Lipases and phenolic aggregates

Characterization, inhibition and interaction

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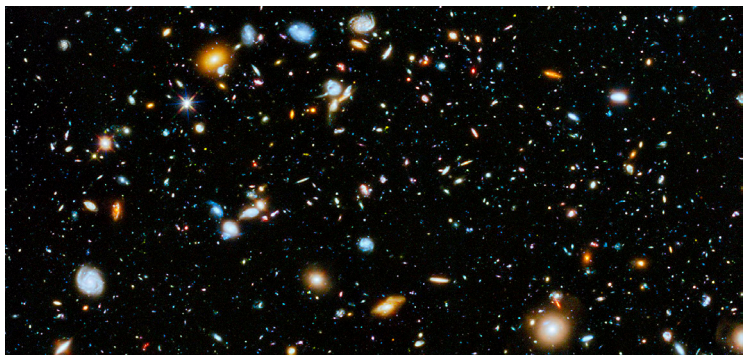

Hubble Ultra Deep Field. Photo taken by NASA and the Space Telescope Science Institute (STScI)

Many of the people who have influenced my life have been marveled with the vastness of the universe, with all the mysteries of celestial bodies, with the visible but impalpable. Contradictorily, their influence has birthed in me a curiosity for another type of universe, a miniature one, invisible but no less fascinating.

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MADE IN SWEDEN

In loving memory of Jorge Bustos

Popular science summary

Did you know that more people die globally from being obese than for being underweight? Although in the past obesity was a first world country problem, nowadays it has become a global problem. In order to address this situation, several approaches have been considered, ranging from natural treatments to sophisticated surgery. Although there are commercial drugs on the market, not everyone can choose this solution. For example, some people with a low income cannot afford the treatments or some people opt for a healthy diet instead. This example has become a popular trend nowadays and several studies have been performed in order to relate food products with obesity. For instance, it has been shown that a diet rich in phenolic compounds can prevent obesity, potentially because of its capability to prevent fat absorption through the inhibition of lipase, the main enzyme responsible for fat digestion.

The inhibition of lipase is a well-known treatment for obesity. Lipases produced by the human body, such as pancreatic lipase, have been the most studied with regards to obesity, while lipases from commensal organisms such as probiotics still remains unknown even though there are studies that relate obesity with probiotics. In the present work, pancreatic lipase and two new potential lipases from probiotic bacteria were studied. For these last two, a broad characterization was performed. The results indicate that both present lipase activity and, as they are active under physiological conditions, they could play a role in fat digestion as pancreatic lipase does.

Previous studies indicate that phenolic compounds interact with lipase at molecular level, but due to their low solubility it is believed that these compounds can form aggregates and present a different kind of interaction with lipase.

The results of this study clearly show that some phenolic compounds do indeed form aggregates in aqueous medium and that these aggregates can influence the reproducibility of conventional experiments. The results also provide an extended view of how phenolic compounds interact with lipase under intestinal conditions. These findings suggest that lipase inhibition by these compounds can happen at both molecular and aggregated levels.

The present work is relevant because phenolic compounds are highly present in drinkable products, such as fruit juices, wines and teas, at concentrations at which

they can form aggregates. Thus, a better comprehension of phenolic compounds and their interaction with lipase could help to understand how diets rich in phenolic compounds could prevent and/or control obesity, for example, the consumption of green tea which is widely known as a fat loss tea.

Abstract

In recent decades, obesity has gone from being a problem of first world countries to a global one. It is considered one of the main risk factors for many serious medical conditions. In order to treat and/or prevent obesity different strategies have been considered, one of the most common being the inhibition of lipase, the enzyme that is mainly responsible for lipid digestion. Even though there is a commercial drug that can inhibit lipase (orlistat), the most accessible treatment for obesity is based on the intake of a healthy diet. For example, some studies show that a diet rich in phenolic compounds is related to a low obesity index and that this can be related to their potential to inhibit lipase.

The obesity studies based on lipase inhibition make use of endogenous lipases, such as gastric and pancreatic lipase, but there have been no studies of exogenous lipases, such as from probiotics, even though there are studies that reveal that probiotics could have an effect on obesity. Thus, in the present thesis, two potential lipases from *Biffidobacterium longum* NCC 2705 and *Lactobacillus rhamnosus* GG were investigated. Both were produced and characterized. The results indicate that both are active under physiological conditions in their monomeric forms. Also, they were able to hydrolyze long-chain acyl groups (>C10), hence they present lipase activity.

The inhibition of lipase by phenolic compounds has been widely investigated. The results indicate that the inhibition takes place at molecular levels when the phenolic compounds are considered soluble. From the literature it is known that some phenolic compounds have low solubility in aqueous systems and they can form aggregates in solution. Thus, understanding the role that these phenolic aggregates play in lipase inhibition could help to comprehend their relevance in lipid digestion.

For the aforementioned, the interaction between pancreatic lipase and phenolic aggregates was also investigated in the present thesis. The results suggest that, among the studied phenolic compounds, flavonoids are more prone to form aggregates in aqueous medium and that the presence of these aggregates in a solution could affect the reproducibility of lipase assays. Three flavonoids were chosen in order to study in detail their interaction with lipase (myricetin, quercetin and EGCG). All of them were able to form aggregates in water and in the presence

of lipase under simulated intestinal conditions. These aggregates have shown to interact with lipase by a sequestering mechanism under the intestinal conditions that were tested, in which it was found that lipase was in its monomeric form. The results of this thesis suggest that lipase inhibition by phenolic compounds can occur both by molecular mechanism, such as non-competitive inhibition, and by a sequestering mechanism when phenolic aggregates are formed.

The phenolic concentrations used in this thesis are common values found in drinkable products, therefore, the results from this thesis suggest that the inhibition by aggregates take place in real conditions.

Resumen

En las últimas décadas, la obesidad ha pasado de ser un problema de los países del primer mundo a ser un problema global. Es considera uno de los principales factores de riesgo para muchas condiciones médicas. Para tratar y / o prevenir la obesidad se han considerado diferentes estrategias, siendo una de las más comunes la inhibición de la lipasa, la principal enzima responsable de la digestión de lípidos. Aunque existe un medicamento en el mercado que puede inhibir lipasa (orlistat), el tratamiento más accesible contra la obesidad es la ingesta de una dieta saludable. Por ejemplo, algunos estudios muestran que una dieta rica en compuestos fenólicos está relacionada con un bajo índice de obesidad y que esto puede estar relacionado con su potencial para inhibir la lipasa.

Los estudios de obesidad basados en la inhibición de lipasa hacen uso de lipasas endógenas, como la lipasa gástrica y pancreática, pero estudios en base a lipasas exógenas, como las provenientes de prebióticos, son aún desconocidos, incluso aunque existen estudios que revelan que los probióticos podrían tener un efecto en la obesidad. Por lo tanto, en la presente tesis, se investigaron dos posibles lipasas provenientes de *Biffidobacterium longum* NCC 2705 y *Lactobacillus rhamnosus* GG. Ambas fueron producidas y caracterizadas. Los resultados indican que ambas son activas bajo condiciones fisiológicas en sus formas monoméricas. Y que son capaces de hidrolizar grupos acilo de cadena larga (> C10), por lo tanto, se puede decir que presentan actividad lipasa.

La inhibición de lipasa por compuestos fenólicos ha sido ampliamente investigada. Los resultados indican que la inhibición ocurre a niveles moleculares cuando los compuestos fenólicos son solubles. Por revisión bibliográfica se sabe que algunos compuestos fenólicos tienen baja solubilidad en sistemas acuosos y pueden formar agregados en solución. Por lo tanto, comprender el papel que juegan estos agregados fenólicos en la inhibición de la lipasa podría ayudar a comprender su relevancia en la digestión de lípidos.

Por lo mencionado anteriormente, la interacción entre lipasa pancreática y agregados fenólicos fue también investigada en la presente tesis. Los resultados sugieren que, entre los compuestos fenólicos estudiados, los flavonoides son más propensos a formar agregados en medio acuoso y que la presencia de estos agregados en solución puede afectar la reproducibilidad de ensayos enzimáticos.

Tres flavonoides fueron seleccionados para estudiar en detalle su interacción con lipasa (miricetina, quercetina y EGCG). Los tres fueron capaces de formar agregados en agua y en presencia de lipasa en condiciones intestinales. Estos agregados interactúan con lipasa mediante un mecanismo de secuestro en las condiciones intestinales probadas, en las cuales se observó que la lipasa pancreática se encuentra en su forma monomérica. Los resultados de esta tesis sugieren que la inhibición de lipasa por compuestos fenólicos puede ocurrir por un mecanismo molecular, como es el caso de la inhibición no-competitiva, y también puede darse por un mecanismo de secuestro en la presencia de agregados fenólicos.

Las concentraciones fenólicas utilizadas en esta tesis son típicos valores encontrados en bebidas comunes, por lo tanto, los resultados de esta tesis sugieren que la inhibición por agregados fenólicos ocurre en condiciones reales.

List of papers

Paper I. Interaction Between Phenolic Compounds and Lipase: The Influence of Solubility and Presence of Particles in the IC_{50} Value.

Bustos, A-S., Håkansson, A., Linares-Pastén, J. A., Peñarrieta, J. M., & Nilsson, L.

Journal of Food Science, 2018, 83(8), 2071-2076.

Paper II. Interaction Between Myricetin Aggregates and Lipase Under Simplified Intestinal Conditions.

Bustos, A-S., Linares-Pastén, J. A., Håkansson, A., & Nilsson, L.

Submitted to PLOS ONE

Paper III. Interaction of Quercetin and Epigallocatechin Gallate (EGCG) Aggregates with Pancreatic Lipase Under Simplified Intestinal Conditions

Bustos, A-S., Håkansson, A., Linares-Pastén, J. A., Peñarrieta, J. M., & Nilsson, L.

