsorption, our results could contribute to the elucidation of cholesterol-reducing

mechanisms of lupin dietary fibres. In future studies, the effects of food processing on the composition and rheological properties of lupin dietary fibre should be addressed to ensure to transferability of the *in vitro* results to complex food matrices.

Our results indicate that bile acid adsorption of lupin cotyledon is not directly attributable to the cell wall polysaccharides. The contribution of dietary fibre polysaccharides to bile acid adsorption, as frequently reported for a variety of dietary fibre sources (Singh et al., 2019), should be considered more critically in future investigations. To improve clarity, the term binding should not be used for interactions between fibres and bile acids unless a molecular interaction is evident. Future studies should focus on adsorptive capacities of further lupin components, like proteins and phytochemicals, to identify relevant structures and mechanisms.

Abbreviations

ADF	acid detergent fibre
ADL	acid detergent lignin
CL	cellulose-lignin fraction
DM	dry matter
GCA	glycocholic acid
GCDC	glycochenodesoxycholic acid
HC	hemicellulose fraction
NDF	Neutral detergent fibre
OHEP	NaOH–EDTA-soluble pectin-like fraction
TCA	taurocholic acid
TCDC	taurochenodesoxycholic acid

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 4: Characterisation of the Molecular Interactions between Primary Bile Acids and Fractionated Lupin Cotyledons (*Lupinus angustifolius* L.) [4]

Abstract

Interactions between bile acids and plant-based materials, and the related feedback mechanisms in enterohepatic circulation, have been considered targets for lowering cholesterol. This study aimed to identify lupin compounds that interact with primary bile acids on molecular level. Lupin cotyledons were fractionated and bile acid adsorbing activities were investigated using *in vitro* digestion, equilibrium dialysis, and kinetic analyses. Protein- and fibre-enriched fractions significantly ($p \leq 0.05$) adsorbed chenodesoxycholic acids (up to 2.33 µmol/100 g dry matter). Alcohol purification showed that bile acid adsorption is independent of protein and fibre structures. Moreover, high adsorption was observed with an alcohol extract (6.97 µmol chenodesoxycholic acids/100 g dry matter) that was rich in phytochemicals, such as flavonoids (1842 mg/100g dry matter). These results suggest the formation of hydrophobic interactions between polyphenols and bile acids. Further studies of molecular mechanisms are required to define the contributions of polyphenols to the cholesterol-lowering actions of lupins.

Keywords: cholesterol; bile acid binding; bile acid excretion; *in vitro* digestion; polyphenol; flavonoid

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1. Introduction

Due to their high plant protein contents, sweet lupins (*Lupinus* L.) are an abundant source of proteins for consumption by the ever-growing human population (Lucas et al., 2015). Promising health benefits of lupins have been demonstrated following addition to human diets. Among these, decreased blood pressure, improved blood lipid profiles, increased insulin sensitivity and favourable alterations of gut microbiota have been observed (Arnoldi et al., 2015). To facilitate the addition of lupins to a variety of food products, lupin seeds can be fractionated by de-oiling, protein extraction, and purification, providing protein and fibre fractions with improved sensory and functional properties (Muranyi et al., 2016).

In recent decades, the effects of lupin flour and lupin protein and fibre isolates on blood lipids have been shown *in vivo* and *in vitro*. Moreover, in a recent exhaustive review of human and animal trials Arnoldi et al. (2015) concluded that both fibre and protein components of lupins have cholesterol-reducing effects, and may act synergistically. Dietary fibre is abundant in lupins, because lupin kernel cell walls contain mostly insoluble fibres, predominantly comprising pectin-like polymers (Naumann et al., 2019b). Globulins are the most abundant proteins in lupin seeds, comprising about 90% of all protein, and albumins account for the other 10%. The α- and β-conglutin classes of proteins are dominant in lupins, and δ- and γ-conglutins are present as minor constituents. γ-conglutin is found in fractions of lupin extracted at acidic conditions, and was shown to have unusual functional and nutritional benefits (Duranti et al., 2008, Arnoldi et al., 2015). In particular, a major role of γ-conglutin in cholesterol reduction was proposed by Radtke et al. (2015).

Bile acid metabolism is considered central to the cholesterol-lowering effects of lupin fibres (Cornfine et al., 2010) and lupin proteins (Bettzieche et al., 2009, Parolini et al., 2012). These lupin components are thought to affect blood lipids by modifying enterohepatic circulation in favour of increased faecal bile acid excretion and stimulated conversion of cholesterols to bile acids (Gunness and Gidley, 2010, Spielmann et al., 2008).

Our recent *in vitro* findings indicate that lupins interact with bile acids through adsorption and the formation of viscous chyme matrices (Naumann et al., 2019a). After *in vitro* digestion of lupin cotyledons, a 50% delay in bile acid release was observed for all primary bile acids, suggesting that bile acids were retained due to the increased viscosity associated with pectic polymers in fibre fractions (Naumann et al., 2019b). Furthermore, we found that the molecular interactions of several dietary fibre-enriched food ingredients (derived from lupin, maize, barley, and oat) are correlated with the hydrophobicity of bile acids (Naumann et al., 2019a). Regardless of the source, fibre increases the adsorption of dihydroxy bile acids more than trihydroxy bile acids. This constant adsorption pattern suggests that hydrophobic

interactions are core to the mechanism of bile acid adsorption. We previously hypothesised that adsorption is mediated by the hydrophobic surface properties of insoluble dietary fibres. To test this hypothesis, we isolated and fractionated cell wall components from lupin seeds, but measured no significant bile acid adsorption by purified lupin fibre fractions. These data indicate that cell wall polysaccharides do not participate in the bile acid adsorbing activities of lupins. Therefore, it remains unclear which lupin fractions are responsible for adsorption of bile acids (Naumann et al., 2019b).

High bile acid binding capacity was previously described for acid soluble proteins that were rich in γ-conglutin (Yoshie-Stark and Wäsche, 2004). The potential roles of proteins in bile acid adsorption are further corroborated by studies of other protein-rich plant materials, including those from soy beans, black beans, and pinto beans (Kahlon and Woodruff, 2002). However, bile acid interactions were also described for edible plants with low protein contents. In recent decades, bile acid-binding capacities have been reported for numerous fruits and vegetables (Singh et al., 2019). For example, Kahlon and Smith (2007) reported high variability of bile acid interactions on equal dietary fibre basis and suggested that these differences in adsorptive capacity reflect the presence of phytochemicals such as phenolic compounds.

The aim of this study was to investigate whether and to what extent lupin dietary proteins and phytochemicals contribute to the adsorption of the primary bile acids tauro- and glycoconjugated cholic acids (CA) and chenodesoxycholic acids (CDCA). To this end, de-oiled lupin kernels were sequentially extracted and interactions of protein and fibre fractions with bile acids were determined *in vitro*. We also applied a pilot-scale procedure to generate protein isolates containing α-, β-, and δ-conglutin (precipitated fraction, LPI-E) and others containing γ-conglutin (ultrafiltrated fraction, LPI-F; D'Agostina et al. (2008); Wäsche et al. (2001)). Bile acid adsorption of protein isolates was then investigated after *in vitro* digestion and was compared with adsorption of lupin cotyledons and their fibre-rich residual fractions. Finally, we purified protein and fibre fractions using repeated alcohol extraction procedures, and assessed the resulting phytochemical extracts for their bile acid adsorption capacities. The present data contribute understanding of the nutritional benefits of lupins and promote their incorporation into foods for human consumption.

2. Materials and methods

2.1. Chemicals and enzyme preparations

Taurocholic acid sodium salt hydrate (CAS 345909-26-4), sodium taurochenodeoxycholate (CAS 6009-98-9), sodium glycocholate hydrate (CAS 338950-81-5), sodium glycochenodeoxycholate (CAS 16564-43-5)), L-α-lecithin (egg yolk, highly purified, CAS 8002-43-5), Folin-Ciocalteu reagent, gallic acid (CAS 149-91-7), pancreatin from porcine pancreas (8 × USP specifications), pepsin from porcine gastric mucosa (3,200–4,500 units/mg protein) and vitexin (4',5,7-trihydroxyflavone 8-C-glucoside, CAS 3681-93-4) were obtained from Merck KGaA (Darmstadt, Germany). All other reagents and chemicals were of analytical grade and were supplied by VWR (Darmstadt, Germany).

2.2. Pre-treatment of lupin seeds

Lupin seeds of *Lupinus angustifolius* L. Boregine (Saatzucht Steinach GmbH & Co. KG, Steinach, Germany) were de-hulled using an underrunner disc sheller (Streckel & Schrader KG, Hamburg, Germany). The hulls were then removed using a zigzag air-classifier (Hosokawa Alpine AG, Augsburg, Germany) and the kernels were flaked using a flaking mill (Streckel & Schrader KG, Hamburg, Germany). Flakes were de-oiled using supercritical carbon dioxide extraction. The resulting white flakes were further processed according to the extraction procedure described in 2.3.

2.3. Extraction of protein and fibre-enriched lupin ingredients

White flakes of lupin kernels were submitted to a two-stage extraction process as described by D'Agostina et al. (2006) and displayed in Figure 1. To isolate oligosaccharides, alkaloids and flavour-intense low molecular weight compounds, the first extraction step was conducted in water under acidic conditions (pH 4.5) at 15°C for 1h, with an S:L ratio of 1:8. Separation was performed using a decanter centrifuge (GEA Westfalia Separator Group GmbH, Oelde, Germany). Extracts were concentrated by ultrafiltration (cut-off 10 kDa, Pall Water, Cortland, NY, USA) and were pasteurised and spray-dried (using a two-fluid nozzle, 180 °C inlet and 80 °C outlet air temperatures, 28 kg/h, APV PSD 58, SPX Flow Technology, Crawley, United Kingdom) to yield the lupin protein isolate F (LPI-F). Aliquots of acid-extracted raffinate were then lyophilised for further analyses. For lyophilisation samples were frozen at −50 °C. Freeze-drying was performed at 5–45 °C for 72 h under a pressure of 1.030 mbar (freeze dryer beta 1-8, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). To isolate the main storage-protein fraction, raffinate was extracted in a second stage that was performed in 2 1-h sessions at neutral pH (pH 7) and at 30°C (S:L = 1:8). Extracts were

precipitated at the isoelectric point of pH 4.5. Residues were then separated using a disk-type separator (GEA Westfalia Separator Group GmbH, Oelde, Germany), and were then pasteurised and spray-dried to yield the protein isolate E (LPI-E). Dietary fibre fractions were obtained by lyophilising raffinate and then grinding it in a centrifugal mill with a 500-µm insert (Hosokawa Alpine AG, Augsburg, Germany).

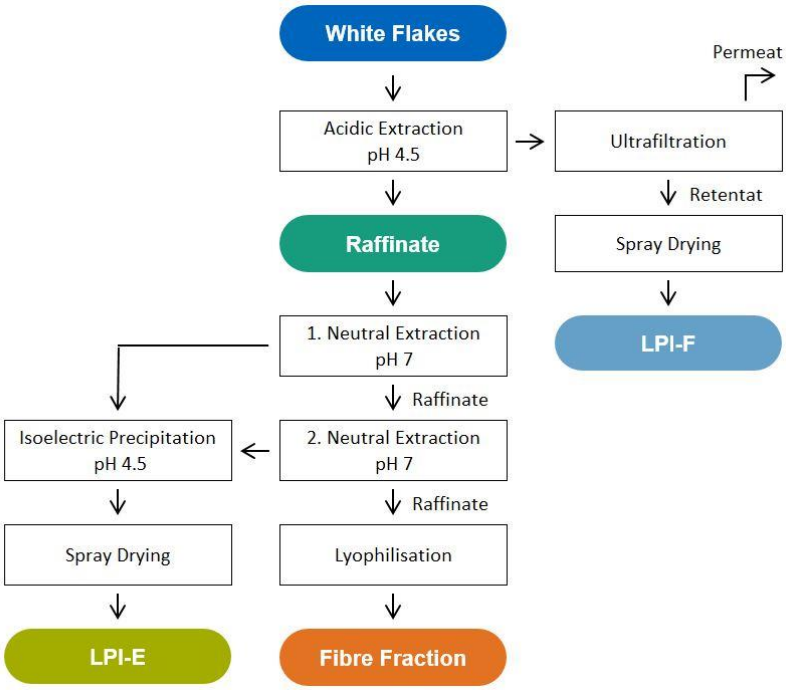

Figure 1. General procedure for extraction and separation of dietary lupin protein isolates (LPI) F and E and fibres from de-hulled, flaked, and de-oiled cotyledons (white flakes) of *Lupinus angustifolius* L. Boregine, adapted from D'Agostina et al. (2006).

2.4. Alcohol extraction of phytochemicals

After suspending 30 g of pre-treated lupin white flakes (2.2) in 300 mL of 80% *v/v* ethanol, extraction was performed with mixing and boiling for 1 h. The alcohol phase was then separated by filtration using a Büchner funnel and blue ribbon filter paper. The extraction procedure was repeated until the alcohol phase was clear, which took five to seven repetitions. The residue was stirred overnight in 500 mL of acetone and was then filtered through a G3 glass sinter filter. The solids were then dried at 40 °C for 24 h and the alcohol insoluble residues (AIR) were determined gravimetrically (n = 3; Fügel et al. (2004)). Alcohol extracts containing phytochemicals were pooled and concentrated to dryness using a rotary evaporator (Rotavapor R-215, BÜCHI Labortechnik AG, Flawil, Switzerland).

2.5. Determination of nutrient composition

Dry matter and ash contents were determined using thermogravimetry at 105 °C and 550 °C with a TGA 601 analyser (Leco, St. Joseph, MI, USA) according to the official methods L 18.00-12 and L 15.00-7, respectively (Deutsches Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2005). Nitrogen (AOAC Official Method 968.06) contents were analysed according to the Dumas combustion method using a Nitrogen Analyzer TruMac N (LECO Instrumente GmbH, Mönchengladbach, Germany) and protein contents were calculated as N × 5.8 (Association of Official Analytical Chemists, 2016). Soluble (SDF) and insoluble dietary fibre (IDF) contents were determined using enzymatic–gravimetry and total dietary fibre was calculated (TDF = SDF + IDF; AOAC Official Method 991.43, Association of Official Analytical Chemists (2016)). Carbohydrate contents were calculated as 100 − (ash + protein + total dietary fibre).

2.6. Determination of total polyphenol contents

Total polyphenol contents were determined in diluted aliquots (50 mg/mL in 70% *v/v* ethanol) of the alcohol extracts (2.4) using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Absorption was measured at 765 nm using a Specord 210 plus spectrophotometer (Analytik Jena, Jena, Germany). Gallic acid was employed as a calibration standard (0.1–1 g/L) and total polyphenol contents were expressed as gallic acid equivalents (GAE).

2.7. Quantification of flavonoids

For quantification of flavonoids the alcohol extracts (2.4.) were diluted to 0.1 g/mL (70% *v/v* ethanol). Flavones were identified and quantified using a reversed-phase high-performance liquid chromatography (HPLC) system (Agilent series 1200), equipped with Open Lab CDS software version 2.3, a G1379 degasser, a G1312B binary gradient pump, a G1367D thermo autosampler, a G1316B column oven, and a G1315C diode-array detector (Agilent, Santa Clara, CA, USA). The column used was a Kinetex® C18 (150 × 4.6 mm i.d.; 2.6 µm particle size) from Phenomenex (Torrance, CA, USA) operated at 30 °C. The binary gradient consisted of water adjusted to pH 2.7 with orthophosphoric acid (solvent A) and a mixture of acetonitrile-water (50:50, *v/v*, solvent B). A binary gradient program was applied from 0% to 50% B in 60 min, followed by flushing with 100% acetonitrile (10 min) and re-equilibration at 100% A (10 min). The injection volume was 10 µL and column flow was 1 mL/min (Siger et al., 2012). Flavones were detected at 335 nm and identified by comparing retention times and diode-array detector (DAD) spectra (200–600 nm) with previous studies (Siger et al., 2012). For quantification of flavones vitexin was employed as calibration standard (0.15–3 mg/mL,

R^2 = 0,997) and molecular weight correction factors were used for their specific calculations (Chandra et al., 2001).

2.8. *In vitro* adsorption studies of primary bile acids

Samples were digested *in vitro* in simulated oral, gastric, and small intestinal digestion environments according to the harmonised INFOGEST protocol (Minekus et al., 2014) with slight modifications: Samples were diluted with ultrapure water to a dry matter concentration of 50% (*w/w*) before *in vitro* digestion, which gives a final dry matter concentration of 6.25% (*w/w*) in the digestion mixture. Due to the low starch contents of lupin cotyledons (Naumann et al., 2019b), oral digestion was applied without added α-amylase. Sodium azide (0.04% *w/v*) was added to all electrolyte fluids. Instead of bile acid extracts or fresh bile (as recommended by Minekus et al. (2014)) a 1:1:1:1 mixture of taurocholic acid (TCA), glycocholic acid (GCA), taurochenodesoxycholic acid (TCDCA), and glycochenodesoxycholic acid (GCDCA) was prepared to a cumulative concentration of 10 mM in the final digestion mixture. For blank samples, the same concentrations of simulated digestion fluids, lecithin, pepsin, pancreatin, and bile were applied but sample aliquot was replaced with ultrapure water.