Submitted to PLOS ONE

Paper IV. Lipase / Acyl-Hydrolase from Human Probiotic Bacteria: *Biffidobacterium Longum* NCC 2705 and *Lactobacillus Rhamnosus* GG (ATCC 53103)

Manasian, P., Bustos, A-S., Pålsson, B., Håkansson, A., Peñarrieta, J. M., Nilsson, L., & Linares-Pastén, J. A.

Manuscript

The Author's Contributions

Paper I. The author was in charge of designing the study, performing the experiments and the corresponding analysis, evaluating the results and was responsible for writing the manuscript.

Paper II. The author planned the study, carried out the experiments and the corresponding analysis (with the exception of the molecular dynamics), evaluated the results and was in charge of writing the manuscript.

Paper III. The author planned the study, performed the experiments and the corresponding analysis, evaluated the results and was in charge of writing the manuscript.

Paper IV. The author designed, performed and analyzed the AF4 experiments, helped with the design of the enzymatic experiments and helped writing the manuscript.

Abbreviations

EGCG	(−)-epigallocatechin-3-gallate
AF4	Asymmetrical flow field flow fractionation
r_h	hydrodynamic radius
MALS	multiangle light scattering
UV	ultraviolet
dRI	differential refractive index
*Bl*Lip	putative enzymes from *Biffidobacterium longum*
*Lr*Lip	putative enzymes from *Lactobacillus rhamnosus*
PRP	proline-rich protein

Table of Contents

1 Introduction

Let's begin…

La inspiración existe, pero tiene que encontrarte trabajando (Pablo Picasso)

Nowadays obesity has become one of the major causes of several chronic diseases, such as cardiovascular diseases, diabetes, and cancer (Lunagariya, Patel, Jagtap, & Bhutani, 2014; WHO, 2018).

In 2016, around 13% of the global adult population were obese, 15% females and 11 % males. Since 1975 and up to 2016, the global incidence of obesity has approximately tripled. Currently, overweight and obesity are not only considered a first world country problem. They have expanded into middle and low-income countries, especially in urban settings. Between 2000 and 2016, the number of overweight children under the age of five has risen by 50% in Africa. Sadly, overweight and obesity are related to more deaths than underweight. Globally, there are more obese people than underweight people (WHO, 2018).

The prevention and treatment of obesity has been highly studied, one of the most common approaches being the inhibition of gastrointestinal lipases, enzymes responsible for the digestion of ingested fat. With this aim, numerous studies have been performed, highlighting that a diet rich in phenolic compounds can be inversely related to obesity (Meydani & Hasan, 2010; Rodriguez-Perez, Segura-Carretero, & Del Mar Contreras, 2019; Vernarelli & Lambert, 2017).

Different ingested products rich in phenolic compounds, such as fruit juices, wines and teas as well as their isolated phenolic compounds have been studied as potential lipase inhibitors (Griffiths, 1986; Nakai et al., 2005; Shimura et al., 1994). The common methods applied to these studies are enzymatic assays, in which reaction products can be quantified using spectrophotometry or fluorescence assays. In these studies, the phenolic compounds are regarded as soluble compounds, assuming that their interaction with lipase takes place at a molecular level. Under the same assumption, several molecular dockings were performed showing the molecular interaction between lipase and specific phenolic compounds (Li et al., 2016; Martinez-Gonzalez et al., 2017; C. Zhang et al., 2018).

However, phenolic compounds have shown poor solubility in aqueous systems (Mota, Queimada, Pinho, & Macedo, 2008), suggesting that they are prone to forming aggregates in conventional enzymatic assays. These findings open the possibility that phenolic compounds can interact with lipase at colloidal levels, besides the molecular interactions that have been reported. Phenolic aggregates have never been studied in the presence of lipase and their comprehension could help to understand the complete mechanism of lipase inhibition and thereby their relevance to obesity.

The inhibition of lipase as a treatment for obesity is based on endogenous lipases, such as pancreatic lipase. However, there have been no studies of exogenous lipases, for instances from the gastrointestinal microbiota including probiotics. Some recent reviews have shown that probiotics could have an effect on obesity. However, despite the abundance of data, there are not conclusive statements and the use of probiotics for treating and/or preventing obesity remains a subject of debate (Brusaferro et al., 2018; Kobyliak et al., 2016). Thus, the production and characterization of these enzymes are important and could form the basis of future obesity studies.

2 Objectives

From the discussion in chapter 1, it is believed that the study of lipases that could be involved in lipid digestion and their interaction with phenolic aggregates is important for understanding their role in obesity treatments. Thus, the objectives of this thesis are as follows.

2.1 General Objective

To study lipases that could be related to lipid digestion and their interaction with phenolic aggregates.

2.2 Specific Objectives

I. To characterize new enzymes from probiotic bacteria that could be involved in lipid digestion.

II. To identify phenolic compounds that could form aggregates in aqueous medium and their effect on lipase inhibition.

III. To study the interaction of the aggregates of myricetin, quercetin and EGCG with pancreatic lipase under simplified intestinal conditions.

3 Background

3.1 Lipases

3.1.1 What are lipases?

Lipases (EC 3.1.1.1, triacylglycerol hydrolases) are lipolytic enzymes that hydrolyze relatively long-chain acyl groups (≥10 carbon atoms) to acylglycerols and fatty acids. Although there are some exceptions, lipases differ from esterases (another lipolytic enzymes) because their activation follows a phenomenon called interfacial activation, which is characteristic of lipases only. This phenomenon occurs due to the active site of the enzyme being covered by a hydrophobic domain (flap or lid) that moves apart in the presence of an interface, for example, hydrophobic organic solvents or triglycerides (K-E. Jaeger, Dijkstra, & Reetz, 1999; Ramnath, Sithole, & Govinden, 2016).

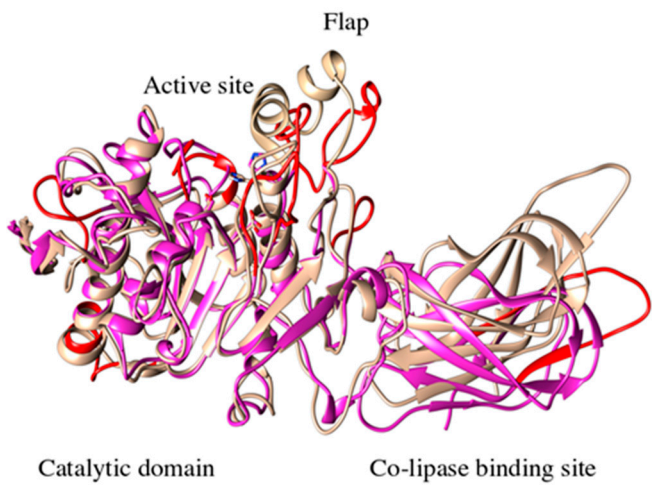

Figure 1. Molecular dynamic simulations of pancreatic lipase based on its crystal structure

3.1.2 Sources and Physiologic Role

Lipases can be found in various living organisms, such as animals, plants and microorganisms, the latter being one the most common source for industry due to its abundance and relatively easy production. Lipases from microorganism are accepted in the food industry, which is not the case for animal lipases because they are not pure enough to be used. Readily available commercial animal lipases are mainly extracted from the pancreas of sheep, cattle and pigs; theses lipases can contain traces of trypsin, hormones and viruses that can modify the flavor and properties when used in the food industry (Casas-Godoy et al., 2018).

Lipases are considered to be ubiquitous enzymes that have several physiological roles. In vertebrates, including humans, lipases are found in the digestive system, in which they control the digestion, transport, absorption and reconstitution of lipids and participate in lipoprotein metabolism (Casas-Godoy et al., 2018; Derewenda, 1994). In the vegetable kingdom, lipases are mainly found in seeds; contributing to the energy reserve tissue and hydrolyzing the triglycerides needed for post germination growth. Its production in plants is induced upon wounding Thus, it is believed that they also play a protective role (Casas-Godoy et al., 2018). In microorganisms, extracellular lipases can be produced; this facilitates the lipids assimilation through the hydrolysis of triglycerides present in the media. The extracellular lipase expression is governed by environmental factors, for example, a carbon source composed of lipids can induce the production of extracellular lipases, helping the microorganism to grow in hostile environments (Casas-Godoy et al., 2018; Fürstenberg-Hägg, Zagrobelny, & Bak, 2013). Due to microorganisms work like host for recombinant proteins, nowadays lipases are also produced as recombinant proteins. The production of recombinant proteins has several advantages over the traditional methods, including reproducibility, high yields and specific protein production (Borrelli & Trono, 2015).

3.1.3 Applications

Lipases are widely used in the industry in different applications because their broad range of substrates, their high selectivity, their capacity to work as catalyst without costly cofactors and because they are stable in organic solvents. In addition, they can also be activated in mild conditions and be easily produced (Bassegoda, Cesarini, & Diaz, 2012; Casas-Godoy et al., 2018).

3.1.3.1 Food industry

In the food industry, lipases are used for the manufacturing of dairy goods such as cheese (Jooyandeh, Amarjeet, & Minhas, 2009). They are also used for the modification of oils and fats (e.g. production of margarine, butter and special

cooking oils) and in the production of baby foods and modified lipids with specific characteristics (e.g., human milk replacement, cocoa butter equivalent, fats with low or high calories, enriched oils) (Guerrand, 2017). Lipases are also applied to produce fragrance compounds and to modify flavors (Casas-Godoy et al., 2018). On the other hand, they also give rise to emulsifiers that enhance flour products such as pasta and bakery as well as in animal feed as additives (Casas-Godoy et al., 2018; Guerrand, 2017).

3.1.3.2 Cleaning agents

Because some lipases are stable at basic pH and high temperatures; they are used as additives. They are normally found in the manufacturing of soap, dry cleaning solvents, dishwashing products, and contact lens cleaning solutions (Hasan, Shah, & Hameed, 2006).

3.1.3.3 Chemicals industry

For the perfume industry, they are employed as scents and to produce surfactants and emollient in cosmetic products (Yvergnaux, 2017). The pharmaceutical industry uses lipases to synthesize pure enantiomers from racemic mixtures (e.g., prostaglandins, hydantoins, anti-inflammatory drugs, and penicillins) (Karl-Erich Jaeger & Eggert, 2002). Their specificity of chiral molecules can also be applied on the agrochemical industry in the use of herbicides (Casas-Godoy et al., 2018).

3.1.3.4 Environmental processes and energy industry

They are used to treat the oil in residual waters and for the degradation of organic debris; for production of biodiesel and bio kerosene as well as for the production of additives incorporated in biodiesel that reduce the viscosity (Hasan et al., 2006; Karl-Erich Jaeger & Eggert, 2002).

3.1.3.5 Medical applications

The level of lipase in the human body can reflect an infection or disease. It is related with high cholesterol levels, with pancreatic disorders and obesity. It is also considered as a drug for digestive illnesses (Casas-Godoy et al., 2018; Hasan et al., 2006).

3.1.4 Lipase and Obesity

Obesity is described as an excessive and unnecessary fat accumulation that can harm health. Its main cause is the energy imbalance between consumed and expended calories.

Different strategies have been developed to treat obesity. The drug industry normally focus on two scenarios: the regulation of food intake and the absorption of dietary fat (Lunagariya et al., 2014). This last scenario is related with pancreatic lipase inhibition, the main enzyme of lipids digestion.

Pancreatic lipase is secreted by the pancreas in the pancreatic juice, that is responsible of triglycerides digestion in the small intestine. Although lingual and gastric lipases partially hydrolyze the dietary triacylglycerols, pancreatic lipase is in charge of the hydrolysis of 50 to 70% of dietary fats (Kim et al., 2016). The hydrolysis products, together with bile salts, form mixed micelles that can be absorbed by enterocytes (Lowe, 2002).

Although endogenous lipases such as pancreatic lipase were highly studied in terms of fat assimilation, ergo obesity, there is a lack of information about exogenous lipases from commensal organisms, such as probiotics. The relationship between probiotics and body weight seems to be strain specific, for instance *Bifidobacterium* and *Lactobacillus* genera are effective, whereas other genera such as *Escherichia coli* can be harmful (Brusaferro et al., 2018). Still, the dosage, long-term effects and detailed mechanism of action are unknown.

For the aforementioned, in the present thesis potential exogenous lipases coming from two well-known probiotic strains were studied: *Bifidobacterium longum* NCC 2705 and *Lactobacillus rhamnosus* GG

3.1.4.1 Bifidobacterium longum NCC 2705

Bifidobacterium longum is an anaerobic bacterium usually found in the intestines of most animals, including humans and insects (Quigley, 2017). Since its discovery, it has been associated with a healthy gastrointestinal tract due to it provides a substantial health benefit to breast fed infants. Nowadays it is one of the most important members of the probiotic bacteria because of its relation with different health benefits; including cholesterol reduction, immune stimulation, diarrhea prevention, mitigation of lactose intolerance symptoms, and cancer prevention (UniProt, 2018).

3.1.4.2 Lactobacillus rhamnosus GG

L. *rhamnosus* GG has been highly studied as a probiotic strain because of its controlled and rapid growth characteristics, its resistance to bile and acid and its adhesion capacity in the gut (Doron, Snydman, & Gorbach, 2005). It is an anaerobic bacterium that reside in the gastrointestinal track, tonsils, oral cavity and vagina (Segers & Lebeer, 2014). It is particularly used for female-related infections (Westerik, Kort, Sybesma, & Reid, 2018). moreover, L. *rhamnosus* GG is widely used in dairy products like cheese, fermented and unpasteurized milk and yoghurt

(Franz et al., 2014; Lazzi et al., 2014), as well as in other kind of drinkable products such as soups and fruit juices (Saxelin, 2008).

It is possible that the production of potential lipases, by these two strains, can play a role in lipid digestion, and this can be related with their effects on obesity, therefore a better understanding of this enzymes is necessary.

3.1.5 Lipase inhibitors

There is only one drug approved by FDA, orlistat, that acts under the pancreatic lipase inhibition mechanism (Guerciolini, 1997). Nevertheless, several natural products have been tested against pancreatic lipase, such as plant extracts, teas, dietary fibers, microbial products, etc. Moreover, isolated molecules from plants and microorganism were extensively studied, resulting in good pancreatic lipase inhibitors (Birari & Bhutani, 2007; Buchholz & Melzig, 2015). Among the tested natural molecules, phenolic compounds are the most studied class of pancreatic lipase inhibitor. Numerous teas and fruits have been widely studied for this purpose. Some examples that gave positive results of pancreatic lipase inhibition are listed below, taken from a review made by Lunagariya et al., 2014.

3.1.5.1 Polyphenols

Galantin, a flavonol from Alpinia galangal (plant belonging to the ginger family); neohesperidin and hesperidin, isolated from Citrus unshiu (tangerine); 3-O-caffeoyl-4-O-galloyl-L-threonic acid, extracted from Filipendula kamtschatica; methyl chlorogenate, isolated from the leaves of Eremochloa ophiuroides; licochalcone A; proanthocyanidin and a flavan (2S)-3',4',7-trihydroxyflavan-(4α→8)-catechin from fruits of Cassia mimosoides; 7-phloroeckol from Eisenia bicyclis (brown algae); 3,3',4,4'-tetrahydroxy-2-methoxychalcone and isoliquiritigenin from Glycyrrhiza glabra roots; caffeic acid, ferrulic acid and luteonil present in different edible products like fruits, peanuts and teas; cassiamin A from Cassia siamea roots; (-)-epigallocatechin-3-O-gallate (EGCG), (-)-epigallocatechin-3,5-digallate, oolonghomobisflavan A and B and oolongtheanin 3'-*O*-gallate mainly found in teas such as oolong tea; extra active polyphenols recognized from oolong tea are assamicain A, prodelphinidin B-2,3,3'-di-*O*-gallate, oo-longtheanin-3'-*O*-gallate, theasinensin D, theaflavin, and theaflavin-3,3'-*O*-gallate.

On the other hand, some oligomeric structures have also been studied. Procyanidins present in apples can also inhibit pancreatic lipase according to the polymerization degree (from dimmer to nonamer) (Sugiyama et al., 2007). Catechins and condensed tannins have also shown lipase inhibition (Yoshikawa, Shimoda, Nishida, Takada, & Matsuda, 2002).

3.1.6 Lipase Inhibition, mechanism

3.1.6.1 Molecular inhibition

It is well known that enzymes in general can experience different kinds of molecular inhibition. The inhibition can be divided in two groups, reversible and irreversible.

Reversible inhibitors interact with the enzymes at non-covalent level, through ionic bonds, hydrogen bonds and hydrophobic interactions. These inhibitors can be removed by dilution or dialysis because there are no chemical reactions when the enzyme binds to the inhibitor. There are three kinds of reversible inhibitors: *Competitive inhibition*, where the inhibitor and substrate have affinity for the active site of the enzyme resulting in a competition that depends on the substrate concentration. *Uncompetitive inhibition*, where the inhibitor interacts only with the substrate-enzyme complex. *Non-competitive inhibition*, where the inhibitor does not bind to the active site of the enzyme, but instead to a separate site reducing the enzyme activity. On the other hand, irreversible inhibitors usually bind the enzyme covalently through functional groups such as aldehydes, alkenes, phenyl sulfonates, or fluorophosphonates among others. The amino acid chains from the enzyme react with these electrophilic groups inhibiting the enzyme permanently. Irreversible inhibition is usually specific and do not incapacitate all proteins.

For lipase, different types of inhibition have been reported.

Orlistat, the only commercial drug that acts on pancreatic lipase as an inhibitor, follows an irreversible mechanism, in which the serine residue of the active site binds covalently to orlistat avoiding the formation of products (Borgström, 1988).

Inhibitors from natural products normally show competitive and non-competitive mechanisms, as is the case of the seven flavonoids extracted from Glycyrrhiza glabra roots that bind to the active site of pancreatic lipase non-covalently (Birari, Gupta, Mohan, & Bhutani, 2011), as well as carnosic acid that was found to be a competitive concentration-dependent inhibitor (Ninomiya et al., 2004). On the other hand, a study has shown that acteoside forms a non-covalent bond with lipase that modify its molecular conformation, which reduce their enzyme activity (Wu et al., 2014). Another study shows that the inhibition of pancreatic lipase by licochalcone A follows a reversible non-competitive mode (Won et al., 2007). Catechins, a big group of lipase inhibitors have also shown a non-competitive inhibition, such is the case of (−)-epigallocatechin-3-gallate (EGCG) (Glisan, Grove, Yennawar, & Lambert, 2017). For other polyphenols, such as quercetin, both, competitive and non-competitive, mechanism have been reported (Martinez-Gonzalez et al., 2017).

3.1.6.2 Inhibition by aggregates

For promiscuous inhibitors, molecules that can inhibit several unrelated enzymes, a different mechanism of inhibition has been proposed. This mechanism suggests that these promiscuous compounds form aggregates in solution, resulting in the sequestering of the enzymes that interact with them. Studies made by electron microscopy indicated that the enzymes interact on the aggregates' surface (McGovern, Helfand, Feng, & Shoichet, 2003).