Aliquots of 4 g of *in vitro* digesta were dialysed using 16-mm Servapor® 12–14-kDa cut-off dialysis tubes (SERVA Electrophoresis GmbH, Heidelberg, Germany) against 36 mL of phosphate buffer (50 mM, pH 7, 0.02% *w/v* sodium azide) using a shaking water bath at 150 rpm and 37°C. At regular time intervals (1, 2, 4, 8, 12, 24, and 48 h) 100-µL aliquots of dialysate were analysed for permeated primary bile acids using HPLC as described by Naumann, Schweiggert-Weisz, Eglmeier, et al. (2019). Parameters of bile acid release, such as concentrations of primary bile acids after reaching equilibrium (C_f) and apparent permeability rate constants (k), were calculated using non-linear regression with the following first-order kinetic Equation 1 (Gunness et al., 2012):

$$C_t = C_f \times (1 - e^{-kt}) \quad (1)$$

Based on equilibrium concentrations (C_f), adsorption capacities of primary bile acids were calculated relative to dry matter concentrations in *in vitro* digesta.

2.9. Statistical analysis

All statistical analyses were performed using R version 3.2.4 (www.r-project.org). Differences were considered significant when $p \leq 0.05$. Homogeneity of variance and normality of distributions were tested using Bartlett and Shapiro–Wilk tests, respectively, before applying

ANOVA and Tukey tests. Regression analyses were performed using SigmaPlot® (version 12.5, Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

In this study, we initially extracted LPI-E and LPI-F and fibre-rich ingredients from de-oiled flakes of cotyledons from *Lupinus angustifolius* L. Boregine in a pilot-scale plant (3.1). These fractions were then examined for their compositions (3.1.1) and bile acid adsorption abilities (3.1.2). In subsequent experiments, cotyledons were repeatedly extracted with boiling ethanol. The resulting alcohol extracts were rich in phytochemicals (3.2.2) and gave residues containing purified protein and fibre (3.2.1), which were assessed for bile acid adsorption (3.2.3).

3.1. Composition and bile acid adsorption of protein and fibre-enriched lupin ingredients

3.1.1. Nutrient composition of protein and fibre-enriched lupin ingredients

Lupin seeds were de-hulled, flaked, and de-oiled to obtain white flakes, which were used as raw material for the production of protein and dietary fibre isolates. The compositions of the fractions from 2.3 are summarised in Table 1.

Table 1. Chemical composition analyses of dry matter (DM), ash, protein, carbohydrate, dietary fibre (total (TDF), insoluble (IDF), and soluble dietary fibre (SDF)) in lupin protein isolates (LPI) F and E, residue remaining after acid extraction (raffinate), and the dietary fibre fraction obtained from de-hulled, flaked, and de-oiled cotyledons (white flakes) of *Lupinus angustifolius* L. Boregine (*n* = 3).

	DM	Ash	Protein	Carbohydrates	TDF	IDF	SDF
Sample	g/100g	g/100g DM	g/100 g DM	g/100 g DM	g/100 g DM	g/100 g DM	g/100 g DM
White Flakes	89.1 ± 0.0 ^{b}	4.6 ± 0.0 ^{d}	41.8 ± 0.1 ^{c}	11.9 ± 2.4 ^{b}	41.7 ± 2.3 ^{c}	38.2 ± 1.0 ^{b}	3.5 ± 2.1 ^{a}
Raffinate	90.5 ± 0.1 ^{d}	3.3 ± 0.0 ^{b}	42.2 ± 0.2 ^{c}	3.8 ± 3.3 ^{a,b}	50.6 ± 3.3 ^{d}	47.5 ± 2.5 ^{c}	3.2 ± 2.1 ^{a}
Fibre fraction	88.5 ± 0.0 ^{a}	1.8 ± 0.0 ^{a}	11.1 ± 0.2 ^{a}	3.7 ± 2.1 ^{a}	83.3 ± 2.1 ^{e}	79.7 ± 2.0 ^{d}	3.7 ± 0.3 ^{a}
LPI-F	89.3 ± 0.1 ^{c}	15.2 ± 0.0 ^{e}	63.1 ± 0.2 ^{b}	4.8 ± 3.7 ^{a,b}	16.9 ± 3.7 ^{b}	2.2 ± 1.2 ^{a}	14.7 ± 3.5 ^{b}
LPI-E	90.8 ± 0.0 ^{e}	3.4 ± 0.0 ^{c}	93.7 ± 0.1 ^{d}	0.0 ± 0.0 ^{a}	4.7 ± 1.1 ^{a}	3.4 ± 0.6 ^{a}	1.3 ± 1.0 ^{a}

Differing letters in columns indicate significant differences; $p \leq 0.05$.

Lupin white flakes comprised equal proportions of protein (41.8 g/100 g dry matter (DM)) and dietary fibre (41.7 g/100 g DM), which was mostly insoluble (38.2 g/100 g DM). After acid extraction, the resulting raffinate samples had similar proportions of protein (42.2 g/100 g DM), but increased dietary fibre contents (50.6 g/100 g DM). These increased dietary fibre contents of raffinate reflect removal of low molecular weight components, such as oligosaccharides, from acid soluble protein fractions. However, dietary fibres were also partially extracted and recovered in LPI-F. The high solubility of dietary fibres in LPI-F (SDF:

14.7 g/100 g DM) indicates that water-soluble dietary fibres accumulated in this fraction. The protein content of LPI-F was 63.1 g/100 g DM, and is described to mainly comprise acid soluble γ-conglutin (Wäsche et al., 2001). In LPI-E, protein contents were higher (93.7 g/100 g DM) and dietary fibre contents were lower (5.0 g/100 g DM). LPI-E protein fractions are described to be enriched in α-, β-, and δ-conglutins, which are highly soluble in neutral and alkaline conditions (Wäsche et al., 2001). The dietary fibre fraction that remained as a residue of the extraction procedure contained 11.1 g/100 g DM of residual protein and was rich in fibre (83.3 g/100 g DM).

3.1.2. *In vitro* bile acid adsorption of protein and fibre-enriched lupin ingredients

We investigated primary bile acid adsorption capacity of lupin cotyledon fractions under *in vitro* conditions that simulated upper gastrointestinal digestion. For all fractions that interacted with bile acids, adsorption was not significantly influenced by the bile acid conjugation with taurine or glycine (two-way ANOVA, $p = 0.1$). Due to its sulfonic acid groups, taurine is more acidic than glycine. Furthermore, the anionic charge of taurine is more distal from the steroid nucleus of bile acid, leading to increased affinity of tauroconjugated bile acids for ionic bonds, as shown for the ion-exchanging resin cholestyramine (Johns and Bates, 1969). Because conjugation had no significant impact on bile acid adsorption in our study, we suggest that ionic bonds do not contribute to the adsorptive properties of lupin cotyledon components.

Diffusion kinetics of bile acid release from *in vitro* digesta containing protein isolates (LPI-F, LPI-E), acidic extracted raffinate and dietary fibre from lupin white flakes are presented in Figure 2 and the resulting adsorption values are listed in Table 2. Because adsorptive capacity was not significantly related to conjugation of bile acids, we summed up data for glyco- and tauroconjugated bile acids and displayed bile acid release kinetics for CA and CDCA only.

We detected no significant differences in adsorption of CA (3, 7, 12 trihydroxy bile acid) between cotyledon fractions and blank digests (Figure 2a & Table 2). However, with the exception of LPI-F, all lupin cotyledon fractions showed significant adsorption of CDCA (3, 7 dihydroxy bile acid, Figure 2b & Table 2). Adsorptive capacity decreased with increasing degrees of hydroxylation, suggesting that hydrogen bonding does not contribute to adsorption. In contrast, higher adsorptive capacity towards CDCA than towards CA indicates the presence of hydrophobic interactions between lupin fractions and bile acids. In addition to lupins, we have previously described adsorptive interactions that are related to hydrophobicity of bile acids for dietary fibre-enriched ingredients derived from barley, oat, and maize and for lignin (Naumann et al., 2019b). Several other studies of dietary fibres from different sources report similar results (Singh et al., 2019).

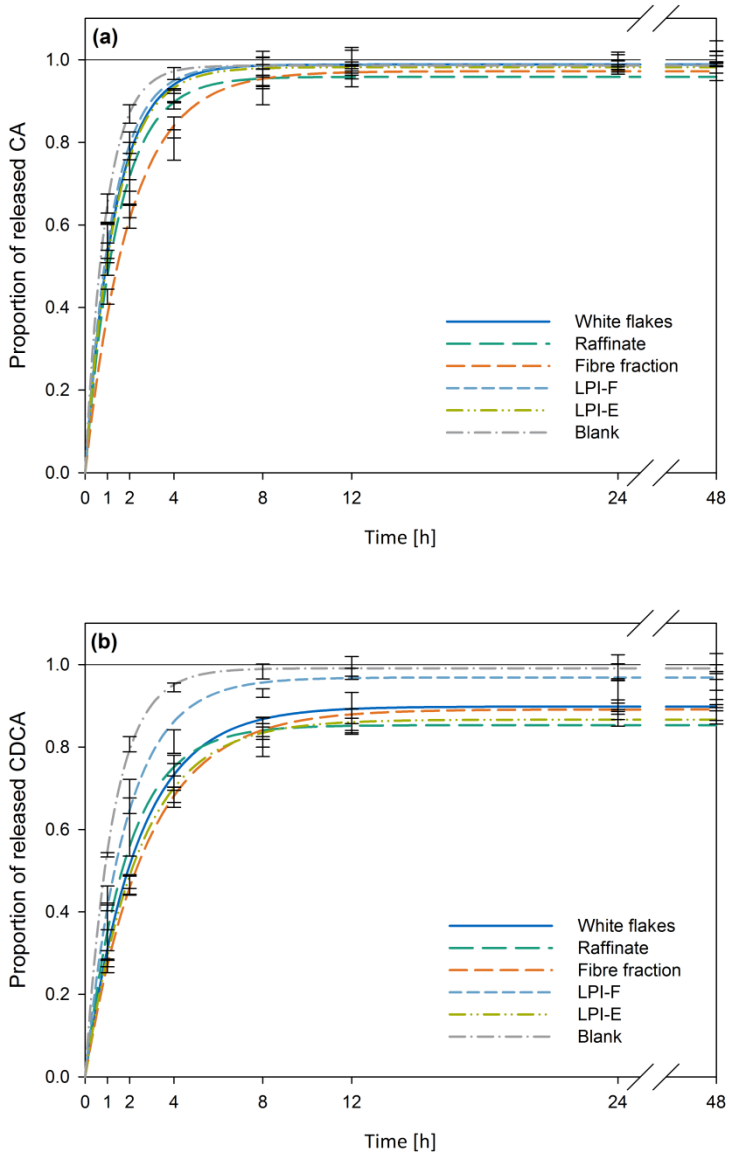

Figure 2. Diffusion kinetics of bile acid release of (a) cholic acids (CA) and (b) chenodesoxycholic acids (CDCA) from *in vitro* digesta containing lupin protein isolates (LPI) F and E, the residue remaining after acid extraction (raffinate), and dietary fibre fraction from de-hulled, flaked, and de-oiled cotyledons (white flakes) of *Lupinus angustifolius* L. Boregine compared with blank digests (*n* = 3).

As described for dietary fibre-enriched ingredients, hydrophobic interactions have been investigated for protein residues of digestion. Iwami et al. (1986), for example, showed that soy protein lowers cholesterol contents in rats and correlated this activity with increasing hydrophobicity of digested protein/peptide residues. Similar binding patterns reported in several other studies suggest that hydrophobic interactions are a common underlying mechanism of bile acid adsorption.

As shown in Table 2, the highest adsorption was detected for acid extracted raffinate (2.33 µmol CDCA/100 mg DM), and was significantly higher than for white flakes

(1.63 µmol CDCA/100 mg DM), indicating that components that adsorb bile acids are not soluble in the first extraction step of protein isolation (Figure 1). In potential agreement, the γ-conglutin acid soluble fraction LPI-F was formerly investigated as a bile acid adsorbent (Yoshie-Stark and Wäsche, 2004), but failed to absorb bile acids in our study. In addition, low molecular weight components such as oligosaccharides and alkaloids are extracted under the acidic conditions of the first extraction step. Consequently, adsorptive components may accumulate in raffinate. Adsorptive capacities of fibre and LPI-E fractions did not differ significantly, despite their differing protein contents (Table 1). Hence, proteins may not be directly involved in bile acid adsorption by these fractions. However, components that adsorb bile acids appear to be associated with proteins in LPI-E and with dietary fibres. We therefore assumed that associated phytochemicals contribute to bile acid adsorption and tested this in further experiments (3.2).

Table 2. Bile acid adsorption is presented as the sum of glyco- and tauroconjugated cholic acids (CA) and chenodesoxycholic acids (CDCA) of *in vitro* digesta containing lupin protein isolates (LPI) F and E, the residue remaining after acid extraction (raffinate), the dietary fibre fraction, alcohol extracts, and alcohol insoluble residues (AIR) from de-hulled, flaked, and de-oiled cotyledons (white flakes) of *Lupinus angustifolius* L. Boregine compared with those of blank digests (*n* = 3).

	Bile acid adsorption [µmol/100 mg DM]	
Samples	CA	CDCA
White flakes	0.19 ± 0.23 [a]	1.63 ± 0.21 [b]
Raffinate	0.65 ± 0.10 [a]	2.33 ± 0.16 [c]
Fibre fraction	0.44 ± 0.15 [a]	1.74 ± 0.26 [b,c]
LPI-F	0.20 ± 0.02 [a]	0.50 ± 0.11 [a]
LPI-E	0.30 ± 0.15 [a]	2.12 ± 0.17 [b,c]
AIR	0.30 ± 0.32 [a]	0.60 ± 0.13 [a]
Alcohol extracts	2.04 ± 0.22 [b]	6.97 ± 0.34 [d]
Blank	0.22 ± 0.46 [a]	0.14 ± 0.38 [a]

Differing letters in columns indicate significant differences; $p \leq 0.05$.

3.2. Composition and bile acid adsorption of phytochemical extracts and residues

3.2.1. Yield and nutrient composition of phytochemical extracts and residues

The present AIR comprised equal proportions of protein (49.8 ± 0.2 g/100 g DM) and dietary fibre (48.4 ± 2.9 g/100 g DM), which was mostly insoluble (41.3 ± 2.4 g/100 g DM). The alcohol extractable fraction of de-oiled lupin cotyledons (white flakes) comprised 21.6 ± 1.9 g/100 g DM. These compositional data are consistent with removal of

oligosaccharides and phytochemicals from de-oiled lupin cotyledons due to alcohol extraction.

3.2.2. Determination of phytochemical composition in alcohol extracts

Polyphenols and saponins are the main phytochemicals of de-oiled sweet lupins. We determined the total polyphenol content by Folin-Ciocalteu assay. The total polyphenol content of alcohol extracts was 745.1 ± 19.6 mg GAE/100 g DM. Accordingly, approximate polyphenol contents of de-oiled and whole lupin cotyledons were 160.6 mg GAE/100 g DM and 145.4 mg GAE/100 g DM, respectively. Previous studies report total polyphenol contents in seeds of *Lupinus angustifolius* L. of between 95.4 and 856 mg GAE/100 g DM (Dueñas et al., 2009, Rumiyati et al., 2013). This great variance can be explained by strong influences of genetic and environmental factors on polyphenol contents (D'Agostina et al., 2008). Polyphenols in *Lupinus angustifolius* L. are categorised as phenolic acids and flavonoids. In a study of seeds of *Lupinus angustifolius* L., Siger et al. (2012) identified the phenolic acids gallic acid (0.06 mg/100 g DM), protocatechuic acid (1.31 mg/100 g DM), p-hydroxybenzoic acid (4.32 mg/100 g DM), caffeic acid (0.07 mg/100 g DM), and p-coumaric acid (0.04 mg/100 g DM). Saponins were reportedly present in lupin cotyledons at 57.3 mg/100 g DM (Petterson and Fairbrother, 1996).

While saponins and phenolic acids are minor constituents, flavonoids are considered predominant phytochemicals of de-oiled sweet lupins. Therefore, we assessed the flavonoid profile using HPLC-DAD. Figure 3 presents the chromatogram and DAD-spectra of the two dominant peaks (retention times 28.5 and 28.9 min) obtained for alcohol extracts.