However, as far as the author knows, this inhibition of lipase by aggregates has not been studied before.

3.2 Phenolic compounds as potential inhibitors

3.2.1 What are phenolic compounds?

Phenolic compounds are an extensive class of secondary metabolites present in the plant kingdom. Chemically speaking, phenolic compounds own an aromatic ring with one hydroxyl group as minimum.

These secondary metabolites are commonly involved in protection against aggression by pathogens and/or UV radiation (Belščak-Cvitanović, Durgo, Huđek, Bačun-Družina, & Komes, 2018). Besides their protective mechanism, some studies have shown that they contribute to plant development (Noel, Austin, & Bomati, 2005) and that they work as signaling agents between plants and some organisms (Vincenzo Lattanzion, 2008).

Phenolic compounds are highly related with antioxidant properties, nevertheless, other properties like antiviral, antibacterial, antidiabetic and anti-inflammatory were also reported. Their consumption in our diet has been also associated with the prevention of several diseases such as obesity, cardiovascular diseases and cancer (Vernarelli & Lambert, 2017; Wang, Li, & Bi, 2018)

3.2.2 Chemical characterization

Phenolic compounds can be classified in different ways, based on source of origin, natural distribution, biological function and chemical structure. This last one can depend on the carbon chain or on the number of rings that the structure has and to the structural elements involved in the binding of these rings among each other. This last classification can slightly vary between authors, therefore in this thesis it will be only mentioned the one reported by Belščak-Cvitanović et al., 2018, that is one of the most common classification of phenolic compounds based in their chemical structure, see figure 2.

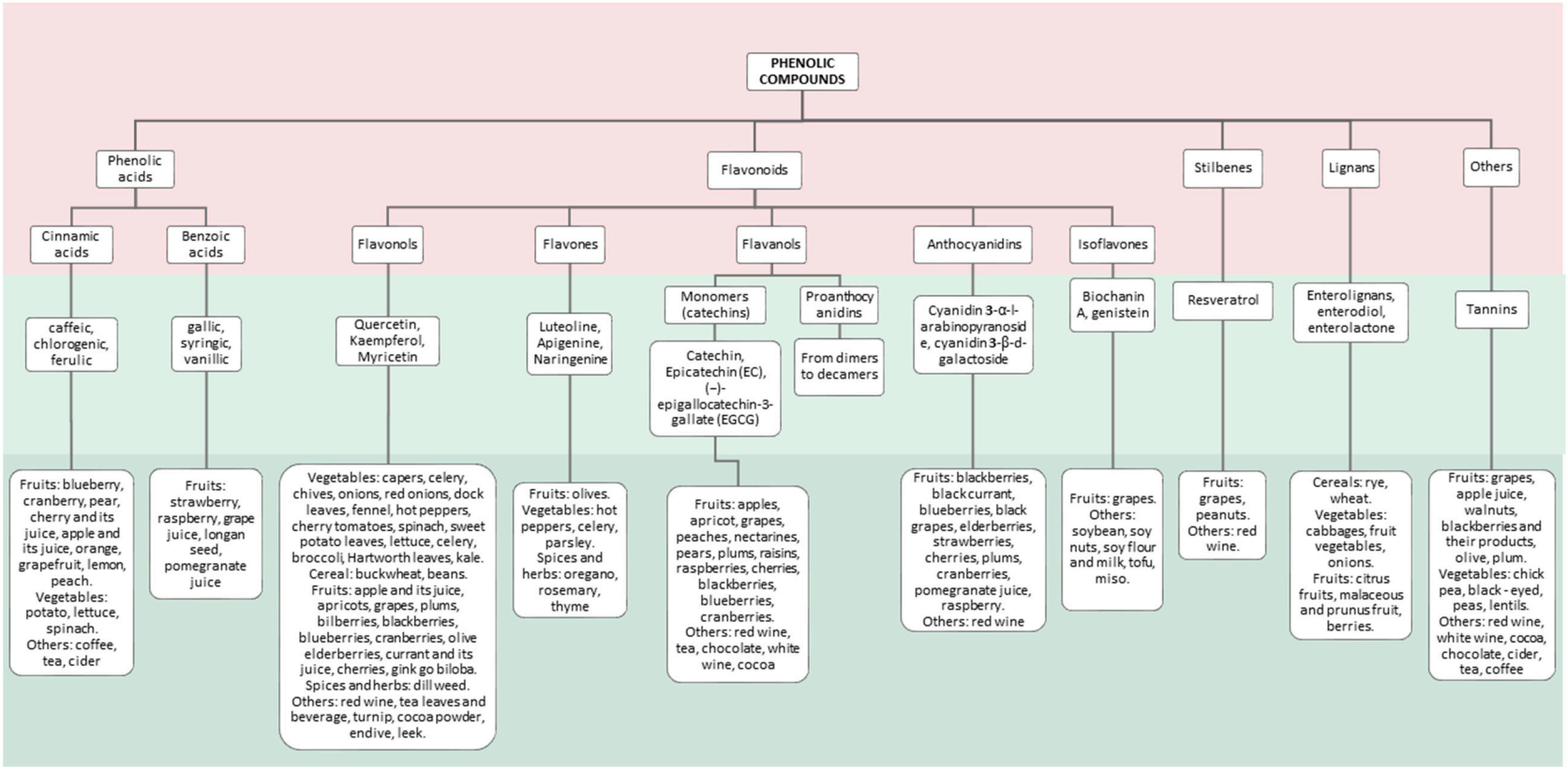

Figure 2. Phenolic compounds classification and dietary sources

The first row, from top to bottom, shows the chemical classification, the second row shows some common examples of the compounds and the third one the most common dietary sources. (Modified from Belščak-Cvitanović et al., 2018.)

Nine phenolic compounds were chosen for this thesis, six flavonoids and three phenolic acids. Their chemical structures are presented in Figure 3.

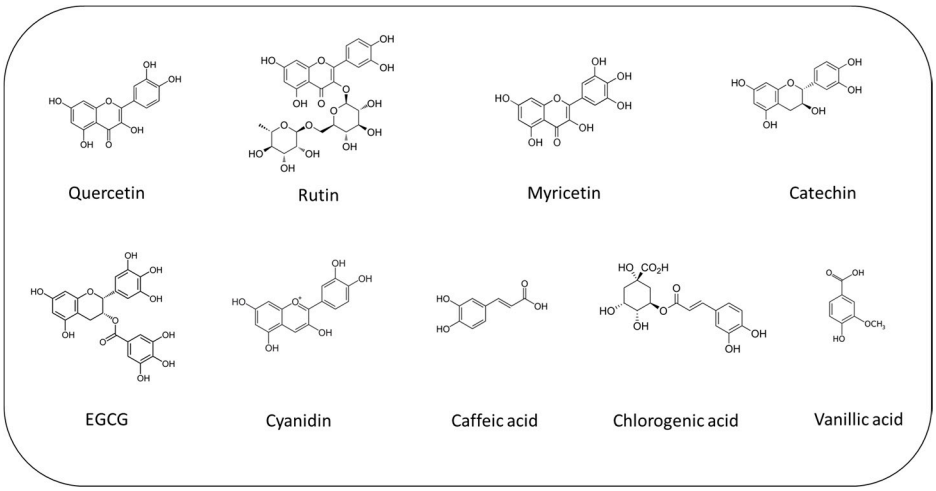

Figure 3. Chemical structure of the phenolic compounds used in this thesis.

3.2.3 Dietary sources

Phenolic compounds are highly present in foods of plant origin and are rarely found in fungi, bacteria and algae; in which flavonoids are almost totally absent (Vincenzo Lattanzion, 2008). There is not a clear trend about their composition, they can be both broadly spread or belong to specific species or families. Even single species can present large variations that depend of genetic factors, maturation stages and environmental conditions (Cheynier, 2005).

Nowadays there is a remarkable information about the content of phenolic compounds in diverse food, whether specific phenolic compounds or total phenols contents are given. This information is possible to find in several databases and reviews published during the last 20 years. One of the most important database is Phenol – Explorer, "the first comprehensive database on polyphenol content in foods with more than 35,000 content values for 500 different polyphenols in over 400 foods" (INRA, 2015). Another relevant database is the one provided by The United States Department of Agriculture (USDA) "Database for the Flavonoid Content of Selected Foods", that covers results for 506 food items divided in five subclasses of flavonoids ([USDA], 2013).

Considering the chemical characterization of phenolic compounds, Figure 2 displays the most significant phenolic characterized in edible plants (Belščak-Cvitanović et al., 2018).

3.2.4 Phenolic compounds as aggregates

It has been reported that some phenolic compounds present poor solubility in water, see table 1, and this fact can be related with their low bioavailability. Despite solubility studies, other authors have found that phenolic compounds can form aggregates in aqueous mediums at low concentrations, for instance, it has been reported that flavonoids like quercetin can form aggregates at 10 μM in Tris buffer, a common buffer used in bioassays (Pohjala & Tammela, 2012). Although it is not proper to compare these buffers with pure water, these findings suggest that phenolic compounds can be as aggregates in enzymatic assays and why not in ingested products such as teas and juices that contain large amounts of phenolic compounds, for example, the amount of EGCG, quercetin and myricetin in green tea can vary from 10 μM to 8000 μM (Medina-Remón et al., 2013).

Table 1. Aqueous solubility, from literature, of the phenolic compounds used in the present study

PHENOLIC COMPOUND	WATER SOLUBILITY (μM)
quercetin	12.6 (37 °C) (K. Zhang et al., 2016)
rutin	565(40 °C) (Peng, Li, & Yan, 2009)
myricetin	19.6 (37 °C) (Hong et al., 2014)
catechin	17328.7 (41.5 °C)(Srinivas, King, Howard, & Monrad, 2010)
EGCG	> 10908.2 (room temperature) (Sigma-Aldrich, 2017)
caffeic acid	1670(25 °C) (Kfoury et al., 2016)
chlorogenic acid	112895.5 (25 °C) ("Showing metabocard for Chlorogenic acid,")
vanillic acid	13000 (35°C) (Noubigh, Abderrabba, & Provost, 2007)

3.2.5 Phenolic compounds and proteins

Several studies have related phenolic compounds with different beneficial biological effects on humans. This property is highly associated with their interaction with a broad variety of proteins, including receptors and enzymes. The unsaturated C-ring of flavonoids is the main responsible of these interactions (Havsteen, 2002).

A covalent and noncovalent interaction can occur between phenolic compounds and proteins, being this last one the most common (Belščak-Cvitanović et al., 2018). Noncovalent interaction involves hydrogen bonds, resulting mainly from electronegative interaction between amino and hydroxyl groups. In aqueous

mediums, these interactions are reinforced by the hydrophobic effect (Jöbstl, O'Connell, Fairclough, & Williamson, 2004).

Depending on the type of protein and phenolic compound, two general phenol-protein complex can be form. Proteins that show an open conformation present multiple binding sites, allowing unspecific bindings along the protein chain. On the other hand, globular proteins have well-defined binding sites that lead to more specific interactions, such is the case of enzymes (Quideau, 2006).

The phenol-protein complex has been highly studied. For example, in some common foods (such as wines, beers, honey and fruit juices) the noncovalent interactions form soluble complexes of colloidal size, leading to haze formation (Quideau, 2006). In contrast, phenolic compounds and mouth proteins form insoluble complexes that are responsible for astringency sensation (Bandyopadhyay, Ghosh, & Ghosh, 2012). However, flavonoids have shown specific affinity with globular proteins, as is the case of digestive enzyme.

4 Methodology

4.1 Lipase selection

Mammalian lipases such as human, bovine, ovine and porcine present high similarity between them: similar amino acid composition, common antigenic determinants and same molecular weight ~ 50kDa. However, only porcine and human lipases are in glycosylated form (de Caro, Bonicel, Pieroni, & Guy, 1981). Therefore, porcine lipase is the most similar to human lipase and due to its low cost it is the one used in paper 1, 2 and 3.

Pancreatic lipase is inhibited by bile salts presents in the organism and by the phospholipids and proteins from dietary lipids. The presence of colipase in the organism avoids this inhibition, restoring lipase activity and allowing an efficient digestion of dietary fats. Therefore, the interaction between pancreatic lipase and colipase is important for lipase activity in the small intestine. Because the present experiments are in vitro and under simplified conditions (no dietary lipids and bile salts), no colipase was used in the present thesis.

4.2 Lipase activity assays

Lipases are a remarkable group of biocatalysts with the capacity to carry out novel and specific reactions in both non-aqueous and aqueous media. The wide utility of lipases demands effective methods for performing screenings and studying specific properties for novel and well-known lipases.

Lipases can act as both as esterolytic and lipolytic enzymes. Thus, lipase activity can be monitored by the release of glycerol from triacylglycerols, fatty acids or fatty acid esters.

Fatty acids released can be measured quantitatively by titrimetry, spectrophotometric assays, fluorescence, and for more specific measurements, chromatographic procedures such as GC and HPLC. The first method, titrimetry, is tedious and time-consuming. Therefore, spectrophotometric and fluorimetric

methods are highly appreciated for their efficiency at the time to perform screenings. Both methods were used in the present work.

4.2.1 Spectrophotometric assays

Esters of p-nitrophenol with different chain-length fatty acids are the most used as substrates. The released p-nitrophenol after the lipolysis is measured at 410 nm by a spectrophotometer (Pencreac'h & Baratti, 1996). Depending on the chain-length, these assays can be water-soluble. p-nitrophenyl palmitate is one of the most common substrates used to determine lipase activity by colorimetric assays. Due to p-nitrophenol lack of absorbance at acidic pH, this assay can only be performed at neutral and alkaline pH. For acidic enzymes, the assay has to be modified, raising the final pH of the mixture and adjusting the absorption coefficients of p-nitrophenol.

4.2.2 Fluorescence assays

When the reaction mixture is colorful, as is the case in paper 1, the spectrophotometric assays present complications. Fluorescence assays are one of the alternatives against these challenges. The most common and new assays use 4-methylumbelliferyl esters as substrate, where the realized compound 4-methylumbelliferone can be detected by fluorescence (Jacks & Kircher, 1967). Fluorescents assays are highly sensitives and less complicated. For instance, it has been shown that in assays using 4-methylumbelliferyl butyrate, the pancreatic lipase activity is not enhanced by colipase and that the use of detergents (that is common in other assays) is not necessary (Roberts, 1985).

4.3 Asymmetrical flow field flow fractionation (AF4)

Even though lipase can be quantified by activity assays as it is described in the previous point, paper 1 shows that the presence of particles in these assays can alter the reproducibility of the results. Therefore, a different technique had to be chosen for the quantification of lipase.

Asymmetrical flow field flow fractionation (AF4) is a gentle separation technique used in the analysis of aggregates and proteins (K.-G. Wahlund & Nilsson, 2012). The separation of analytes by AF4 consist in the diffusion properties of the analytes. Depending of the detectors connected to the system, different properties can be determined, such as radius and molar mass (Håkansson, Magnusson, Bergenståhl, & Nilsson, 2012; K.-G. Wahlund & Nilsson, 2012). The detectors used

in this thesis are: multiangle light scattering (MALS), ultraviolet (UV) and differential refractive index (dRI). Compared to size exclusion chromatography (SEC) the lack of a stationary phase in AF4 can reduce the loss of analyte and decrease breakdown of aggregates by high shear forces.

The separation in an AF4 take place in a thin ribbon-like channel lacking of packing material, where the liquid carrier is pumped creating a laminar flow through the channel. Once the laminar flow is formed, the sample in injected onto the channel and its accumulated near to the bottom wall. This wall is a semi-permeable membrane with specific pore sizes. The accumulation occurs thanks to an extra field, named crossflow, perpendicular to the longitudinal flow (channel flow) that take place along the channel (see Figure 4). The cross flow applies a force that push the analytes against the bottom wall. When the cross flow is reduced or removed completely, the analytes spread above the bottom wall at different velocities (K. G. Wahlund & Giddings, 1987). In Brownian mode, the smaller analytes diffuse faster reaching higher average height from the bottom wall, that results in higher transport velocity once that the longitudinal flow is applied. Therefore, small analytes will elute first than large analytes.

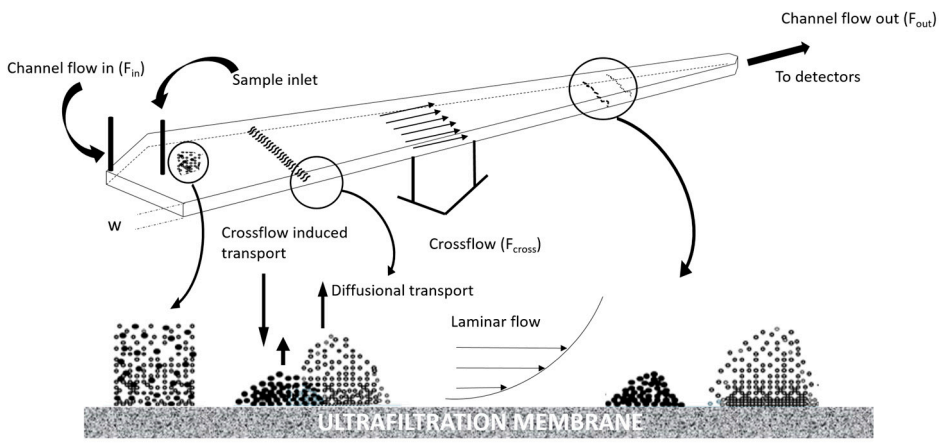

Figure 4. Scheme of asymmetrical flow field fractionation (AF4). Image from Wahlund & Nilsson, 2012. Copyright(2012) Springer-Verlag/Wien

One of the parameter that is possible to be obtained with the AF4 is the hydrodynamic radius (r_h), called as well the Stokes radius. This value can be determined combining the Stokes-Einstein equation with the diffusion coefficient obtained from the geometry of the channel (K.-G. Wahlund & Nilsson, 2012). The resulting equation is as follows:

$$r_h = \frac{V^0 kT}{w^2 \pi \eta t^0 F_{cross}} t_r \qquad (1)$$

where V^0 is the geometric volume of the channel, k is the Boltzman constant, T the absolute temperature, w the channel thickness, η the dynamic viscosity of the solvent, t^0 the void time, F_{cross} the volumetric crossflow and t_r the retention time of the analyte.

4.4 Simulated in vitro intestinal conditions

Although *in vivo* methods are considered the best standards for gastrointestinal studies, *in vitro* methods are still widely used because of their several advantage; they can be performed rapidly, they are less laborious and less expensive, and do not present ethical restrictions. *In vitro* gastrointestinal studies are also highly reproducible and, because they offer the possibility to control all the parameters, they are suitable for specific studies such as the mechanical factors during the digestive process and the understanding of digestive enzymes in the organism among others.

There is an international consensus for standardized static *in vitro* digestion methods proposed by INFOGEST (Minekus et al., 2014), an international network working in food digestion with more than 380 researchers from 40 countries (Dupont, 2011).

The method proposed by this consensus covers oral, gastric and intestinal phases, but due to the objective in this work is to study lipase, only the intestinal phase was considered. The simulated in vitro intestinal conditions in the present thesis are a simplifyed version of a "typical example" propoused by INFOGEST, see Table 2.