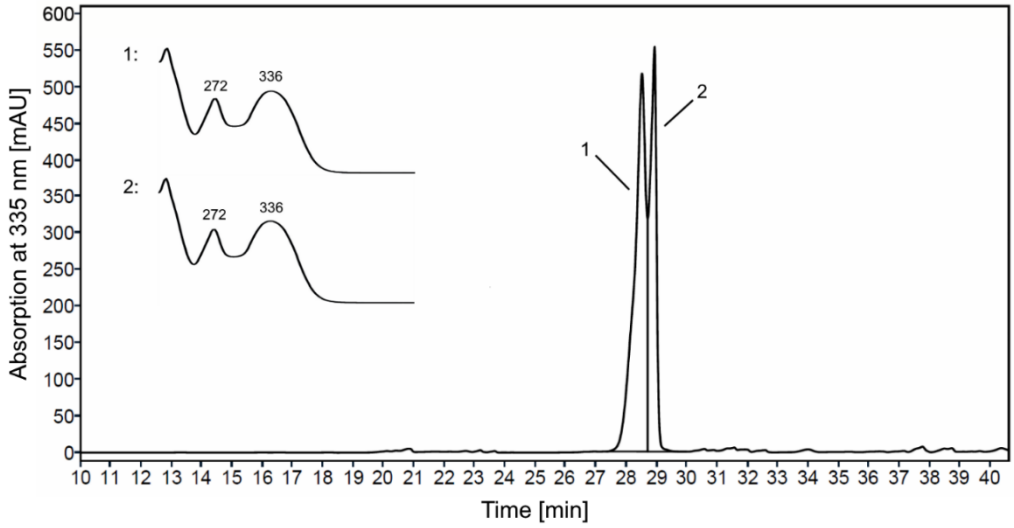

Figure 3. HPLC chromatogram of alcohol extracts recorded at λ = 335 nm and DAD spectra of two main flavonoids of *Lupinus angustifolius* L. Boregine (1: apigenin-6,8-di-C-β-glucopyranoside, 2: apigenin 7-O-β-apiofuranosyl-6,8-di-C-β-glucopyranoside).

On the basis of retention time and DAD-spectra the two main flavonoids correspond to the apigenin C-glucosides described by Siger et al. (2012). The main flavonoids in their study were the apigenin derivatives apigenin-6,8-di-C-β-glucopyranoside and apigenin 7-O-β-apiofuranosyl-6,8-di-C-β-glucopyranoside (29.0 mg/100 g DM and 41.9 mg/100 g DM of lupin seeds, given as vitexin equivalents). In our study, the content in the alcohol extracts was 1080 mg/100 g DM for apigenin-6,8-di-C-β-glucopyranoside and 762 mg/100 g DM for apigenin 7-O-β-apiofuranosyl-6,8-di-C-β-glucopyranoside. Accordingly, approximate total contents of these flavonoids for whole lupin cotyledons were 359 mg/100 g DM. The higher observed contents of flavonoids in our study in comparison to the study of Siger et al. (2012) can be explained by the use of de-hulled seeds in our study and the differences in the distribution and content of flavonoids in seed coat and cotyledon (Zhong et al., 2018). Further studies on the influence of agricultural and environmental conditions and genetic variability are needed to more accurately characterise the structure of lupin phytochemicals.

3.2.3. *In vitro* bile acid adsorption of phytochemical extracts and residues

Because bile acid conjugation did not significantly affect adsorptive capacity (two-way ANOVA, $p = 0.1$), we present data for glyco- and tauroconjugated CA and CDCA. Diffusion kinetics of bile acid release from *in vitro* digesta containing alcohol extracts and AIR from lupin white flakes are presented in Figure 4 and corresponding adsorption data are shown in Table 2.

AIR did not adsorb bile acids significantly (Figure 4 and Table 2), as indicated in our initial experiments (3.1) showing that neither fibre nor proteins are directly involved in bile acid adsorption. In contrast, alcohol extracts showed high adsorptive capacity, at 2.04 µmol/100 mg DM for CA and 6.97 µmol/100 mg DM for CDCA (Figure 4 and Table 2). Considering the alcohol-soluble portion of white flakes (21.6 ± 1.9 g/100 g DM), we suggest that the components of this alcohol-soluble fraction are responsible for the CDCA adsorption by white flakes (1.63 µmol CDCA/100 mg DM, Table 2).

During alcohol extraction processes, oligosaccharides and phytochemicals were removed from cotyledons and recovered in alcohol extracts. After removing oligosaccharides from white flakes in our first experiment (3.1), bile acid adsorption by the acidic extract raffinate was significantly higher (2.33 µmol CDCA/100 mg DM, Table 2) than for white flakes containing oligosaccharides (1.63 µmol CDCA/100 mg DM, Table 2). Therefore, oligosaccharides are presumably not directly involved in bile acid adsorption by lupin cotyledon components.

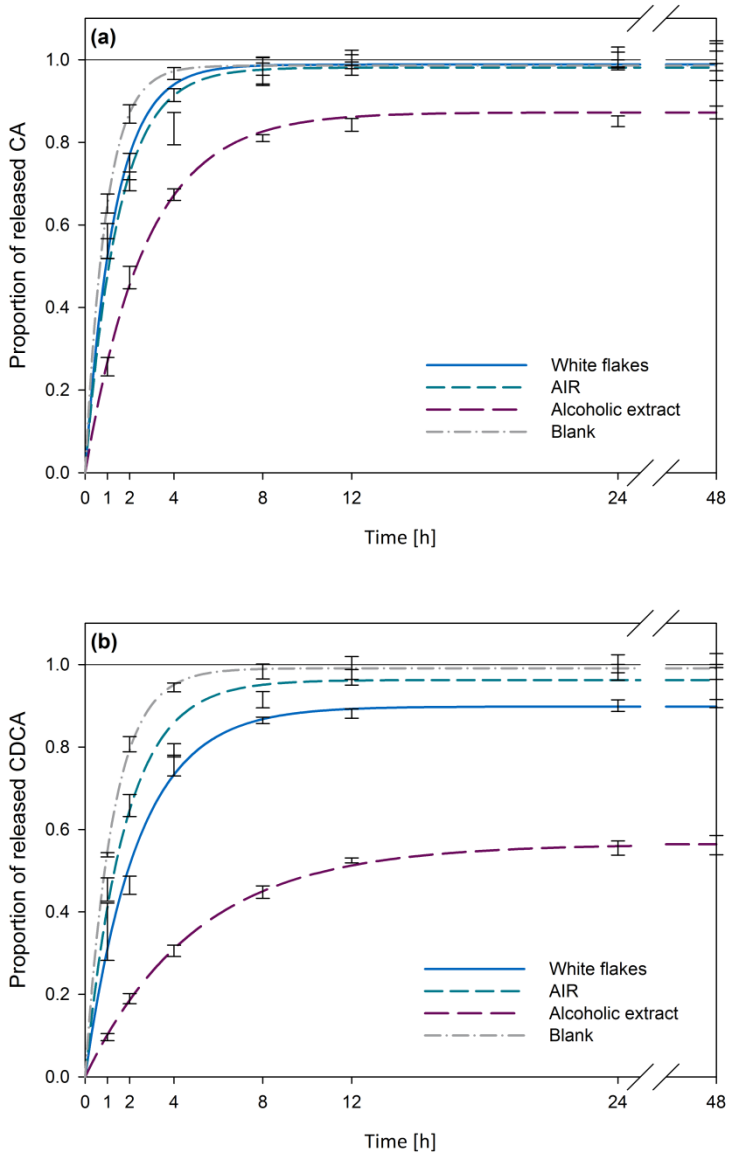

Figure 4. Diffusion kinetics of bile acid release of (a) cholic acids (CA) and (b) chenodesoxycholic acids (CDCA) from *in vitro* digesta containing alcohol extracts and alcohol insoluble residues (AIR) of de-hulled, flaked, and de-oiled cotyledons (white flakes) of *Lupinus angustifolius* L. Boregine. All data were compared with those from blank digests (*n* = 3).

In a recent publication by Yang et al. (2018), high bile acid binding capacities of up to 90% were reported for solvent extracts from kale, which contained numerous flavonoids (mostly derivatives of kaempferol and quercetin). Flavonoids of grape seeds are mostly procyanidins and were shown to increase faecal bile acid excretion and hepatic bile acid biosynthesis by Heidker et al. (2016). Hamauzu and Suwannachot (2019) also suggested that non-extractable polyphenols contribute to bile acid binding capacities of persimmon fruits. Ogawa et al. (2016) proposed a novel mechanism for the cholesterol-lowering activities of green tea polyphenols based on nuclear magnetic resonance analyses, and suggested that catechins and oolongtheanins form hydrophobic spaces that adsorb bile acids. These interactions between

polyphenols and bile acids reduce micellar solubility of cholesterol, and thereby decrease absorption and increase faecal excretion. Similar physiological outcomes were reported for extracts from black bean seed coats, which are rich in flavonoids (mainly quercetin 3-O-glucoside) and saponins (mainly soyasaponin Af; Chavez-Santoscoy et al. (2014)). Subsequent incorporation of these extracts into the diet promoted cholesterol and bile acid excretion in a mouse model, suggesting synergistic effects of flavonoids and saponins. Saponins are a group of amphiphilic glycosides and have been considered as cholesterol-reducing agents since the 1950s. However, as recently reviewed by Zhao (2016), the molecular mechanisms behind these effects remain unclear.

Based on our results, we assume that polyphenols, such as flavonoids, interact with bile acids through hydrophobic interactions. In addition to the present results, we observed bile acid adsorption by lignin in our previous study (Naumann et al., 2019b). Polyphenols are known to be associated with dietary fibre and protein, which show enhanced interactions in alkaline medium (Tscherch et al., 2013). Therefore, polyphenol–bile acid interactions may mediate the bile acid adsorption observed for lupin cotyledons and their dietary fibre and protein isolates (LPI-E).

4. Conclusions

Herein, we investigated the contributions of lupin proteins and phytochemicals to adsorption of primary bile acids *in vitro*. Except for the γ-conglutin rich protein isolate LPI-F, all lupin fractions adsorbed about 10 %–15 % of CDCA. But we were unable to correlate adsorptive capacity with the abundance of protein and dietary fibre in lupin. In subsequent adsorption studies we purified these components using alcohol extraction and revealed a high adsorptive capacity (up to 44%) for all primary bile acids in the alcohol extracts. These extracts are rich in phytochemicals, which were identified predominantly as flavonoids derived from apigenin.

The present *in vitro* data provide some basic indicators of the roles of polyphenols in bile acid adsorption. To determine the nature of these interactions more precisely, further fractionation procedures are required to identify the dominating adsorptive phytochemicals and underlying mechanisms. In particular, the present hypothesised hydrophobic interactions need to be confirmed using more hydrophobic secondary bile acids. Future studies of adsorption patterns are also required in other plant materials. In addition, the proposed polyphenol–bile acid interaction and its participation in cholesterol-reducing actions must be assessed *in vivo*.

Abbreviations

AIR	alcohol insoluble residue
CA	cholic acid
CDCA	chenodesoxycholic acid
DAD	diode-array detection
DM	dry matter
GAE	gallic acid equivalents
GCA	glycocholic acid
GCDCA	glycochenodesoxycholic acid
HPLC	high-performance liquid chromatography
LPI	lupin protein isolate
TCA	taurocholic acid
TCDCA	taurochenodesoxycholic acid

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER 5: Effects of Extrusion Processing on the Physiochemical and Functional Properties of Lupin Kernel Fibre [5]

Abstract

Lupin kernel fibre is an underutilised by-product of lupin protein isolation rich in insoluble dietary fibre. By means of extrusion technology, insoluble fibres can be converted in soluble fibres, which are considered the most effective dietary fibre fraction for human health. Lupin kernel fibre was processed at various barrel temperatures, feed moistures, and screw speeds. The physiochemical (dietary fibre composition, colour, water, and oil binding capacities) and functional (viscosity and bile acid interactions) properties of the lupin fibres after extrusion were evaluated compared to the non-extruded fibre. Due to extrusion processing, the soluble fraction of dietary fibre was increased from 1.9 g/100 g dry matter to up to 37.7 g/100 g dry matter, water binding capacity was increased by up to 95%, while oil binding capacity significantly decreased. Moisture content, followed by barrel temperature were identified as the most relevant extrusion parameters to influence physiochemical properties. To estimate effects of extrusion on fibre functionality, extrudates were digested under simulated gastro-intestinal conditions. Viscosity of *in vitro* digesta was increased for most extruded fibres. Accordingly, diffusion of bile acids was decreased, which may improve cholesterol lowering properties. Molecular interactions of dietary fibre with bile acids were not affected by the extrusion treatment. The results indicate that extrusion caused solubilisation of hydrophilic pectin-like polymers, which exhibit high hydration properties and thus increase the viscosity at physiological conditions. These findings suggest that extrusion could be a practical technology to enhance health benefits of lupin kernel fibre.

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Keywords: Dietary fibre; bile acid binding; cholesterol; *in vitro* digestion; viscosity; water binding

1. Introduction

Human evolution was accompanied with the consumption of plant foods rich in dietary fibre. However, the introduction of agriculture and industrialisation resulted in the emergence of a high caloric 'Western diet' rich in refined grains, sugars, and animal products (Cordain et al., 2005). As a result of this development, dietary fibre intake is far below the recommendations, which range from 25 to 35 g/day, subject to country specific guidelines (Lunn and Buttriss, 2007). Dietary fibre consumption is associated with numerous health benefits. The lack of fibre in the current Western diet may therefore contribute to the predisposition of the modern population to chronic diseases (Mann and Cummings, 2009).

With regard to their nutritional effectiveness, dietary fibres are differentiated according to their water solubility and associated physiochemical and functional properties. Health benefits of soluble fibre are mainly related to their water binding capacity, viscosity, and fermentability by gut microbiota. Since the intake of soluble fibre increases viscosity in the digestive tract, water-soluble fibres have been associated with prolonged gastric emptying, reduced rate of starch digestion and glucose absorption. These effects result in enhanced satiety and reduction of glycaemic response, making these kind of fibres a part of diet plans to treat Type 2 diabetes (Fuller et al., 2016, Kumar et al., 2012). Viscous fibres are further described to lower blood cholesterol levels, one of the major biomarkers for cardiovascular disease (Gunness and Gidley, 2010). The conversion of highly fermentable soluble fibres to short chain fatty acids in the colon is further associated with cancer protection and anti-inflammatory activities (Fuller et al., 2016). In contrast, insoluble fibres are described to be metabolically inert, less fermentable and have only slight effects on viscosity within the gastrointestinal tract. Health benefits of insoluble fibre are therefore mainly resulting from increased bulk volume and decreased transit time associated with the increased dietary fibre supply. These effects increase satiety, decrease saturated fat intake and are further associated with reduced risk for cardiovascular disease, diabetes and colorectal cancer (Ul Ain et al., 2019, van Bennekum et al., 2005).

Lupin seeds are a good source of dietary fibre. The dietary fibre fraction of de-hulled seeds of *Lupinus angustifolius* L. accounts for 37.8 g/100 g dry matter (DM) and is predominantly insoluble (34.6 g/100 g DM). Lupin kernel fibre is reported to be composed of the monosaccharides galactose (67.6%), arabinose (11.5%), uronic acids (8.1%), glucose (7.6%) and xylose (2.6%) (Naumann et al., 2019b). The structure of the principal dietary fibres from

the kernels of *Lupinus angustifolius* L. are described as 1–4-linked long-chain galactans and highly branched 1–5-linked arabinans, which are linked to the rhamnosyl residues of a rhamnogalacturonan backbone (Cheetham et al., 1993). Lupin kernel fibres are referred to as pectin-like non-starch polysaccharides. Apart from pectins, this group also includes rhamnogalacturonans, arabinans, galactans, arabinogalactans, glucomannans, and galactoglucomannans (Belo and De Lumen, 1981, Selvendran and MacDougall, 1995). The group of pectin-like polymers includes water-soluble and insoluble fibres. Insoluble pectin-like polymers, in contrast to most other insoluble fibres, were demonstrated to be fermentable by human gut microbiota (Fechner et al., 2014, Klosterbuer et al., 2011). Lupin fibres have been shown to promote various health benefits in humans, including cholesterol-lowering properties, improvement of colonic functions and beneficial alterations of risk factors for colorectal cancer. These health benefits were mainly attributed to an increase of faecal mass, reduction of transit time and fermentation of the fibre to short chain fatty acids, which reduces faecal pH and concentrations of secondary bile acids (Fechner et al., 2013, Fechner et al., 2014).