Table 2. *In vitro* simulated intestinal condition

Parameter	Typical example (INFOGEST)	Simulated intestinal conditions (thesis)
pH	7	7
Temperature (°C)	37°C	37°C
Simulated Intestinal Fluid composition (mM):		
KCl	3.4	3.4
KH_2PO_4	0.4	0.4
$NaHCO_3$	42.5	42.5
NaCl/Km	19.2	19.2
$MgCl_2(H_2O)_6$	0.16	0.16
HCl	4.2	4.2
$CaCl_2(H_2O)_2$	0.3	0.3
Enzymes	Pancreatin (amylase, lipase, and protease)	Lipase
Fresh bile (mM)	160	-

5 Lipases involved in lipids digestion

Three lipases were studied in the present work. The first one, pancreatic lipase, is a well-known enzyme for the role that plays in lipid digestion. For this enzyme only the size and oligomerization were studied (under *in vitro* intestinal conditions). The other two are enzymes from typical probiotic bacteria that were never been studied before, therefore a broader characterization was performed, since they will become the base for future studies.

The physicochemical parameters of pancreatic lipase were investigated in paper 2, where a molecular modelling was performed based on the crystallographic structure of pancreatic lipase. The results from this simulation give a theoretical r_h of 3.3 nm and 2.6 nm of radius of gyration. The experimental results from AF4 agree with the theoretical ones, with a r_h of 3.1 nm and 50 kDa of molecular weight, value previously reported for monomeric lipase (Birner-Grunberger, Scholze, Faber, & Hermetter, 2004). These results suggest that pancreatic lipase is in its monomeric form under intestinal conditions.

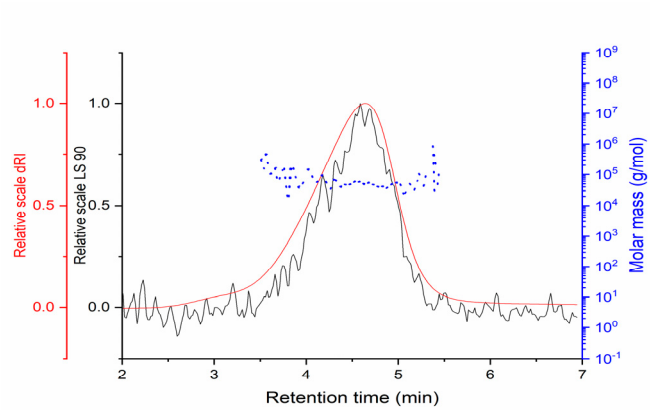

Figure 5. AF4-LS-dRI fractogramas of pancreatic lipase. Average molecular weight: 50 kDa

Figure 5 shows typical AF4 fractogram for the molecular mass characterization. The retention time, that correspond to the top of the peak, as well as the peak height of the concentration detector (dRI in this case) was used as reference for lipase quantification in Figure 7.

The other two lipases (from probiotic bacteria) were produced and characterized in paper 4. The first step was to search for any putative extracellular lipase in the genome of *Biffidobacterium longum* NCC 2705 and *Lactobacillus rhamnosus* GG. The results from the genome analysis only shows one putative lipase/acyl-hydrolase for each strain. From now on these putative enzymes will be named *Bl*Lip and *Lr*Lip from *Biffidobacterium longum* NCC 2705 and *Lactobacillus rhamnosus* GG, respectively. Both enzymes were successfully expressed by *E. coli* BL21 and purified by Ion-Metal Affinity Chromatography (IMAC). The parameters characterized for these two enzymes are shown in Table 3, where the kinetic constants as well as the stability over time and the optimal temperature were measured using *p*NP-laurate as substrate.

Table 3. Parameters characterized for BlLip and LrLip

Km is the Michaelis constant, Vmax the maximum velocity, Kcat the catalyst rate constant and Ki is the inhibition constant.

Parameter	*Bl*Lip	*Lr*Lip
Optimal pH	-	7 - 8
Optimal temperature at pH 7	-	37 – 40°C
Stability over time at	-	80 % decay of activity after 6 days
Kinetic constants		
Km (mM)	2.56	11.4
Vmax (U/mg enzyme)	0.05	5.8
Kcat (s^{-1})	0.04	2.6
Kcat/Km (s^{-1}/mM)	0.02	0.22
Ki (mM)	-	1.57
Theoretical molecular weight (genoma analisys)	52 kDa	27 kDa
Experimental molecular weight (AF4)	53 kDa	-
hydrodynamic radii of monomer (r_h)	3.6 nm	3 nm
Oligomeric form at pH 7	monomer	monomer
Relative substrate selectivity		*p*NP-butyrate (C4) 100% *p*NP-acetate (C2) 70% *p*NP-laurate (C12) 35%

From Table 3 it is possible to see that the optimal pH and temperature for *Lr*Lip correspond to the physiological conditions. The results also indicate that the typical kinetic constants were successfully determined for *Bl*Lip. On the other hand, for *Lr*Lip it was complicated to determine precise kinetic constants because the enzyme seems to suffer substrate inhibition, hence why, an inhibition constant was calculated. Regarding to the structural analysis, the experimental molecular

weight calculated for *Bl*Lip is consistent with the theoretical one and shows that the active oligomer form is a monomer, as it is for *Lr*Lip and pancreatic lipase. The calculated size (r_h) indicate that *Bl*Lip (3.6 nm) is larger than pancreatic lipase (3.3 nm) and this in turn more than *Lr*Lip (3nm).

The substrate selectivity indicates that *Lr*Lip has affinity for short chain esters rather than large chains. Short and large chain fatty acid esters are present in plant and animal lipids. A common example is bovine milk, that contains both short and long fatty acids esterified with glycerol (Lindmark Månsson, 2008). Although in the present work only simple substrates were tested, it would be important to study the activity of both enzymes with natural substrates in order to understand the roll of these proteins in edible products as well as in the gastrointestinal tract.

In contrast with pancreatic lipase, the crystallographic structure for *Lr*Lip has not yet been determined, therefore, several of the loops predicted by the molecular modeling could be unmodeled regions that difficult the characterization of the active site. To face this challenge, the model was superimposed with a crystallographic structure of aesterase, resulting in the prediction of 3 potential catalytic amino acids: Ser21, His222 and Asn93 (see paper 4, figure 8). The molecular modelling for *Bl*Lip is not reported because it did not reach the validation parameters.

Among the lipases presented in this point, pancreatic lipase was used for the following section due to its commercial availability.

6 Phenolic aggregates as inhibitors of pancreatic lipase

6.1 Identification of phenolic aggregates

In order to identify phenolic aggregates and how they can affect lipase assays, the IC_{50} value (inhibitor concentration where the enzymatic activity is reduced by half) of nine phenolic compounds (three phenolic acids and six flavonoids) were studied in paper 1. For this, a fluorescent assay was selected with the aim to choose an adequate technique to measure lipase inhibition without the interference of the intrinsic color of phenolic compounds.

The phenolic compounds chosen for this study are highly present in fruits and teas and the maximum concentration studied falls into concentrations found in the mentioned edible products.

The results from paper 1 indicate that between the phenolic compounds chosen, there is two dominating groups, those that aggregate and those that do not. Quercetin, myricetin, EGCG and catechin (all of them belonging to the flavonoid family) can form aggregates at very low concentrations, therefore they belong to the first group. In contrast with the compounds that do not aggregate, these flavonoids affect the reproducibility of the IC_{50} value, leading to false positive results. In agreement with Pohjala et al., flavonoids were more prone to form aggregates over phenolic acids. Pohjala et al., as well as McGovern et al., have shown that the addition of detergents such as Triton X-100 can control the aggregates formation and should be considered in enzymatic assays, but as one of the aims of this study is to understand the aggregation behavior, no detergents were used in this work. Instead, an alternative technique, AF4, that can be used for proteins characterization was considered (see methodology section).

The finding of aggregates at low concentrations (10 μM) suggest that, besides the molecular inhibition of lipase by phenolic compounds, there can be another kind of interaction, an aggregates-lipase interaction. Therefore, three of the flavonoids, that aggregate, were selected (quercetin, EGCG and myricetin) and studied in detail. Besides these compounds belong to the flavonoids family, they can present

different properties and the methods applied for their study can vary between each other.

6.2 Interaction between phenolic aggregates and pancreatic lipase under simplified intestinal conditions

Although paper 1 shows that myricetin, quercetin and EGCG can form aggregates in aqueous solution, this fact can change in different solutions, as is the case of the intestinal solution. Thus, the first step was to detect aggregate of the mentioned phenolic compounds under intestinal condition. For this, the optical density of different solutions was measured, assuming that a change in optical density suggests aggregate formation.

Four different solutions were prepared: 1) *Control solution*, that is the analyte in water; 2) *salt solution*, that contain the ions presents in the small intestine; 3) *low lipase solution* and 4) *high lipase solution*, that contain the ions of the intestine together with lipase at two different concentrations. All these solutions are a simplified version of the INFOGEST solution proposed for the small intestine, where only lipase was used as enzyme. For myricetin only solutions 1 and 4 were studied (see paper 2 and 3 for more details).

To have a general view of the aggregate formation, the flavonoid concentration was plotted against the optical density after 2 hours of incubation (time corresponding to intestinal digestion) (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, Carrière, et al., 2014). Although different flavonoid concentrations were tested (see paper 2 and 3), the difference between results is easier to appreciate at the highest concentration tested (1000 μM), see Table 4.