In addition to lupin flour, the kernel fibre fraction obtained as a by-product of protein isolation of dehulled lupin seeds can be incorporated into food as functional ingredients. The processed lupin kernel fibre is rich in dietary fibre (total dietary fibre content: 83.3 g/100 g DM). It further contains residual protein (11.1 g/100 g DM) and associated substances, such as polyphenols (Naumann et al., 2020). In contrast to most cereal fibres, processed lupin kernel fibres have a smooth texture, a neutral taste, and a white colour. These properties ensure the sensory acceptability of the kernel fibre fraction and thus increase the application potential in fibre enrichment of food formulations (Hall et al., 2010).

Apart from the composition, metabolic and physiological benefits of dietary fibre ingredients further depend on the process applied during fibre isolation or during food processing. As most plant-based fibres are predominantly insoluble, efforts have been made to convert insoluble into soluble fibre in order to enhance their physiological effectiveness (Ul Ain et al., 2019). Extrusion cooking is a widely used process to change physiochemical properties of food components, which was reported to increase the solubilisation of cell wall constituents, in particular of pectin-like polymers (Redgwell et al., 2011). While conversion of insoluble to soluble fibre induced by the extrusion process has been extensively studied and reported (Ul Ain et al., 2019), some data on modification of physiological relevant physiochemical properties is contradictory. Some studies describe reduced water binding capacity due to extrusion cooking, e.g. for lupin seed hulls (Zhong et al., 2019) and pea hulls (Ralet et al., 1993). On the other hand, water binding was reported to be increased in wheat bran (Wu et al., 2018) and orange pomace (Huang and Ma, 2016). Increased water binding and viscosity have been

credited for beneficial physiological responses in the upper gastrointestinal tract. Viscous fibre may alter gastric emptying, slow down glucose diffusion and absorption in the small intestine and interfere with the enterohepatic circulation of bile acids by reduction of bile acid reabsorption in the terminal ileum (Gunness et al., 2016, Mackie et al., 2016). Some studies indicate increased interactions between bile acids and dietary fibres after extrusion processing. However, the intermediate mechanism of interaction was not elucidated (Camire et al., 1993, Huth et al., 2000). Additionally, the physiochemical properties of dietary fibres are strongly dependent on the extrusion conditions (Liu et al., 2019).

Most studies dealing with the interaction of bile acids and fibre-enriched ingredients allow a classification of the interactions into two possible mechanisms. One of the mechanisms is based on the direct molecular association of bile acids with dietary fibre structures or associated substances. The other mechanism is based on the higher viscosity of the chyme due to the dietary fibres, which leads to a reduced mobility of the bile acid micelles (Gunness and Gidley, 2010, Naumann et al., 2018). For *in vitro* digested lupin kernel fibre viscosity was increased and bile acid diffusion was decreased compared to a blank sample. However, the viscosity increasing effect of lupin kernel fibre was significantly lower than for other fibre ingredients, such as a barley ingredient enriched in β-glucan (Naumann et al., 2019a). For these barley ingredients, the EFSA allows a health claim for cholesterol reduction, which is mainly based on the increased viscosity by β-glucan (EFSA NDA Panel, 2011). Increasing the viscosity of lupin kernel fibre by targeted processing, could thus improve its cholesterol-lowering properties. In addition to the viscosity related effects on bile acid retention in simulated gastrointestinal conditions, a bile acid sequestering effect on molecular scale was revealed *in vitro* for lupin kernel fibre ingredient (Naumann et al., 2019a). Using sequential extraction procedures, the molecular interaction was shown to be independent of dietary fibre structures and was associated with an alcohol extract rich in flavonoids (Naumann et al., 2020, Naumann et al., 2019b). In accordance with recent results described for other feedstocks, polyphenols associated with the lupin kernel fibre fraction may interact with bile acids on molecular scale (Ogawa et al., 2016, Chambers et al., 2019, Hamauzu and Suwannachot, 2019). Thermomechanical treatments of fibre preparations rich in polyphenols could change their content and bioavailability (Schmid et al., 2020). Therefore, it must be investigated to what extent the molecular interactions of bile acids and polyphenols found *in vitro* are influenced by extrusion.

The aim of this study was to evaluate the impact of extrusion processing on the physiochemical and functional properties of lupin kernel fibre. The lupin kernel fibre ingredient, obtained after protein isolation of dehulled, deoiled lupin seeds, was extruded with varying temperature, moisture, and screw speed. The effect of extrusion processing

conditions on dietary fibre composition, colour, water binding, and oil binding capacity of the lupin kernel fibre ingredient was investigated. An *in vitro* gastrointestinal digestion was performed to degrade digestible fractions of the lupin kernel fibre ingredient and to simulate the physiological conditions of human small intestine. We assessed viscosity of *in vitro* digesta and investigated bile acid interactions in the upper gastrointestinal tract using dialysis as a simplified absorption model of the unstirred water layer. By this means we aim to contribute towards understanding if extrusion processing can improve the health benefits of lupin dietary fibre.

2. Material and methods

2.1. Chemicals and Enzyme Preparations

L-α-lecithin (egg yolk, highly purified, CAS 8002-43-5), pancreatin from porcine pancreas (8 × USP specifications), and pepsin from porcine gastric mucosa (3,200–4,500 units/mg protein) were purchased from Merck KGaA (Darmstadt, Germany). All other reagents and chemicals used were of analytical grade and supplied by VWR (Radnor, PA, USA).

2.2. Production of lupin kernel fibre

Lupin seeds of *L. angustifolius* L. Boregine were kindly provided by Saatzucht Steinach GmbH & Co. KG (Steinach, Germany). Lupin seeds were processed based on the method of D'Agostina et al. (2006) as described in detail in Naumann et al. (2020). In brief, lupin seeds were de-hulled, flaked, and de-oiled. After sequential acid and neutral extractions of proteins, lupin kernel fibre was obtained from the raffinate separated by decanter centrifugation of the extracts. The raffinate was lyophilised and ground (< 500 µm) to obtain the lupin fibre fraction. In the present study, the non-extruded lupin kernel fibre ingredient is used as a reference.

2.3. Extrusion processing of lupin kernel fibre

The lupin kernel fibre ingredient obtained after lupin protein extraction (section 2.2) was processed by extrusion. A laboratory, co-rotating twin screw extruder (Haake Rheocord, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with a screw diameter of 16 mm, a smooth barrel, a length–diameter ratio of 25:1 and a 3.5 mm circular die was used to perform extrusion processing. The extruder was equipped with a twin screw gravimetric feeder type KCM (Coperion K-tron, Coperion GmbH, Stuttgart, Germany). To obtain the target total moisture during extrusion, distilled water (ambient temperature) was pumped (Alpha 50 Plus, ECOM, Prague, Czech Rep.) into the top of the extruder barrel while operating (130 mm downstream from the centre of the feed port). Water addition was calculated based on the

dry matter content of lupin kernel fibre ingredient (93.2 ±0.1g/100g), which was analysed by thermogravimetry at 105 °C using a TGA 601 analyser (Leco, St. Joseph, MI, USA) following the official method L 18.00-12 (Deutsches Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2005). The screw profile was built with different screw elements as described in detail in Osen et al. (2014). The barrel is divided into 5 temperature zones that can be set at different temperatures independently. A full experimental plan with variation of temperature (25 °C, 100 °C, 150 °C), moisture (20%, 50%), and screw speed (200 rpm, 400 rpm) was applied. The limits of the experimental plan were chosen to avoid clogging of the extruder. The mass flow rate was kept constant at 1 kg/h. For extrusion at 25 °C, all extruder zones were tempered equally. For extrusion at 100 °C and 150 °C, the barrel temperatures of the first three zones were increased in stages from 40 °C in the first zone, 60 °C and 80 °C, respectively, in the second and 80 °C and 100 °C, respectively, in the third zone. The last two zones were evenly tempered to 100 °C and 150 °C, respectively. The specific mechanical energy (SME) was calculated from the screw speed n (rpm), motor torque τ (Nm), and mass flow rate MFR (kg/h) as described in Equation 1 (Godavarti and Karwe, 1997).

$$\mathrm{SME}\ [\mathrm{kJ/kg}] = (2\pi \times \mathrm{n} \times \tau)/\mathrm{MFR} \quad (1)$$

Once the extruder reached steady state conditions, samples were collected, lyophilised, and ground. For lyophilisation, samples were frozen at −50 °C. Freeze-drying was performed at 5–45 °C for 72 h under a pressure of 103 Pa (freeze dryer beta 1-8, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Samples were ground using a ZM-200 mill (Retsch GmbH, Haan, Germany) to pass through a 500 µm screen.

2.4. Colour

A DigiEye Imaging System V2.60 (VeriVide Ltd., Leicester, United Kingdom) was used to take pictures of milled extrudates at defined lighting conditions (illuminant D 65) and CIE L*C*h°-values were evaluated.

2.5. Dietary fibre composition

The soluble (SDF) and insoluble dietary fibre content (IDF) was determined following the enzymatic–gravimetric method AOAC 991.43 (Association of Official Analytical Chemists, 2016). Total dietary fibre content (TDF = IDF + SDF) and the soluble proportion (SDF/TDF) were calculated.

2.6. Oil binding capacity

Oil binding capacity (OBC) was determined as described by Lin et al. (1974) with slight modifications. 3.0 g of the sample were dispersed with 20 mL of corn oil in a 25-mL conical graduated centrifuge tube. After centrifugation at 700 *g* and 20 °C for 15 min, the volume of free oil was determined. Oil binding capacity was expressed as the amount of oil bound by one gram of the sample on dry matter basis.

2.7. Water binding capacity

The water binding capacity (WBC) was determined according to the AACC Method 56-20.01 after suspension and hydration in deionised water and centrifugation at 20 °C and 1000 *g* for 15 min (AACC International, 2009).

2.8. *In vitro* digestion

In vitro digestion was performed prior to analyses of viscosity (section 2.9) and bile acid interactions (section 2.10). Samples were diluted to obtain a final dry matter concentration of 6.25% in the *in vitro* digesta. Simulation of oral, gastric, and small intestinal digestion was based on the harmonised INFOGEST protocol (Egger et al., 2016, Minekus et al., 2014) with slight modifications. Due to the low content of starch in lupin (Naumann et al., 2019b), α-amylase was omitted during oral digestion phase. To avoid microbial growth, sodium azide was added to all electrolyte fluids to reach a concentration of 0.02% in the final digestion mixture. Taurocholic acid, glycocholic acid, taurochenodesoxycholic acid, and glycochenodesoxycholic acid were mixed (1:1:1:1) and added during small intestinal digestion to obtain a final concentration of 10 mM in the digesta. Additionally, a blank digestion was carried out: The sample was replaced by demineralised water, simulated digestion fluids, lecithin, pepsin, pancreatin, and bile acids were added following the same protocol.

2.9. Viscosity measurement

The viscosity of *in vitro* digesta (section 2.8) was measured at constant temperature of 37 ± 0.1 °C using a rotational rheometer (Physica MCR 301; Anton Paar, Graz, Austria) equipped with a parallel plate geometry (diameter: 25 mm, shear gap: 1 mm PP25-SN23060). Prior to the measurements, samples were sheared at a shear rate of 5 s^{-1} for 20 s and allowed to rest for 20 s. The viscosity η was monitored as a function of the shear rate, which was analysed in a logarithmic scale ranging from 0.01–1000 s^{-1} (26 measurement points, measurement point duration ranged from 20 to 1 s). Applying the same measurement conditions, viscosity η was measured separately at shear rates of 5 s^{-1} and 15 s^{-1} (measurement point duration 10 s).

2.10. Bile acid interactions

Equilibrium dialysis of *in vitro* digested samples (section 2.8) was used to evaluate bile acid interactions based on the parameters of bile acid release kinetics. 4 g of *in vitro* digesta were filled into dialysis tubings (16 mm Servapor® 12–14 kDa, SERVA Electrophoresis GmbH, Heidelberg, Germany) and dialysed against 36 mL of phosphate buffer (50 mM, pH 7, 0.02% sodium azide). Permeating bile acids were analysed in 100 µL aliquots taken after 1, 2, 4, 8, 12, 24, and 48 hours of dialysis using high-performance liquid chromatography as described in detail in Naumann et al. (2019a).

Bile acid release kinetics were assessed (SigmaPlot®, version 12.5, Systat Software Inc., San Jose, CA, USA) using non-linear regression applying the first-order kinetic described in Equation 2 (Gunness et al., 2012, Macheras et al., 1986).

$$C_t = C_f \times (1 - e^{-kt}) \quad (2)$$

k: Apparent permeability rate constant
C_f: Equilibrium concentration

Retardation of bile acid diffusion due to viscous dietary fibres results in decreasing apparent permeability rate constants (k). Molecular interactions can be recognised by decreased equilibrium concentrations (C_f) displaying decreased total release of bile acids (Naumann et al., 2018). Bile acid adsorption (µmol/100 mg DM) was calculated from equilibrium concentrations (C_f) of the samples compared to the blank digestion.

2.11. Statistical Analysis

Results are expressed as mean ± standard deviation. R version 3.2.4 was used for statistical evaluation (www.r-project.org). Homogeneity of variance (Bartlett test) and normal distribution (Shapiro-Wilk test) were tested prior to ANOVA with post-hoc Tukey test. Three-way ANOVA was performed to evaluate interacting effects of extrusion parameters (independent variables: temperature (25 °C, 100 °C, 150 °C), moisture (20%, 50%), and screw speed (200 rpm, 400 rpm)). Pearson correlation coefficient (*r*), coefficient of determination (R^2) and *p*-value (ANOVA) were used to indicate correlations and to evaluate their significance. Samples were considered significantly different and correlations were considered significant for $p \leq 0.05$.

3. Results and discussion

3.1. Extrusion

As indication for extruder response, the SME was calculated from the data generated following a full factorial experimental design with varying temperature (25 °C, 100 °C, 150 °C), moisture (20%, 50%), and screw speed (200 rpm, 400 rpm). Therefore, the extrusion of lupin kernel fibre generated SME values between 1348 kJ/kg and 10118 kJ/kg as displayed in Table 1.

Table 1. Extruder response of specific mechanical energy (SME) and CIE L*C*h° values (n = 5) of extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.).

Sample	T [°C]	MC [%]	n [rpm]	SME [kJ/kg]	Kernel fibre/ Extrudates	L*	C*	h°
Ref.	-	-	-	-		88.5 ± 1.0 ^{g}	11.8 ± 1.1 ^{a}	90.8 ± 0.9 ^{f}
1	25	50	200	1659		84.6 ± 0.7 ^{d,e,f}	15.3 ± 1.7 ^{b,c}	84.4 ± 5.2 ^{c,d,e}
2	25	50	400	3100		85.2 ± 2.2 ^{e,f}	13.1 ± 0.7 ^{a,b}	87.3 ± 3.3 ^{e,f}
3	25	20	200	5481		86.4 ± 0.6 ^{f,g}	14.2 ± 0.8 ^{a,b,c}	86.1 ± 3.7 ^{d,e,f}
4	25	20	400	7897		74.0 ± 0.7 ^{b}	27.5 ± 1.3 ^{f}	79.5 ± 1.6 ^{b,c}
5	100	50	200	1371		86.5 ± 1.1 ^{f,g}	13.8 ± 1.3 ^{a,b}	90.1 ± 1.6 ^{e,f}
6	100	50	400	2898		85.1 ± 0.9 ^{e,f}	14.4 ± 0.5 ^{b,c}	85.8 ± 3.8 ^{d,e,f}
7	100	20	200	3325		83.2 ± 1.3 ^{d,e}	18.3 ± 1.2 ^{d}	80.4 ± 1.5 ^{b,c,d}
8	100	20	400	10118		78.3 ± 1.5 ^{c}	22.9 ± 1.1 ^{e}	79.1 ± 1.0 ^{b,c}
9	150	50	200	1348		81.8 ± 1.3 ^{d}	16.4 ± 1.0 ^{c,d}	77.8 ± 1.5 ^{e}
10	150	50	400	2851		84.5 ± 2.0 ^{d,e}	14.6 ± 1.4 ^{b,c}	86.1 ± 4.7 ^{a,b,c}
11	150	20	200	4569		73.3 ± 1.9 ^{b}	27.0 ± 1.0 ^{f}	76.8 ± 0.3 ^{b}
12	150	20	400	8897		60.5 ± 1.9 ^{a}	32.0 ± 0.9 ^{g}	65.7 ± 0.6 ^{a}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

No significant effect of the barrel temperature on SME was detected ($p > 0.05$). Three-way ANOVA revealed the SME to be influenced by moisture content ($p < 0.01$) and screw speed ($p < 0.01$), showing significant interaction ($p < 0.05$) of both parameters. On average, SME doubled by doubling the screw speed, which is in line with the direct dependence of SME on speed described in Equation 1. SME showed threefold value by decreasing the moisture content from 50% to 20%, which indicates an increased mechanical load due to increased viscosity in the extruder barrel.