Table 4. Aggregates formation

Optical density after two hours of incubation at 37ºC. The results for quercetin and EGCG are expressed as the average of at least triplicates ± one standard deviation and for myricetin as the average of duplicates ± the pooled standard deviation. * Named Myricetin – water in paper 4. ** Named Myricetin – intestinal solution in paper 4

SOLUTION	QUERCETIN (1000 μM)	EGCG (1000 μM) $\times 10^2$	MYRICETIN (1000 μM)
Control*	0.57±0.08	0.07±0.15	0.09±0.04
Salt	0.14±0.05	2.3±0.28	
Low lipase	0.30±0.03	1.70±0.15	
High lipase**	0.02±0.01	1.62±0.26	0.032±0.001

An interesting observation from Table 4 and more clearly visualized in paper 2 – Fig 1 and paper 3 – Fig 2 is that the aggregate formation of quercetin and myricetin

is favored in control solution than in the other solutions, while for EGCG the opposite behavior occurs. In Table 4 all the results are significantly different compared to the blank, see paper 2 and 3, thus we can assume that the three flavonoids tested can aggregate under the studied solutions at the highest concentration. Therefore, 1000 µM of flavonoid concentrations was used from now on. The aggregate formation of phenolic compounds has been reported before, but not in presence of lipase and intestinal conditions. For instances, Pohjala et al., have shown that flavonoids can aggregate in different buffers and at different NaCl concentrations.

The finding that the chosen flavonoids aggregate under simulated intestinal condition lead to a new curiosity. How do these aggregates interact with pancreatic lipase? As the first approach to investigate the enquiry, their change in morphology was studied by optical microscopy for two of the aggregates, quercetin and EGCG, see paper 3.

The results from paper 3 show that quercetin aggregates can be detected by optical microscopy in all the solutions tested; while for EGCG, aggregates in the *control solution* were not detected with this instrument.

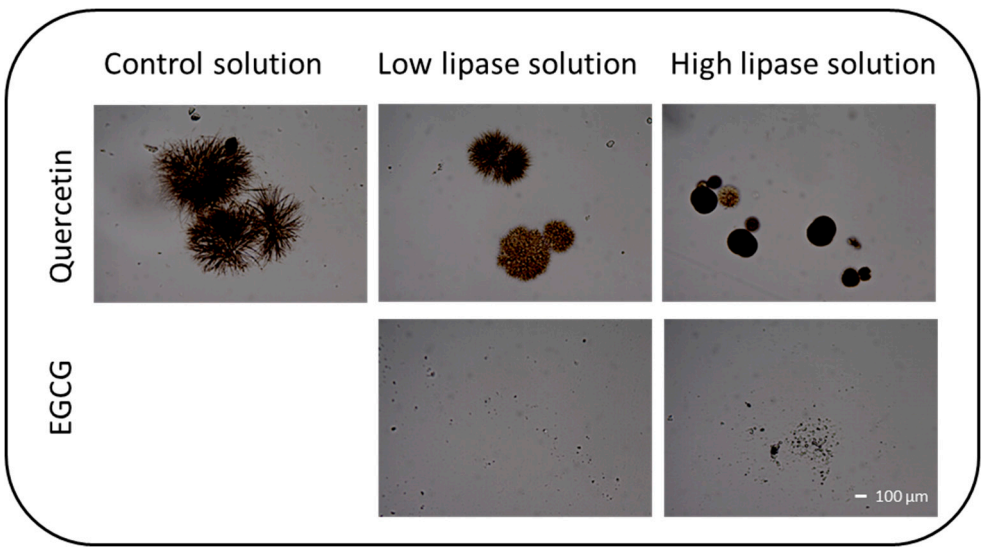

Figure 6. Optical microscopy images of Quercetin and EGCG aggregates in different solutions.
Magnifications of 400 X for all the images. 1000 µM of flavonoid concentration in each sample.

Focusing in lipase-aggregates interaction (under intestinal condition), figure 6 (as well as paper 3), shows that quercetin form large aggregates in water (control solution) with 1200 µm of longitudinal diameter approximately and a jagged

surface. The size of these aggregates is reduced in the presence of the other solutions, being smaller at the highest concentration of lipase tested. In addition, the presence of the enzyme seems to modify the jagged structure of the aggregate making them look smoother (Figure 6: Quercetin in lipase solutions). On the other hand, aggregates for EGCG were not detected in water, while in the simulated intestinal condition they were found at low and high lipase solution with diameters lower than 100 µm.

In the case of myricetin, no aggregates were found with optical microscopy under the simulated intestinal condition. As optical microscopy was not enough to study these particles, an AF4 method was applied for their characterization. In paper 2 and Table 4 it is possible to see that myricetin aggregates at high lipase solution and although the morphology of these aggregates was not visualized, their r_h were measured, see table 5. Myricetin aggregates in the control solution were not detected.

Table 5. Hydrodynamic radius

SUBSTANCE NAME	r_h (nm)
Small myricetin aggregates	1.4
Large myricetin aggregates	≥ 100
Myricetin–lipase aggregates	2.3 – 4.4

For quercetin and EGCG aggregates the radii is in the order of µm, while for myricetin in nm. This justifies why myricetin particles could not be detected by optical microscopy.

Table 5 also shows the size of aggregates formed by myricetin and lipase. This result will be discussed later.

The morphology results agree with those obtained by optical density, but still the interaction between these aggregates with lipase remains unclear, therefore another approach was considered. Can these aggregates precipitate together with lipase?

In order to detect if any enzyme, in this case lipase, precipitate together with aggregates, the enzyme under study can be quantified after removing the aggregates that interact with it. For this, enzymatic activity assays are the most common, but as the presence of phenolic aggregates can affect the reproducibility of lipase activity assays (see paper 1), in this study we used AF4 to quantify lipase (see Fig 4).

In paper 2 and 3 the remnant lipase in solution was measured after its interaction with different concentration of the studied aggregates (quercetin, EGCG and

myricetin). The aggregates were removed by filtration before the analysis. The final lipase in solution were reported as relative peak height of the concentration detector used (UV or dRI), where a decrease of peak height means that the phenolic aggregates precipitate together with lipase, resulting in a sequestering of the enzyme. For this experiments, high lipase solution was used in order to obtain a detection limit for lipase.

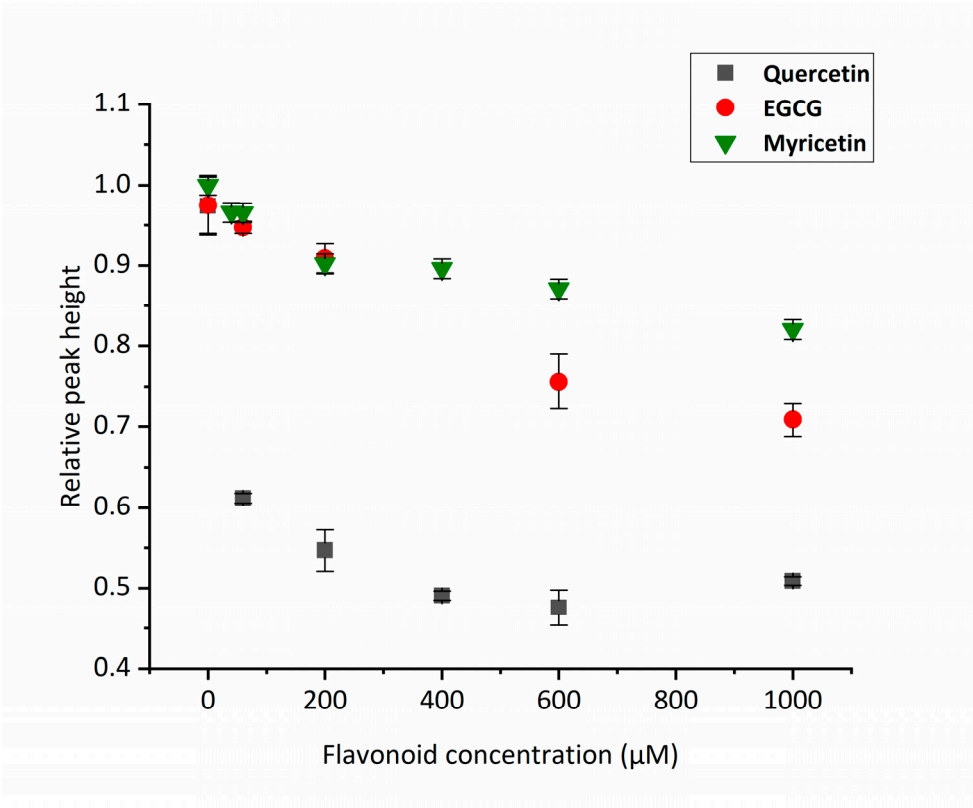

Figure 7. Remnant lipase after the interaction with quercetin, EGCG and myricetin.
The error bars represent one standard deviation of triplicates

Figure 7 shows that quercetin, EGCG and myricetin can sequester lipase under the simplified intestinal conditions tested; being quercetin the phenol that sequester the most, up to 50 %, followed by EGCG, 30 % and by myricetin, 20 %. Although McGovern et al., has shown that some aggregates (including quercetin) can sequester lactamase enzyme and this process can be reversible (McGovern, Caselli, Grigorieff, & Shoichet, 2002), this has never been reported for lipase.

6.2.1 Lipase inhibition mechanisms

From literature it is known that phenolic compounds can inhibit lipase by molecular mechanisms, interacting on specific sites (see section 3.1.6). On the other hand, the results from this thesis suggest that lipase inhibition, by phenolic compounds, can also occur at colloidal levels, by sequestering mechanisms.

6.2.1.1 Molecular mechanism

Figure 8 shows a typical mechanism that some phenolic compounds follow at molecular levels (Glisan et al., 2017; Martinez-Gonzalez et al., 2017). In this situation, the inhibitor binds an allosteric site of lipase that lead to a modification of its active site and prevents a proper substrate-lipase interaction, hence the enzymatic activity decreases.