Colour of lupin kernel fibre was significantly affected by the extrusion process and varied depending on the operational parameters (Table 1). Greatest changes in colour were observed for samples extruded at high temperature and low moisture (11 and 12) in comparison to the reference. Lightness (L*) decreased from 88.5 to 73.3 and 60.5 for extrudates 11 and 12, respectively. Chroma (C*) indicates saturation and increased from 11.8 to 27.0 and 32.0 for extrudates 11 and 12, respectively. Hue angle (h°) changed from 90.8 to 76.8 and 65.7 for extrudates 11 and 12, respectively. Thus the colour quality was changed from yellow to yellow-red. Similar colour changes were described for extruded wheat bran, citrus bran, and lupin hull fibre (Redgwell et al., 2011, Wu et al., 2018, Zhong et al., 2019). The pronounced effect of high temperature and low moisture during extrusion suggests that the increase in darkness, colour saturation and redness of lupin fibre during extrusion could be attributed to Maillard reaction, caramelisation or degradation and polymerisation of polyphenols (Guy, 2001, Zhong et al., 2019).

3.2. Dietary fibre composition

As presented in Table 2, the lupin kernel fibre ingredient, obtained after protein isolation from lupin, is mostly composed of dietary fibre (82.4 g/100 g DM), which is predominantly insoluble (80.5 g IDF/100 g DM).

Extrusion processing significantly influenced the dietary fibre profile of lupin kernel fibre. Depending on the parameters of the extrusion processing, dietary fibre composition shifted towards a higher proportion of soluble fibre. In comparison to the non-extruded reference (1.9 g SDF/100 g DM) dietary fibre solubility was significantly increased up to 37.7 g SDF/100 g DM (for extrudate 11, T: 150 °C, MC: 20%, n: 200 rpm). High soluble fibre contents were further achieved for extrudate 4 (31.1 g SDF/100 g DM) and extrudate 8 (31.0 g SDF/100 g DM), extruded at 25 °C and 100 °C, 20% moisture and 400 rpm, respectively. These results are in line with former optimisation trials aiming to increase the soluble fibre content of orange pomace (Huang and Ma, 2016) and the soybean residue of soymilk and tofu production (Jing and Chi, 2013).

Table 2. Dietary fibre composition (insoluble dietary fibre (IDF), soluble dietary fibre (SDF), total dietary fibre (TDF = IDF + SDF)) of extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.) (*n* = 3).

	T	MC	n	IDF	SDF	TDF	SDF/ TDF
Sample	[°C]	[%]	[rpm]	[g/100g DM]			[%]
Ref.	-	-	-	80.5 ± 0.3 ^{e}	1.9 ± 0.6 ^{a}	82.4 ± 0.3 ^{c,d,e}	2.3 ± 0.7 ^{a}
1	25	50	200	79.1 ± 1.6 ^{e}	6.5 ± 2.8 ^{a,b}	85.6 ± 1.5 ^{d}	7.5 ± 3.1 ^{a,b}
2	25	50	400	66.9 ± 6.4 ^{d}	16.2 ± 4.2 ^{b,c,d}	80.4 ± 2.3 ^{b,c,d,e}	20.0 ± 4.7 ^{b,c}
3	25	20	200	53.8 ± 3.0 ^{c}	19.2 ± 4.2 ^{c,d,e}	72.9 ± 1.3 ^{b,c}	26.2 ± 5.3 ^{c,d}
4	25	20	400	55.5 ± 3.2 ^{c}	31.1 ± 4.3 ^{f,g}	86.6 ± 5.0^{e}	36.1 ± 6.4 ^{d}
5	100	50	200	74.7 ± 4.4 ^{d,e}	7.2 ± 1.6 ^{a,b}	81.8 ± 4.0 ^{c,d,e}	8.8 ± 2.0 ^{a,b}
6	100	50	400	72.6 ± 3.5 ^{d,e}	7.2 ± 2.0 ^{a,b}	79.8 ± 4.5 ^{b,c,d,e}	8.9 ± 2.1 ^{a,b}
7	100	20	200	49.9 ± 4.9 ^{c}	25.3 ± 6.1 ^{d,e,f}	75.2 ± 4.7 ^{b,c,d}	33.5 ± 6.8 ^{d}
8	100	20	400	52.4 ± 2.9 ^{c}	31.0 ± 5.5 ^{f,g}	83.4 ± 3.2 ^{c,d,e}	37.0 ± 5.4 ^{d}
9	150	50	200	67.6 ± 3.2 ^{d}	12.3 ± 0.5 ^{a,b,c}	79.9 ± 3.2 ^{b,c,d,e}	15.4 ± 0.9 ^{b,c}
10	150	50	400	67.7 ± 0.9 ^{d}	11.6 ± 1.1 ^{a,b,c}	79.3 ± 0.9 ^{b,c,d,e}	14.6 ± 1.3 ^{a,b,c}
11	150	20	200	32.4 ± 1.9 ^{b}	37.7 ± 5.6 ^{g}	70.0 ± 6.2 ^{b}	53.6 ± 3.9 ^{d}
12	150	20	400	18.3 ± 1.5 ^{a}	29.3 ± 5.3 ^{e,f}	47.6 ± 3.8 ^{a}	61.2 ± 6.0 ^{e}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

IDF and SDF were significantly influenced by all operational parameters of extrusion processing. Extrusion processing led to a modification of insoluble fibre into soluble fractions. The increase in the soluble proportion (SDF/TDF) correlated with a reduction in lightness (L*) of the samples ($r = -0.88$, $R^2 = 0.78$, $p < 0.001$). Three-way ANOVA revealed this redistribution of IDF to SDF (with constant TDF) to be influenced by the extrusion parameters moisture ($p < 0.001$) and screw speed ($p < 0.01$), while no significant influence of barrel temperature ($p > 0.05$) was observed. Our results thus indicate that the redistribution of IDF to SDF for lupin kernel fibre is associated with mechanical rather than thermal effects, which is in line with the findings of Ralet et al. (1990). Accordingly, we found SDF to increase with increasing SME, which depended on moisture content and screw speed during extrusion. A pronounced effect of moisture content on SME was found, which is in agreement with former studies describing citrus fibre and lupin seed hull extrusion processing (Redgwell et al., 2011, Zhong et al., 2019).

Statistical analysis revealed TDF to be influenced by temperature and screw speed of the extrusion process ($p < 0.001$). While no significant difference to the reference was observed for extrudates 1–10, TDF significantly decreased for samples 11 and 12. Transformation of IDF to SDF with constant TDF was already described by a number of researchers, e.g. recently for rice bran and wheat bran (Andersson et al., 2017, Vasanthan et al., 2002). For extruded lupin kernel fibre processed at high barrel temperature (150 °C) and low moisture content (20%), a

distinct effect on dietary fibre composition was evident. In contrast to extrudates 1–10, TDF decreased to 70.0 g/100 g DM and 47.6 g/100 g DM for extrudates 11 and 12, respectively. This TDF degradation was significantly influenced by temperature and screw speed, which indicates a thermal effect in addition to the mechanical effects described for extrudates 1–10. Thermal treatment thus may have led to additional breaks of glycosidic bonds, converting insoluble polysaccharides to smaller fractions (Ul Ain et al., 2019, Wolf, 2010). Especially for extrudate 12, this degradation caused an increase in low molecular weight molecules too small to be detected as dietary fibre (Redgwell et al., 2011).

3.3. Oil binding capacity

The OBC of lupin kernel fibre (2.28 mL/g DM) was significantly reduced due to the extrusion processing (OBC extrudates: 1.09–1.87 mL/g DM, Figure 1). While moisture content during extrusion had no significant effect on OBC ($p > 0.1$), screw speed and temperature showed a significant impact on OBC ($p < 0.001$). All operational parameters showed significant interacting effects ($p < 0.01$).

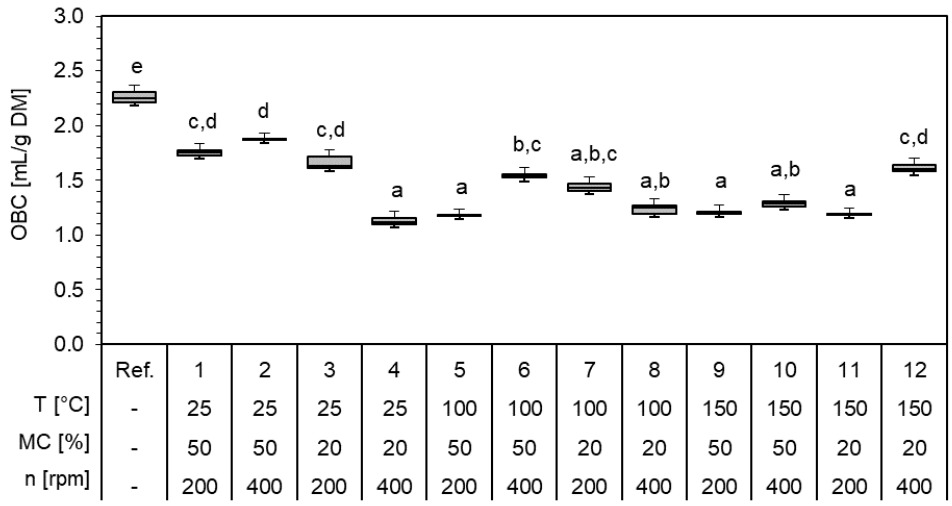

Figure 1. Oil binding capacity (OBC) of extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.). Different letters indicate significant differences on a $p \leq 0.05$ level basis ($n = 3$).

Significant decreases of OBC due to extrusion processing were already described for orange pomace (Huang and Ma, 2016), apple pomace (Liu et al., 2019) and rice bran (Rafe et al., 2016). Oil retention by dietary fibres is mainly related to their surface properties, but may also be influenced by overall charge density and the hydrophilic nature of the constituents (Dhingra et al., 2012, Elleuch et al., 2011, Fleury and Lahaye, 1991). The reduction in OBC may thus be associated with a decrease in the surface area and total pore volume due to structural changes

induced by the extrusion processing. Furthermore, the decrease in OBC may be related to an increase of hydrophilic sites and/or decrease of lipophilic sites (Huang and Ma, 2016), which could be linked to depolymerisation of dietary fibre or change in chemistry or conformations of proteins (residual protein content in the lupin fibre ingredient: 11.1 ± 0.2 g/100 g DM, Naumann et al. (2020)). The higher OBC of extrudate 12 further indicates a potential contribution of thermal processing and/or Maillard conjugation, which is described to increase surface hydrophobicity (Joubran et al., 2017).

3.4. Water binding capacity

When investigating the physiological functionality of dietary fibres, it is important to consider the water associated with the fibre (Robertson and Eastwood, 1981). The WBC represents the ability of dietary fibre to retain free water in the gastrointestinal tract. This physiochemical property is of great relevance for fibre functionality as it accounts for satiety effects and faecal bulking and determines the induction of fermentation in the gut (Chen et al., 2018, Guillon and Champ, 2000).

Water binding to lupin kernel fibre was significantly affected by extrusion processing as influenced by moisture content and temperature (Figure 2). WBC of non-extruded lupin fibre was 7.71 ± 0.08 mL/g DM and was increased up to 15.04 ± 0.43 mL/g DM for extrudate 11 extruded at 150 °C, 20% moisture and 400 rpm.

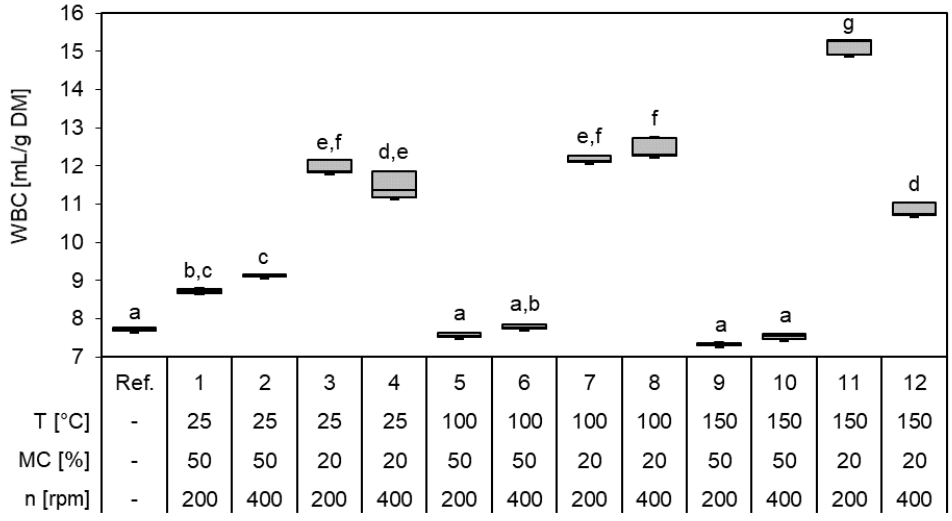

Figure 2: Water binding capacity (WBC) of extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.). Different letters indicate significant differences on a $p \leq 0.05$ level basis ($n = 3$).

The kinetic of water uptake is controlled by structural fibre characteristics and the chemical affinity of fibre components towards water. Consequently, water may be held in capillaries formed by the insoluble fibres due to surface tension strength or water may interact with molecular components of fibre by hydrogen bonding or dipole interactions (Chaplin, 2003, López et al., 1996). Extrusion processing is described to solubilise cell wall constituents, in particular hydrophilic pectin-like polymers, which exhibit high hydration properties (Redgwell et al., 2011, Tosh and Yada, 2010). In our study, we observed a redistribution of IDF to SDF at nearly constant TDF for extrudates 1–11 (section 3.2). The increase in the soluble proportion (SDF/TDF) for extrudates 1–11 was found to correlate directly proportional with the increase in WBC (r = 0.94, R^2 = 0.88, p < 0.001), as displayed in Figure 4. This indicates that the increase in WBC is associated with the structural and chemical changes of the fibres caused by mechanical and thermal processing. The deviation in WBC observed for extrudate 12 can be explained by the drastic reduction in TDF associated with high thermal and mechanical loads during extrusion (Table 2). The extrusion thus leads to the destruction of the dietary fibre matrix to smaller sugar molecules, which exhibit lower hydration properties.

Previous studies suggest that extrusion processing may increase the water absorption of proteins (Brnčić et al., 2011). Changes in the WBC of the residual protein contained in the lupin kernel fibre ingredient, may thus add to the observed increase in WBC after extrusion processing. However, the WBC reported for a lupin protein concentrate (2.8 mL/g DM; Peters et al. (2017)) is lower than the range observed for the extrudates and reference of lupin kernel fibre ingredient (7.7–15.0 mL/g) in the current study. We therefore assume that the changes in WBC are mainly associated with changes in the dietary fibre fraction.

Our results are in line with improved hydration properties observed after extrusion for wheat bran (Wu et al., 2018), orange pomace (Huang and Ma, 2016), carrot residues (Gao et al., 2015), and barley meal (Huth et al., 2000). However, water binding was reported to be reduced due to extrusion processing of lupin seed hulls (Zhong et al., 2019) and pea hulls (Ralet et al., 1993). These conflicting findings may be explained by the source and composition of the extruded fibre materials. Solubilisation of hemicellulosic and pectin-like polymers, occurring in a number of fibre sources, was repeatedly associated with increased hydration properties (Ng et al., 1999, Redgwell et al., 2011). On the other hand, plant hull materials mainly consist of structural components rich in cellulose (Pfoertner and Fischer, 2001). Therefore, water binding of hull fibres is primarily linked to structural parameters like porosity (Ulbrich and Flöter, 2014). Extrusion is described to degrade cellulose fibrils, which may thus result in decreased WBC due to a reduction of the water bound in capillary structures (Dang and Vasanthan, 2019, Redgwell et al., 2011, Ulbrich and Flöter, 2014).

3.5. Viscosity

The viscosity increase induced by fibres is determined by the ability of polysaccharides to thicken when mixed with liquids due to physical entanglements between the polysaccharide components (Dikeman and Fahey, 2006). Viscous fibres have been associated with changes in blood sugar and cholesterol concentrations, prolonged gastric emptying, and increase in transit time through the intestinal tract (Mälkki, 2001). An *in vitro* digestion protocol using physiological enzymes at 37 °C was used to estimate the effect that extrusion processing would have on small intestinal viscosity. Therefore, the viscosity of *in vitro* digesta obtained from lupin kernel fibre and its extrudates was measured as a function of the shear rate. Figure 3 shows the flow curves determined after *in vitro* digestion of extruded lupin fibre (illustrated for extrudates 9–12) in comparison to the non-extruded reference.

For all samples, a division of the viscosity curve into two ranges was observed. In the range of low shear rates (< 0.1 s^{-1}), the curves showed a Newtonian plateau. This flow behaviour can be explained by the superimposition of partial disentanglements and re-entanglements of polymeric fibres occurring at low-shear conditions. At higher shear rates, a similar pattern of shear thinning was observed for all extrudates and the reference. The shear thinning is likely induced by orienting and partial disentanglement of fibre polymers due to the shearing forces (Dikeman and Fahey, 2006, Dikeman et al., 2006, Guillon and Champ, 2000).

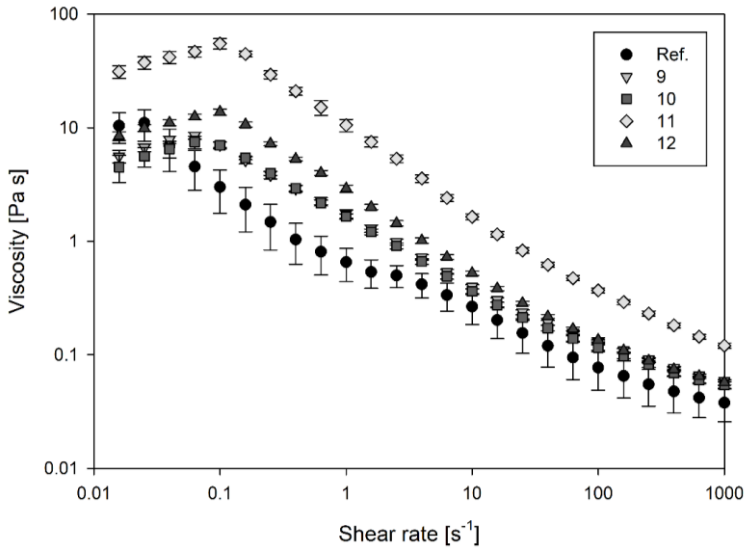

Figure 3: Viscosity of *in vitro* digesta containing extrudates (extruded at 150 °C with varying moisture content (MC) and screw speed; 9: 50% MC, 200 rpm; 10: 50% MC, 400 rpm; 11: 20% MC, 200 rpm, 12: 20% MC, 400 rpm) in comparison to non-extruded lupin kernel fibre (Ref.).

The viscosity of *in vitro* digesta obtained from lupin kernel fibre and its extrudates was compared at two shear rates (5 s^{-1} and 15 s^{-1},Table 3), which were chosen to simulate the small shearing forces in the gastrointestinal tract (Gunness et al., 2012, Naumann et al., 2018). Viscosity was significantly influenced by the moisture content during the extrusion process ($p < 0.001$). While screw speed and temperature had no significant impact on viscosity, all operational parameters showed significant interacting effects ($p < 0.001$).

Viscosity increased due to extrusion process, which was especially significant after extrusion with low moisture content and increased SME (Figure 4, Table 3). Highest viscosity was observed for extrudate 11, which was significantly higher than for all other samples. Viscosity of extrudate 12 differed from all other samples extruded at 20% moisture content, which indicates an additional effect of high temperature (150 °C) processing on viscosity.

Table 3. Viscosity at shear rates 5 s^{-1} and 15 s^{-1} of extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.) (n = 3).

	T	MC	n	Viscosity [Pa s]	
Sample	[°C]	[%]	[rpm]	5 s^{-1}	15 s^{-1}
Ref.	-	-	-	0.44 ± 0.17 ^{a}	0.23 ± 0.08 ^{a}
1	25	50	200	1.26 ± 0.17 ^{b,c}	0.55 ± 0.07 ^{c,d}
2	25	50	400	1.43 ± 0.04 ^{c}	0.62 ± 0.02 ^{d}
3	25	20	200	2.23 ± 0.08 ^{d,e}	0.93 ± 0.04 ^{e,f}
4	25	20	400	2.25 ± 0.24 ^{e}	1.04 ± 0.11 ^{f}
5	100	50	200	0.47 ± 0.06 ^{a}	0.24 ± 0.02 ^{a}
6	100	50	400	0.57 ± 0.03 ^{a}	0.28 ± 0.01 ^{a}
7	100	20	200	2.47 ± 0.09 ^{e}	1.05 ± 0.05 ^{f}
8	100	20	400	1.91 ± 0.07 ^{d}	0.84 ± 0.02 ^{e}
9	150	50	200	0.66 ± 0.07 ^{a}	0.34 ± 0.04 ^{a,b}
10	150	50	400	0.57 ± 0.04 ^{a}	0.29 ± 0.02 ^{a}
11	150	20	200	3.17 ± 0.05 ^{f}	1.30 ± 0.03 ^{g}
12	150	20	400	0.99 ± 0.06 ^{b}	0.45 ± 0.03 ^{b,c}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

Regarding the physiological effects of dietary fibres, it is crucial to understand process impacts on the viscosity increasing effect of dietary fibres. Yet, available studies show that extrusion does not have a uniform effect on viscosity. Some authors reported no influence of extrusion on viscosity e.g. for soluble fibre from nodes and lotus root (Chen et al., 2018). On the other hand, other authors could show that apparent viscosity of citrus fibre and sesbania gum increased due to extrusion processing (Gourgue et al., 1994, Li et al., 2019). In addition,

viscosity after extrusion was reported to decrease due to depolymerisation in citrus peel fibre and oat bran (Redgwell et al., 2011, Tosh et al., 2010).

In our study, viscosity was increased for all extrudates at conditions simulating physiological conditions in the small intestine. For extrudates 1–11 increasing viscosity correlated linearly with a rise in the soluble proportion of the fibre ($r = 0.93$, $R^2 = 0.86$, $p < 0.001$), which was linked with a coherent increase in WBC (Figure 4). Correspondingly, a linear correlation between viscosity and WBC was found ($r = 0.97$, $R^2 = 0.93$, $p < 0.001$). Non-extruded lupin kernel fibre is predominantly composed of insoluble pectin-like polymers (Naumann et al., 2019b). Increased viscosity after extrusion treatment may therefore be explained by the solubilisation of pectin-like polymers, which exhibit high hydration properties and thus increase the viscosity after *in vitro* digestion. These results are in line with the findings of Ng et al. (1999), who investigated the effects of extrusion processing on the physicochemical properties and microstructure of onion cell walls. The authors concluded that extrusion increased the solubility of pectin-like polymers and hemicelluloses, which was accompanied by an increase in the swelling capacity of the cell wall material. The varying impacts of extrusion processing on the viscosity described for different types of dietary fibre suggest that the influence of extrusion on the viscous and functional properties of dietary fibres is strongly dependent on their initial compositional and physiochemical properties as well as the preservation of viscosity-relevant structures during solubilisation of IDF.

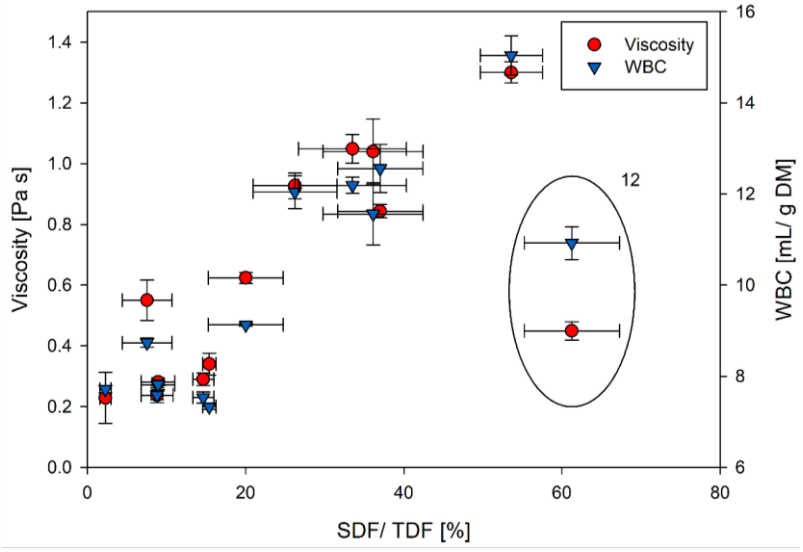

Figure 4: Viscosity (at shear rate 15 s^{-1}) and water binding capacity (WBC) as a function of the soluble proportion (SDF/TDF) of extrudates derived from lupin kernel fibre.

In addition to the solubility, the viscosity of fibre solutions is determined by the molecular weight of the fibre polymers (Wolever et al., 2010). Accordingly, Ng et al. (1999) observed a broadening of the molecular weight distribution in the water-soluble fraction of the extruded onion cell walls, while the mean molecular weight remained unchanged. This indicated an increase in the water soluble fibre fraction. In another study focusing on oat bran, extrusion caused an increase in the yield of soluble fibre, which was associated with an increase in high-molecular weight SDF. The structural changes in SDF after extrusion processing were described as shift in the ratios of the 1–3 and 1–4 chemical bonds in SDF fraction of oat bran (Zhang et al., 2011, Zhang et al., 2009).

In order to improve the functionality of extruded fibre ingredients, extrusion processing should be optimised by evaluating not only the increase in solubility but also the changes in molecular weight distribution and/or viscosity of the extruded samples. This is corroborated by two studies focusing on the influence of extrusion on viscosity and molecular weight distribution of β-glucan, the soluble fibre fraction of oat and barley. Honců et al. (2016) revealed that, under optimal operating conditions for barley, extrusion caused a shift and increase of the molecular weight of β-glucan in the soluble extracts of barley flours, which was associated with transformation of IDF to SDF and formation of additional SDF due to transglycosidation (Vasanthan et al., 2002). In addition, Tosh et al. (2010) investigated the extrusion processing of oat bran cereals under optimal and harsh processing conditions and evaluated the properties of physiological β-glucan extracts obtained by *in vitro* digestion followed by centrifugation. While the extracted portion of β-glucan in the supernatant of unprocessed oat bran was 38.7%, it was increased to 66.8% when processed under optimal extrusion conditions and reached to up to 100% in samples extruded under harsh conditions. The increase in β-glucan solubility was accompanied with a decrease in molecular weight from 2,537,000 g/mol in unprocessed bran extracts to 2,213,000 g/mol and 211,000 g/mol for samples extruded under optimal and harsh conditions, respectively. By microscopic examination is was then shown that the loss of molecular weight at harsh extrusion conditions are due to depolymerisation, loss of cell wall integrity and dispersion of β-glucan. Thus, viscosity in physiological extracts, which was 2900 mPa s after optimal extrusion, dropped to 131 mPa s after harsh extrusion. Accordingly, the harsh processing conditions of extrudate 12 in our study may have led to severe depolymerisation, which explains the decrease of functionality regrading hydration properties and viscosity.

3.6. Bile acid interactions

In general, there are the two plausible mechanisms of interactions between dietary fibres and bile acids. Either a direct interaction at a molecular level and/or reduction of bile diffusion by entrapment of the bile acids in viscous polymer networks (Gunness et al., 2010). Both types of interactions can be assessed by kinetic evaluation of bile acid release by means of *in vitro* digestion, equilibrium dialysis, and high-performance liquid chromatography bile acid analysis (Naumann et al., 2018). Bile acid interactions were investigated for all extrudates in comparison to the lupin kernel fibre reference. Bile acid interactions of primary bile acids were evaluated based on the kinetic analysis of bile acid passage across a dialysis membrane.

In Figure 5, kinetic analysis of bile acid release is displayed exemplified for cholic acid and chenodesoxycholic acid release (summarised for glyco- and tauroconjugated bile acids) from *in vitro* digested extrudate 8 in comparison to the blank digestion and the non-extruded reference.

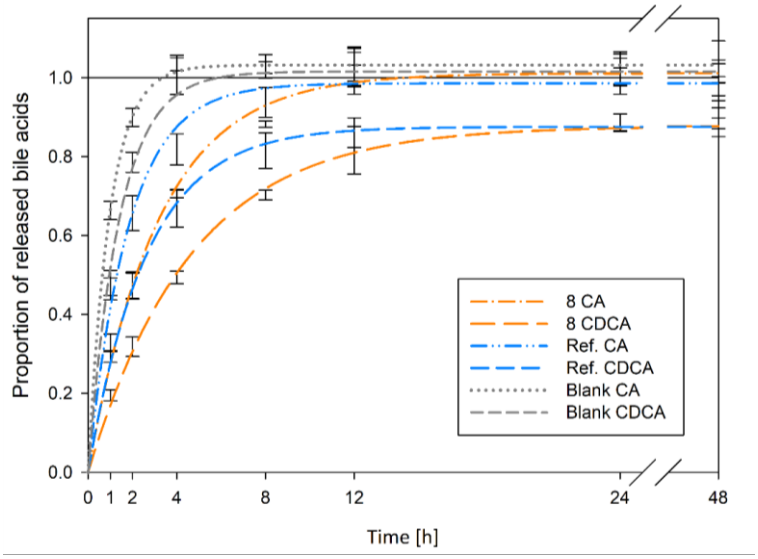

Figure 5. Diffusion kinetics of bile acid release (summarised for glyco- and tauroconjugated cholic acid (CA) and chenodesoxycholic acid (CDCA)) of *in vitro* digested extrudate (sample 8, extruded at 100 °C, 20% moisture content, and screw speed of 400 rpm) in comparison to blank digestion and non-extruded lupin kernel fibre (Ref.).

3.6.1. Bile acid interactions on molecular level

The equilibrium concentration of *in vitro* bile acid release inversely corresponds to adsorptive interactions at molecular level (Figure 5). Equilibrium concentrations of cholic acids were not significantly different in comparison to blank digestion for all extruded samples and the reference. Thus, no significant adsorption of cholic acids was detectable. For the reference

and all extruded samples chenodesoxycholic acids were significantly adsorbed, which can be recognised by the reduced equilibrium concentrations in comparison to blank digestion. The differences in the molecular interactions with cholic acids compared to chenodesoxycholic acids support the hypotheses of a hydrophobic linkage between the fibres and the bile acids, which was already supposed by several authors (Camire and Clydesdale, 1981, Drzikova et al., 2005, Dziedzic et al., 2015, Naumann et al., 2019a, Trautwein et al., 1999). Yet, the exact mechanism of interactions on molecular level remains to be elucidated.

Adsorption of chenodesoxycholic acids by extruded lupin kernel fibre ranged from 1.17 µmol/100 mg DM to 2.00 µmol/100 mg DM. However, no significant differences were observed between the reference (adsorption of chenodesoxycholic acids: 1.97 ± 0.11 µmol/100 mg DM) and all extruded samples. Extrusion thus did not affect molecular interactions between lupin fibre and bile acids in a significant manner. A proposed mechanism to explain molecular interactions between dietary fibres and bile acids is the adsorption of bile acids due to insoluble dietary fibre surface properties (Gunness et al., 2010, Kahlon and Woodruff, 2003, Singh et al., 2019). In our current study, we observed a transformation of IDF to SDF due to the extrusion processing (section 3.2). Although IDF was drastically reduced, no difference in molecular interaction was detectable for the extrudates. Our results thus contradict the hypothesis of a direct linkage between bile acids and IDF structures. These findings are in line with our former study focusing on purified and fractioned lupin fibres (Naumann et al., 2019b). As a direct participation of the cell wall polysaccharides in the bile acid adsorption of lupins could be disproven, molecular interactions could be caused by components associated with the fibre fraction (Dongowski, 2007, Kahlon and Smith, 2007, Sayar et al., 2006). In our recent research study focusing on sequential extractions of lupins to elucidate adsorptive components, a potential contribution of phytochemicals, e.g. flavonoids was indicated (Naumann et al., 2020). The results of the current study suggest that the mechanical and thermal load during extrusion have not influenced the molecular interactions with bile acids in a significant manner, which could be attributed to the high thermostability described for flavonoids (Im et al., 2011). However, further studies are required to understand the contribution of these components to the cholesterol-lowering action of lupins.

3.6.2. Bile acid interactions as related to viscosity

The second mechanism of bile acid interaction is the formation of viscous polymer networks. Increased viscosity reduces bile acid diffusion and the apparent permeability rate constant, which can be calculated by kinetic fitting of the bile acid release curves. The apparent permeability rate constant was reduced due to the extrusion of lupin kernel fibre (Table 4).

Bile acid retardation was influenced by bile acid conjugation ($p < 0.05$) and bile acid hydroxylation ($p < 0.001$): Release rates decreased for glycocholic acid > taurocholic acid > glycochenodesoxycholic acid > taurochenodesoxycholic acid, which can be explained by the micellar properties (critical micelle concentrations) of the different bile acids as described in detail in Naumann et al. (2019a).

Three-way ANOVA showed that the apparent permeability rates were significantly influenced by the moisture content ($p < 0.001$) and the temperature ($p < 0.01$) during extrusion processing. An interaction effect was significant for temperature and screw speed ($p < 0.05$). In particular, significant retardation of bile acid release was detected for the samples extruded at 100 °C or 150 °C at a moisture content of 20% (extrudates 7, 8, 11, 12). For all analysed bile acid species, bile acid release was slowed by up to 45% in comparison to the untreated reference. Compared to the blank digestion value, this corresponds to a reduction in the bile acid release rate of up to 72%. The improvement of bile acid interactions due to extrusion processing is in line with the results of Camire et al. (1993) focusing on potato peels and Huth et al. (2000) investigating extrudates derived from barley.