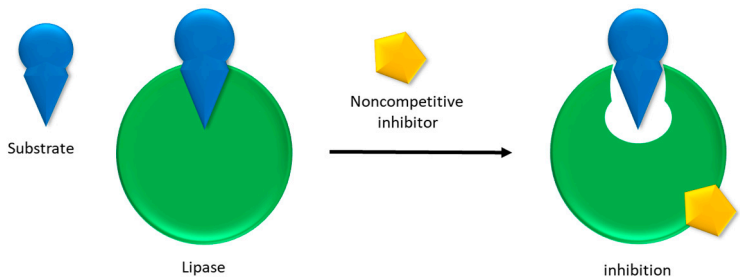

Figure 8. Schematic ilustration of noncompetitive inhibition

6.2.1.2 Sequestering mechanism

Regarding the aggregate formation, in previous sections it has been revealed that phenolic aggregates can inhibit lipase by sequestering mechanism, but although Fig 5 gives an idea of how this mechanism can occur (the morphology of the aggregates is modified in the presence of lipase, see figure 6), the detailed mechanism still remains unclear. McGovern et al., (2003) have studied a similar mechanism for β-Lactamase in presence of substrates that aggregate. Their results indicate that β-Lactamase is adsorbed onto the aggregate surface, however the incorporation of the enzyme in the aggregate structure could not be discarded.

Therefore, the model suggested in this thesis (Figure 9) is based on the one proposed by McGovern et al.

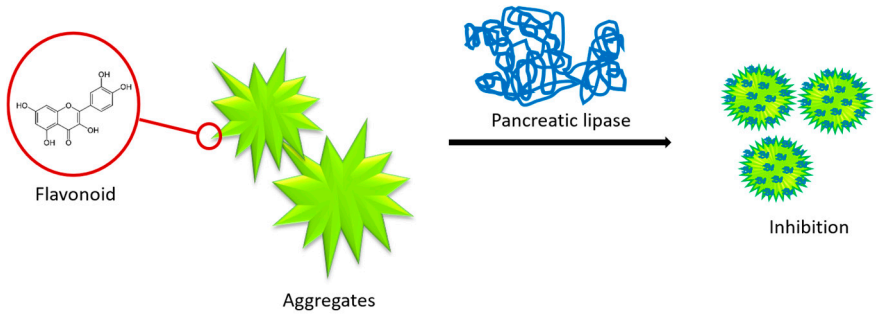

Figure 9. Schematic illustration of pancreatic lipase sequestered by phenolic aggregates

As we mentioned before, the phenolic concentrations in this thesis are common concentration found in food products. Besides, it is known that phenolic compounds are particularly absorbed in the small intestine (Velderrain-Rodriguez et al., 2014), where pancreatic lipase is secreted. Now, for phenolic compounds to form aggregates in the small intestine, they should be released from the food matrix by chemical, enzymatic and/or mechanical forces. This process occurs along the whole gastrointestinal tract, being more complex for solid food than liquid, therefore it is difficult to assume that phenolic compounds from edible products can aggregate in the small intestine. On the other hand, drinkable products such as teas, wine and juices are more likely to reach the small intestine without undergoing many modifications. Phenolic compounds are normally present as glycosides in food products, but due to enzymatic reactions they reach the intestine as aglycones that results in a solubility reduction (Hollman, 2004), hence they became more prone to form aggregates.

Some studies have suggested that the sequestering mechanism produced by phenolic aggregates can be reversible in the presence of surfactants (Pohjala & Tammela, 2012; Ryan, Gray, Lowe, & Chung, 2003), hence their use is highly recommended at the time to perform *in vitro* assays. But, what would happen in real conditions? Some edible products like juices and soups could contain natural and/or artificial surfactants, so it is likely that this sequestering mechanism could be affected by these surfactants, this is less likely in teas and plant infusions. In fact, one of the most well-known natural products, rich in phenolic compounds, is green tea and has been associated with weight loss treatments (Jurgens & Whelan, 2014).

On the other hand, for myricetin another mechanism can be also considered. The results from section 5 indicate that the r_h of monomeric lipase is 3.1 nm, while myricetin–lipase aggregates present a r_h from 2.3 to 4.4 nm (table 3). This finding suggest that a monomeric lipase could be covered by myricetin molecules, see Fig 10.

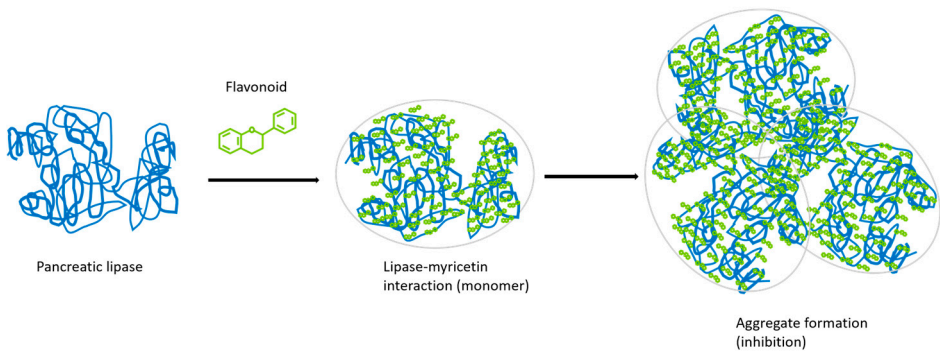

Figure 10. Schematic illustration of pancreatic lipase surrounded by myricetin molecules

This type of mechanism has been proposed before for proline-rich proteins (PRPs) in presence of catechins and tannins (Jöbstl et al., 2004; Siebert, 1999). These phenolic compounds bind several sites in PRPs producing changes in their conformation. At low phenolic compounds concentration, the protein contracts and lose their random coil conformation that leads to a decreasing of protein size. At higher phenolic compounds concentration, a cross-linking phenomenon takes place and aggregates can be formed (Jöbstl et al., 2004).

Although lipase is not a PRP, and their conformation is not open like random coil proteins, it is likely that this mechanism also occurs between lipase and myricetin. Myricetin could bind several sites of lipase forming monomeric lipase-myricetin structures that could aggregate later on and finally precipitate. Thus, this last mechanism suggests that the enzyme is part of the aggregate structure and not only is adsorbed by the flavonoid aggregates surface as it is shown in figure 9.

7 Conclusions

La la la la la ♪♫

Two new putative lipase/acyl-hydrolases from *Lactobacillus rhamnosus* GG and *Biffidobacterium longum* NCC 2705 were successfully produced and characterized. Because both of them hydrolase a large chain substrate (C12), they can be considered within lipase family. These new lipases are active under physiological conditions and, as well as pancreatic lipase, their active oligomer form is a monomer. On the other hand, lipase from *Lactobacillus rhamnosus* GG shows the highest substrate affinity respect to *p*NP-laurate. These findings suggest that both putative lipase/acyl-hydrolases can be involved in lipid digestion under physiological conditions.

Respect to the interaction between lipase and phenolic aggregates, we can conclude that some phenolic compounds can form aggregate in aqueous mediums, such as the intestinal solution. Among the phenolic compounds tested, flavonoids are more prone to form aggregates compared to phenolic acids. The presence of these aggregates disturbs the reproducibility of lipase assays and should be taken into consideration for future studies. On the other hand, the three types of aggregates tested in this study (Quercetin, EGCG and myricetin) have shown a common characteristic, to be able to sequester lipase under intestinal conditions, resulting in a different kind of lipase inhibition compared with those that have been reported before. Hence, lipase inhibition by phenolic compounds can also occur at colloidal level. The results from this study also indicate that, independent of the kind of flavonoid, it is enough that the flavonoid can form aggregates in order to sequester lipase.

8 Future research

Although there are several studies of phenolic compounds, there is a lack of information about phenolic aggregates. The results of this thesis suggest that phenolic aggregates play a role in lipid digestion. However, more studies are needed to comprehend their effect in digestion.

- In the present work, only intestinal conditions were considered. It would be important to study the digestion of phenolic aggregates along the whole gastrointestinal tract. From a morphologic and enzyme inhibition point of view, including all the digestive enzymes.
- Natural surfactants secreted by the intestinal tract could have an effect on phenolic aggregates. Thus, these effects should be investigated.
- The source of phenolic compounds can play an important role in aggregate formation, therefore, it would be interesting to analyze phenolic aggregates in food products, for example, green tea.

On the other hand, the new two enzymes (from probiotics) have been successfully produced and characterized. However, more studies are needed to complete their characterization and to understand their potential uses.

- In this thesis only synthetic substrates have been tested. Therefore, natural substrates should also be analysed, such as natural triacylglycerides found in food products.
- The role of these enzymes in the food industry and as catalysts in green chemistry is also an interesting subject of study. Therefore, it could be an interesting continuation of this thesis.

9 References

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As soon as I saw this section I thought: either it has to be very long so that it includes all those who contributed during the course of my PhD, or rather it has to be very short and concise. The PhD, beyond being a degree to obtain, becomes a life experience, so to write acknowledgements will not be enough to thank everyone who made this journey possible. Also, as memory can be fragile, I fear forgetting to mention someone so I will go for the second option.

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Well, that was all about specific people and as laziness urges, we now move on to general acknowledgements. Many thanks to the administration from the Food Technology department in Lund for their availability to help with any inconvenient situations. Thanks also to all the friends I met throughout my PhD (in Sweden, Bolivia and Korea) for the great shared experiences and the necessary help during difficult times. I also want to thank my Bolivian friends because sometimes a good conversation in your native slang can be more relaxing than hours doing yoga.

Sometimes I think that the more grateful I am, the more personal the 'thank you' has to be. This goes to my closest friends; who despite being included in the general acknowledgements is ironic I don't mention your names. Well… that is why you have a special dedication handmade on the first page of the thesis ;)

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