Table 4: Apparent permeability rate constants (k) of kinetic bile acids release analysis (glycocholic acid (GCA), taurocholic acid (TCA), glycochenodesoxycholic acid (GCDCA), taurochenodesoxycholic acid (TCDCA)) of *in vitro* digested extrudates (following a full factorial experimental design with varying temperature (T), moisture (MC), and screw speed (n)) in comparison to non-extruded lupin kernel fibre (Ref.) (*n* = 3).

	T	MC	n	k [h^{-1}]			
Sample	[°C]	[%]	[rpm]	GCA	TCA	GCDCA	TCDCA
Blank	-	-	-	1.07 ± 0.09 ^{c}	1.01 ± 0.01 ^{c}	0.78 ± 0.08 ^{c}	0.66 ± 0.08 ^{c}
Ref.	-	-	-	0.57 ± 0.08 ^{b}	0.53 ± 0.05 ^{b}	0.38 ± 0.04 ^{b}	0.38 ± 0.06 ^{b}
1	25	50	200	0.47 ± 0.08 ^{a,b}	0.46 ± 0.09 ^{a,b}	0.32 ± 0.04 ^{a,b}	0.31 ± 0.04 ^{a,b}
2	25	50	400	0.43 ± 0.03 ^{a,b}	0.42 ± 0.03 ^{a,b}	0.30 ± 0.03 ^{a,b}	0.29 ± 0.01 ^{a,b}
3	25	20	200	0.40 ± 0.07 ^{a,b}	0.39 ± 0.07 ^{a,b}	0.28 ± 0.05 ^{a,b}	0.27 ± 0.04 ^{a,b}
4	25	20	400	0.38 ± 0.03 ^{a}	0.37 ± 0.03 ^{a,b}	0.26 ± 0.04 ^{a,b}	0.25 ± 0.03 ^{a}
5	100	50	200	0.45 ± 0.05 ^{a,b}	0.43 ± 0.07 ^{a,b}	0.32 ± 0.06 ^{a,b}	0.33 ± 0.04 ^{a,b}
6	100	50	400	0.45 ± 0.01 ^{a,b}	0.45 ± 0.01 ^{a,b}	0.32 ± 0.00 ^{a,b}	0.32 ± 0.02 ^{a,b}
7	100	20	200	0.35 ± 0.03 ^{a}	0.35 ± 0.03 ^{a}	0.24 ± 0.03 ^{a}	0.24 ± 0.04 ^{a}
8	100	20	400	0.32 ± 0.02 ^{a}	0.31 ± 0.01 ^{a}	0.22 ± 0.01 ^{a}	0.21 ± 0.01 ^{a}
9	150	50	200	0.38 ± 0.02 ^{a}	0.37 ± 0.01 ^{a,b}	0.26 ± 0.02 ^{a,b}	0.26 ± 0.02 ^{a,b}
10	150	50	400	0.46 ± 0.11 ^{a,b}	0.44 ± 0.11 ^{a,b}	0.33 ± 0.06 ^{a,b}	0.31 ± 0.05 ^{a,b}
11	150	20	200	0.32 ± 0.01 ^{a}	0.32 ± 0.01 ^{a}	0.22 ± 0.03 ^{a}	0.22 ± 0.01 ^{a}
12	150	20	400	0.34 ± 0.03 ^{a}	0.34 ± 0.02 ^{a}	0.25 ± 0.03 ^{a}	0.24 ± 0.02 ^{a}

Along the column, different letters indicate significant differences on a $p \leq 0.05$ level basis.

The values achieved for apparent permeability rates at favourable extrusion conditions (extrudates 7, 8, 11, 12) were 0.31–0.35 h^{-1} for cholic acids and 0.21–0.25 h^{-1} for chenodesoxycholic acids. These values are comparable to the values measured for a β-glucan enriched barley ingredient (0.33 h^{-1} for cholic acids and 0.24 h^{-1} for chenodesoxycholic acids (Naumann et al., 2019a)). For these barley based products, the EFSA allows a health claim for cholesterol reduction, which is mainly based on the increase in viscosity induced by β-glucan (EFSA NDA Panel, 2011). The available *in vitro* results thus suggest that comparable functionality can be achieved by targeted extrusion modification of lupin fibre. Further *in vivo* studies are needed to confirm these *in vitro* results and ensure the transferability to cholesterol-lowering actions.

The apparent permeability rate constant of *in vitro* bile acid release is inversely controlled by the viscosity of the *in vitro* digesta, which was corroborated by our current results. As discussed for increasing viscosity for extrudates 1–11 in section 3.5, the decrease in apparent permeability rate constants correlated linearly with a rise in the soluble proportion of the fibre ($r = -0.85$, $R^2 = 0.73$, $p < 0.001$). The influence of extrusion processing on the increased viscosity and the retardation of bile acid diffusion can therefore be explained by the solubilisation of hydrophilic pectin-like polymers, which exhibit high hydration properties and thus increase the viscosity after *in vitro* digestion.

4. Conclusions

In our study, extrusion technology was successfully applied to modify the composition and physiochemical properties of lupin kernel fibre. A significant redistribution of IDF to SDF was observed, while only small changes in TDF were detected for most extrudates. The alteration of the dietary fibre composition was linked with the SME and temperature of the extrusion process and is in line with the findings of several studies focusing on different fibre sources. In addition, the physiochemical properties including water binding capacity, viscosity increasing capacity and interaction with bile acids was improved in comparison to the untreated sample. Pronounced effects of moisture content on SME, the dietary fibre transformation of IDF to SDF, the water binding capacity and viscosity were observed. The improvement in fibre functionality may thus be mainly caused by the mechanical stress induced by the extrusion process. We suggest that the processing causes solubilisation of hydrophilic pectin-like polymers, which exhibit high hydration properties and thus increase the viscosity after *in vitro* digestion. Our results indicate that extrusion processing following favourable parameters may be a promising technology to enhance fibre functionality of lupin and widen possible uses of that fibre source. Further extrusion processing studies are needed to integrate extrusion technology into the processing of the lupin kernel fibre fraction

obtained as a residue of protein isolation. In addition, the extrusion conditions must be optimised by a higher variation of the extrusion parameters and the influence of extrusion processing on the sensory properties and the application potential of the lupin kernel fibre ingredient has to be considered.

To ensure transferability of our results, extrusion effects on functionality need to be studied and parameters need to be optimised for further fibre sources. In addition to the fibre composition, the maintenance of functionality-defining properties, such as molecular weight, hydration properties and, viscosity must be assessed. Moreover, the improved fibre functionality after extrusion and its participation in cholesterol-reducing and glucose-regulating actions must be confirmed in *in vivo* studies. As a strong impact of extrusion on solubility and hydration properties of the fibres was observed, the influence on fibre fermentability by gut microbiota should be the focus of future studies.

Abbreviations

DM	dry matter
IDF	insoluble dietary fibre
OBC	oil binding capacity
SDF	soluble dietary fibre
SME	specific mechanical energy
TDF	total dietary fibre
WBC	water binding capacity

Declaration of Interest

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Concluding Remarks

The significance and functions of bile acids in physiological processes have been progressively enlightened over the last decades. Bile acids act as detergents facilitating the digestion and absorption of lipids. The synthesis of bile acids takes place in the liver from cholesterol and represents the main pathway for removing excess cholesterol from the body (Lairon, 2001). Recent studies indicate that bile acid pool size and composition contribute to the regulation of gut microbial community structures (Ridlon et al., 2014). Additionally, bile acids support glucose regulation and energy homeostasis, they are involved in several cellular signalling pathways and are ligands for numerous nuclear hormone receptors (Hanafi et al., 2018). The elucidation of these physiological contributions raised scientific attention towards understanding how the diet modulates bile acid metabolism.

Plant-based food components are considered to interact with bile acids during upper gastrointestinal digestion (Singh et al., 2019). By increasing the transfer rates of bile acids from the small intestine into the colon, these interactions may modulate the bile acid pool size and composition affecting metabolic processes involved in health and disease states. A further understanding of the interactions between bile acids and plant compounds is thus needed in order to recognise related changes of bile acid profiles as a measure of physiological homeostasis (Li and Chiang, 2014).

To elucidate mechanisms behind physiological effects of plant compounds on bile acid metabolism, many studies hitherto focused on isolated plant compounds, such as dietary fibres. However, investigations of isolated dietary fibre structures often conflict with results achieved for more complex fibre enriched ingredients or food matrices. This may be caused by interfering activities from other plant compounds and alterations during food processing. Furthermore, due to limitations of *in vivo* investigations and application of *in vitro* methods differing strongly from physiological conditions, only limited conclusions about underlying mechanisms of interaction between bile acids and plant compounds can be concluded from previous literature. Thus, more detailed studies focusing on bile acid interactions with plant compounds are needed to deepen understanding of mechanistic principles and structure-function relationships.

1. *In vitro* analysis of bile acid interactions

The investigation of interactions between bile acids and plant compounds poses a challenge for research. Due to alterations of bile acids in the human colon and deviating bile acid profiles in many animal models (Chiang, 2009), physiological outcomes of *in vivo* studies provide limited conclusions about the underlying mechanisms. Therefore, a variety of *in vitro* studies were conducted in the last decades (Singh et al., 2019). However, many *in vitro* studies lack comparability and transferability of the results to physiological processes. For instance, *in vivo* studies repeatedly indicate a pronounced effect of viscosity and molecular weight of, e.g. , oat beta-glucan on bile acid excretion (Wolever et al., 2010). Otherwise, some *in vitro* studies reported inverse dependencies of increasing bile acid retention capacity for reduced viscosities and molecular weights (Kim and White, 2010, Sayar et al., 2005).

In a comparison of common *in vitro* methodologies (CHAPTER 1), it was found that these discrepancies between *in vitro* and *in vivo* results might be related to an underestimation of viscous effects in certain *in vitro* assays. Our results suggest that by using methods based on centrifugation exclusively adsorptive effects on bile acids could be determined. The comparison of the results also indicate that the adsorptive effects were underestimated by the centrifugation based approach. Nevertheless, the term ´bile acid binding´ is frequently and erroneously used regardless of the underlying mechanisms. This is partly responsible for the fact that *in vitro* analyses do not lead to conclusive conclusions and contradict *in vivo* results. In addition, diffusion kinetics of a bile acid mixture across a dialysis membrane were demonstrated to include both molecular interactions and viscosity effects. Following this method, a standardised *in vitro* digestion protocol is applied, which includes the addition of bile acid mixtures of typical physiological concentrations (Minekus et al., 2014). The *in vitro* digesta are dialysed as a simplified absorption model of the unstirred water layer. First-order diffusion kinetics are analysed and evaluated to differentiate between viscosity-related and permanent molecular interactions. The comparability of our results, obtained for various dietary fibre-enriched food ingredients in CHAPTER 1 and 2, with *in vivo* studies, in particular with ileostomy studies, underlines the advantages of the *in vitro* method used to study interactions with bile acids.

Our findings showed that bile acid interactions should be assessed holistically to account interactive effects like the interaction of soluble and insoluble compounds, the impact of porosity and adsorptive properties. Suitable *in vitro* methods can act as an initial indicator on components and structures responsible for reduced reabsorption of bile acids in the small intestine. By differentiating adsorptive and viscous effects, the *in vitro* dialysis method described in CHAPTER 1 helps to define relevant parameters for prospective future studies

focusing on the modification of bile acid metabolism. To link the properties of plant compounds and physiological outcomes, clinical studies should be supplemented with targeted *in vitro* and/or *ex vivo* analysis, rheological characterisation and structural techniques to elucidate mechanistic principles. To increase understanding about viscosity-related interactions future studies should include *ex vivo* analysis, i.e. Ussing chamber experiments as a closer approximation of physiological bile acid absorption processes (Gunness et al., 2014). To shed further light on molecular interactions with bile acids, combinations of structural studies (e.g. nuclear magnetic resonance, small angle X-ray and neutron scattering) with stoichiometric and calorimetric analyses should be performed. The combination of transdisciplinary approaches will allow mechanism-based prediction of potential in-body modification of bile acid metabolism.

2. Mechanisms of interaction between bile acids and plant compounds

Most studies, which explain the interaction of plant compounds and bile acids, allow a classification of the interactions into two possible mechanisms. Either increased viscosity during digestion results in reduced micellar mobility of bile acids, or bile acids and plant compounds are associated or complexed at the molecular level (Gunness and Gidley, 2010). Viscous as well as molecular interactions between plant digestive products and bile acids are described to differ depending on the bile acid structures (Camire et al., 1993, Huth et al., 2000, Drzikova et al., 2005, Dziedzic et al., 2015). Bile acids mainly abundant in human bile include glyco- and tauroconjugated cholic acids, chenodesoxycholic acids, and desoxycholic acids, which differ in conjugation and hydroxylation. In CHAPTER 2, the viscosity-related and molecular interactions as influenced by fibre sources were investigated focusing on the structural properties of the main bile acids abundant in human bile.

Increased viscosity after gastrointestinal digestion is mainly generated by indigested plant polymers, namely dietary fibre. Using the *in vitro* dialysis model established in CHAPTER 1, bile acid diffusion was revealed to decrease in the order cholic acids > chenodesoxycholic acids > desoxycholic acids (CHAPTER 2). For highly viscous samples, like citrus and apple pectin, the bile acid release rate for desoxycholic acid was reduced by up to 55% compared to cholic acid. In general, diffusion rates decrease with decreasing temperature and increasing viscosity and/or particle radius as defined by the Stokes–Einstein equation (Miller and Walker, 1924). Stating that the temperature and viscosity during digestion are influenced equally for all bile acids, the diffusion rate depends solely on the radius of the diffusing bile acid micelles. This radius is controlled by the critical micelle concentration and aggregation number, which defines the number of monomers within a micelle. These parameters are summarised for the main bile acids abundant in the human bile acid pool in Table 1.

Table 1: Critical micelle concentration (CMC), aggregation number (N_{agg}), and hydrophobicity of main conjugated bile acids abundant in the human bile acid pool.

Bile acid	CMC [mM][1]	N_{agg}[1]	Hydrophobicity[2]
Glycocholic acid	4	9	
Taurocholic acid	3–18	3–7	
Glycochenodesoxycholic acid	1–2	15	
Taurochenodesoxycholic acid	0.9–7	5–26	
Glycodesoxycholic acid	1–2	13–16	
Taurodesoxycholic acid	2–3	12–19	

[1] as summarised by Parker et al. (2014) taken from Madenci and Egelhaaf (2010).
[2] taken from Heuman (1989).

The critical micelle concentration decreases in the order cholic acids > chenodesoxycholic acids > desoxycholic acids, while the aggregation number increases for the opposite order. These findings indicate that micellisation depends on the hydrophobic effect, aiming at minimising the hydrophobic surface, and on the hydrogen binding, which is determined by the number, location and orientation of the hydroxyl groups (Madenci and Egelhaaf, 2010). Consequently, desoxycholic acid forms micelles at lower concentrations and includes more monomers compared to cholic acid, which could explain the significant decrease observed in bile acid diffusion for desoxycholic acid within viscous matrices (CHAPTER 2 & 3). Micellar properties of bile acids further depend on solution parameters, such as pH and ionic strength. Due to their low pK_α conjugated bile acids are almost completely dissociated during digestion. Thus, changes within the physiological pH range do not markedly change the ionisation state and micellisation of bile acids (Madenci and Egelhaaf, 2010). By increasing the ionic strength, the electrostatic repulsion between micelles is reduced, and aggregation is thus favoured and critical micelle concentration decreased. This is especially relevant when comparing results derived for different *in vitro* conditions, emphasising the need of standardised protocols. The influence of micelle formation on the bile acid release demonstrated in CHAPTER 2 illustrates the limitation of many studies using concentrations below the critical micelle concentration (Gunness and Gidley, 2010).

In addition, molecular interactions between primary and secondary bile acids and dietary fibre enriched ingredients from barley, oat, maize and lupin were revealed in CHAPTER 2. These interactions caused a constant sequestration of bile acids, which was independent of the rheological properties of the preparations. For instance, the investigated oat preparation consisted of high amounts of insoluble fibre (92.6 g/100 g dry matter) and low amounts of soluble dietary fibre (1.4 g/100 g dry matter). Consequently, the viscosity measured after *in vitro* digestion did not significantly differ in comparison to a blank sample without fibre. Nevertheless, the oat preparation significantly adsorbed bile acids (up to 2.6 µmol/100 g dry matter). This is in line with the study performed by Zacherl et al. (2011), who investigated the

bile acid binding capacity of heat damaged oat fibre. Although the viscosity of the fibre was almost completely lost due to the thermal treatment, a dose-dependent bile acid binding of up to 26% was observed. The studies conducted in CHAPTER 2 repeatedly revealed a hydrophobicity dependent pattern of molecular interaction, showing increased interaction of desoxycholic acids > chenodesoxycholic acids > cholic acids. These results indicate that hydrophobic interactions are core to the molecular interactions with plant compounds.

In contrast to previous studies that focused on bile acid interactions associated with bile acid species, the bile acid interactions evaluated in CHAPTER 2 were examined in a differentiated manner to distinguish influences caused by adsorptive and viscous properties. By this means, both viscosity-related effects and molecular interactions could be shown to be increased for dihydroxy bile acids (desoxycholic acids > chenodesoxycholic acids) compared to trihydroxy bile acids (cholic acids). Secondary bile acids, especially desoxycholic acid, are associated with a number of disease phenotypes and accumulate in the bile acid pool, if a 'Western diet' low in dietary fibre is consumed (Ridlon et al., 2014). Our results suggest that the extent of the interaction of desoxycholic acid with plant compounds is increased by its hydrophobicity and micellar properties. The elucidation of this increased interaction in comparison to primary bile acids is especially important as the human liver is unable to undertake 7α-hydroxylation of secondary bile acids (Ridlon et al., 2006). However, dialysis represents a simplified model to simulate absorption. Therefore, the *in vitro* data obtained in this thesis need *in vivo* verification to ensure transferability to physiological processes. The difficulty in predicting physiological responses, especially of viscous polysaccharides, get clearer when looking at the multitude of factors influencing physiological viscosity. These include the influence of viscous polysaccharides on gastric emptying, changes of the mucous layer, small intestinal peristaltic, and intestinal transit time (Gidley and Yakubov, 2019). Additionally, the activation and mechanisms of bile acid transporters are not covered by the *in vitro* model applied in this thesis and need to be focused in further studies. Furthermore, the concentrations of dietary fibre enriched ingredients or isolated fractions applied in the *in vitro* model (CHAPTER 1–5) were kept constant and might conflict with applicable concentrations in actual food matrices. Therefore, the influence on the techno-functional properties induced by fibre fortification need to be considered in future studies, which should include real food matrices. By this means the understanding of bioactive properties related to bile acid interactions of fibre enriched foods should be strengthened both from a nutritional and also from a technological point of view.

3. Structure-function relationships of interactions between bile acids and lupin seed fractions

Interactions of bile acids with plant compounds rich in insoluble fibre were found for a number of different feedstocks, including barley, oat, rice, wheat, soybean, lupin and maize as reported in CHAPTER 1 & 2 as well as in previous studies from Kahlon and Woodruff (2003) and Zhang et al. (2011). These interactions were mostly independent of viscosity, and increased in dependance on bile acid hydrophobicity. The findings indicated that hydrophobic interactions of insoluble fibres with bile acids could be related to the fibre structures. This hypothesis was examined in CHAPTER 3 focusing on cell wall polysaccarides of lupin cotyledon and hull. Fibres were isolated by proteolytic enzyme treatments followed by alcohol extraction. Purified fibres were sequentially extracted to separate pectin-like, hemicellulosic, and lignocellulosic structures. Bile acid interactions were investigated *in vitro* applying the model developed in CHAPTER 1. In this study, none of the purified fibre fractions showed a significant molecular interaction with bile acids. Therefore, a major role of lupin cell wall polysaccharides in molecular bile acid interaction was excluded. Based on these results, the frequently reported bile acid binding capacity of dietary fibre polysaccharides should be considered critically in future investigations. In particular, stating 'bile acid binding' properties should be avoided unless a molecular interaction is evident.

As a major role of lupin cell wall polysaccharides in bile acid adsorption was disproven in CHAPTER 3, additional studies were focused on adsorptive capacities of further lupin components to identify relevant structures and mechanisms. High adsorbing capacities were previously reported for lupin proteins and hydrolysates, which partly exceeded the values reported for cholestyramine, an strong ion exchange resin that forms insoluble complexes with bile acids (Yoshie-Stark and Wäsche, 2004). In this study, the free bile acids were separated by centrifugation and measured by a photometric assay. Thus, no details on the molecular mechanisms can be elucidated from the results (Macierzanka et al., 2019). Using the *in vitro* model established in CHAPTER 1 contradictory results were obtained for lupin protein isolates obtained by a pilot scale procedure in CHAPTER 4. A small but significant bile acid adsorption of chenodesoxycholic acids was found for an isoelectrically precipitated lupin protein isolate, but the adsorption was strongly diminished after additional alcoholic purification. These results indicated contributions from substances associated with the protein and fibre fractions such as polyphenols. Considering the alcohol-soluble portion of lupin, our results suggest that the components of the alcohol-soluble fraction are responsible for the bile acid adsorbing capacity observed for lupin seeds. On the basis of high-performance liquid chromatography and diode-array detector spectra analysis, the main polyphenolic structures included in this extract are two flavonoids described in an earlier study by Siger et

al. (2012) corresponding to the apigenin C-glucosides: apigenin-6,8-di-C-β-glucopyranoside and apigenin 7-O-β-apiofuranosyl-6,8-di-C-β-glucopyranoside. These results suggest the formation of hydrophobic interactions between flavonoids and bile acids. In potential agreement with this hypothesis, we observed bile acid adsorption by lignin, which was used as reference material in CHAPTER 3.

The *in vitro* data presented in CHAPTER 3 & 4 provide some basic indicators of the roles of polyphenols in bile acid adsorption. To determine the nature of these interactions more precisely, further fractionation procedures are required to identify the dominating adsorptive structures and underlying mechanisms. Future studies of adsorption patterns are also required on other plant materials. Only recently, Li et al. (2019) described the formation of a new complex composed of proanthocyanidins and bile acids. Interactions were characterised by turbidity, particle size, microstructure, and physicochemical condition analyses, which indicated that the binding occurred through hydrogen bonding and hydrophobic interactions. Furthermore, the stability and digestion properties of bile acid emulsions were analysed, suggesting that the observed complex formation may inhibit lipid digestion and reduce fat absorption. Taking these studies into account, our results indicate a similar mechanism of bile acid interaction for lupin flavonoids, which need analytical validation to ensure transferability. Furthermore, the interference with lipid absorption should be given attention to increase understanding on how interactions between bile acids and polyphenolic structures modify dietary fat digestion and absorption.

4. Bile acid interactions and influences on health

Research to investigate the complex interaction between the synthesis of bile acids in the liver, the function of bile acids as signalling molecules, and the intestinal microbiome is at an early stage (Ridlon et al., 2014). It is therefore not possible to draw direct conclusions on the development of diseases and the maintenance of health based solely on interactions between plant components and bile acids. Nevertheless, these interactions may cause an interference with the enterohepatic circulation of bile acids, which plays a core role in nutrient absorption, metabolic regulation, and homeostasis (Chiang, 2013). Plant compounds show variations in interaction with different bile acid species (as shown in CHAPTER 2). Both viscosity-related and molecular interactions have been described to be increased for dihydroxy bile acids (chenodesoxycholic acid and desoxycholic acid) compared to trihydroxy bile acids (cholic acid). Interactions with plant compounds may thus alter the bile acid composition, resulting in a more hydrophilic bile acid pattern. Interactions between bile acids and plant compounds may further partially prevent reabsorption of bile acids, which results in changes of the bile acid pool size, an excess excretion, and accumulation of bile acids in the colon. These changes

(demonstrated in CHAPTER 2–4) may possibly affect health aspects currently associated with bile acid metabolism.

It is evident that the conversion of cholesterol into bile acids is responsible for the turnover of a major fraction of cholesterol (about 500 mg per day) in humans (Chiang, 2013, Lairon, 2001). Thus, bile acid metabolism is directly linked to blood cholesterol levels (Stamler et al., 2000). Interactions between plant compounds and bile acids may reduce the reabsorption rates of bile acids back into the enterohepatic circulation and cause a depletion of bile acids in the liver (Gunness and Gidley, 2010). Due to the negative feedback regulation of bile acid synthesis, interactions with plant compounds may cause an increase in the conversion of cholesterol to primary bile acids. Accordingly, activations of cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid synthesis, were decribed after diet interventions with plant compounds such as highly viscous apple pectin (Parolini et al., 2013) or tangeretin, a flavonoid derived from citrus peel (Feng et al., 2020). These studies indicate that the viscosity-related or molecular interactions described for these plant compounds and revealed for lupin seeds fractions in CHAPTER 1–4, may contribute to lowering blood cholesterol levels. Plant compounds, especially polyphenols, are further described to change the micellar properties of bile acids. By this means, the micellar solubility of cholesterol and phosphatidylcholin is reduced, and emulsion interface properties are changed (Ogawa et al., 2016, Shishikura et al., 2006). Interaction-induced changes in these properties may modify dietary fat digestion and absorption.

Bile acids exert a variety of activities beyond their classical role as fat emulsifiers. Bile acids were identified as endogenous ligands of farnesoid X receptor (FXR) – a transcriptional regulator of bile acid, glucose, lipid, and energy metabolism. Bile acids are differently potent in activating FXR in the order of chenodesoxycholic acids > lithocholic acids = desoxycholic acids > cholic acids (Shin and Wang, 2019). Compositional changes of the bile acid pool may result in a variation in the activation of FXR and consequently affect its regulating function in the metabolism. FXR regulates gene expressions that are involved in the synthesis, uptake, secretion, and intestinal absorption of bile acids, which is reflected in the total bile acid concentrations in the gall bladder (Di Ciaula et al., 2017). This feedback mechanism depending on bile acid concentrations is important in preventing a potentially harmful expansion of the bile acid pool (Holt et al., 2003). FXR also plays an important role in lipid and glucose homeostasis, as recently discussed in detail by Shin and Wang (2019).

Bile acids research further revealed that bile acids activate the G protein-coupled receptor TGR5, also showing a potency dependence on bile acid hydrophobicity. Amongst others, TGR5 causes the secretion of the gut hormone glucagon-like peptide-1 (GLP-1) (Lefebvre et al.,

2009). GLP-1 induces the stimulation of insulin secretion and the retardation of gastric emptying, thus contributing to the inhibition of appetite (Kuipers and Groen, 2014). Interestingly, increased faecal bile acid concentrations by capsulated ileo-colonic delivery of conjugated bile acids were recently shown to increase GLP-1 and improve glucose homeostasis. Thereby, the authors contributed to understanding the effects of bile acids on the human pathophysiology of obesity and diabetes (Calderon et al., 2020).

Recently emerging research aims to clarify the complex interactions between the liver, the bile acids, and the gut microbiome. For instance, the size and composition of the bile acid pool is proposed to add to the regulation of microbial community structures in the gut (Ridlon et al., 2014). Perturbations in the equilibrium between the diet, the gut microbiome, and the bile acid pool size and composition can result in disease states (Ridlon et al., 2014). In particular, high concentrations of secondary bile acids, resulting from microbial transformation of primary bile acids, are reported to promote carcinogenesis in the colon (Nguyen et al., 2018, O'Keefe, 2016). Changes in the bile acid pool are linked to cardiac dysfunctions, liver diseases, biliary stones development, and diabetes. Inflammation, apoptosis, and cell death may be caused by cytotoxicity induced by microbial changes of bile acid structures (Chiang, 2013, Hanafi et al., 2018, Vasavan et al., 2018, Dosch et al., 2019). Hydrophobicity is an important determinant of the cytotoxicity of bile acids (Zeng et al., 2019). As plant compounds show increased interaction for hydrophobic bile acids (CHAPTER 2–4), bile acid interactions, e.g., by adsorption, may change the availability of cytotoxic bile acids in the colon (Funk et al., 2008).

5. Effects of extrusion processing on the interactions between bile acids and lupin compounds

Only minor importance has so far been attached to the impact of processing on the bile acid interactions of plant compounds. Using lupin kernel fibre as an example, influences of thermo-mechanical extrusion processing on the physiochemical and functional properties were investigated in CHAPTER 5. Extrusion processing caused a significant change of insoluble to soluble dietary fibre, while only small changes in total fibre content were detected. These results are in line with the findings of several studies focusing on alteration of the dietary fibre composition by extrusion of different fibre sources (Ul Ain et al., 2019). We suggest that the processing of lupin kernel fibre caused solubilisation of hydrophilic pectin-like polymers, which exhibited high hydration properties and thus increased the viscosity after *in vitro* digestion. Due to the changes in rheological properties induced by extrusion processing, retention of bile acid micelles was increased *in vitro*. Molecular bile acid interactions were not

affected, which is in line with the high thermostability of flavonoids hypothesised to be the lupin compound responsible for bile acid adsorption.

Our results indicate that extrusion processing following favourable parameters may be a promising technology to enhance fibre functionality of lupin and widen possible uses of that fibre source. However, further extrusion processing studies are needed to integrate extrusion technology into the processing of the lupin kernel fibre fraction obtained as a residue of protein isolation. In addition, the influence of extrusion processing on the sensory properties and the application potential of the lupin kernel fibre ingredient has to be considered.

To ensure transferability of our results, extrusion effects on functionality need to be studied and parameters need to be optimised for further fibre sources. Most studies applying extrusion processing to improve fibre functionality focused purely on the functional categorisation of dietary fibres based on solubility (Gidley and Yakubov, 2019). The results of CHAPTER 5 corroborate that the maintenance of relevant properties, such as molecular weight, hydration properties, and viscosity must be considered in order to understand influences on the physiological functionality of fibre. Moreover, the improved fibre functionality after extrusion and its participation in cholesterol-reducing and glucose-regulating actions must be confirmed in the frame of *in vivo* studies.

As a strong impact of extrusion on solubility and hydration properties of the fibres was observed in CHAPTER 5, the influence on fibre fermentability by gut microbiota should be the focus of future studies. Dietary fibre and polyphenol intake as well as changes in bile acid profiles are directly related to microbial shifts and the activity of the gut microbiota (Ghaffarzadegan et al., 2019, Ghaffarzadegan et al., 2018). Only a few members of the family *Coriobacteriaceae*, *Clostridiaceae*, *Lachnospiraceae*, or *Ruminococcaceae* are known to produce secondary bile acids (Devlin and Fischbach, 2015). The formation of bioactive metabolites, such as short chain fatty acids and phenolic acids, is associated with compositional changes of the gut microbiota influencing the formation of secondary bile acids (den Besten et al., 2013). The removal of secondary bile acids from the enterohepatic circulation by bile acid interactions and the suppression of the transformation of primary to secondary bile acids in the colon could therefore have a synergistic effect on the bile acid metabolism and associated health and disease states. Future studies thus need to focus on the interplay between dietary fibre, bile acids and the microbiome to elucidate the cascade of events related to the health promoting effects of plant compounds.

6. Final Conclusions

A fundamental understanding of the mechanisms of the interaction between primary and secondary bile acids and plant compounds is needed to improve the overall understanding of how diet modulates bile acid metabolism. Two main mechanisms of interaction can be concluded from the current state of research. Bile acids and plant compounds are either associated or complexed at a molecular level or increased viscosity reduces micellar mobility of bile acids. For both interaction mechanisms, an increased affinity towards hydrophobic bile acids was revealed in this thesis. On the one hand, the constant pattern observed for molecular interactions indicates a common underlying mechanism based on hydrophobic interactions. On the other hand, dependency of bile acid retention on bile acid hydrophobicity in viscous matrices may be linked to the micellar properties of bile acids.

Due to the similar influence of viscosity-related and molecular interactions on the reduction of bile acid reabsorption, the differentiation of these effects in *in vivo* studies is impaired. To close the gap between the interaction mechanisms focused in this thesis and the observed physiological outcomes, collaborative research activities through transdisciplinary approaches are required. *In vitro* approaches mimicking the physiological environment in the small intestine as well as structural techniques offer potential to elucidate the mechanistic principals of interactions in more detail. To verify if *in vitro* results accurately reflect complex *in vivo* scenarios, targeted *in vivo* studies should be conducted based on, and accompanied by, *in vitro* assessments. Future research further needs to clarify the complex interplay between the interaction of plant compounds and bile acids, the microbial changes of bile acids, the fermentation of indigestible plant compounds, and the consequences on the gut microbiome–bile acid axis.

There is growing evidence that phytochemicals, especially polyphenols, may contribute to bile acid sequestering effects of plant-based ingredients and foods. Polyphenols are known to be associated with plant proteins and dietary fibres. Further research thus needs to address combined mechanisms of interactions between bile acids and these plant compounds incorporated in intact food matrices, especially focusing on the influence of digestion on the stability and bioaccessibility of polyphenols. Furthermore, consequences resulting from mechanical, thermal, and chemical treatments need to be considered to enable the development of strategies for improved plant food processing.

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