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The effect of rutin on the stool metabolome

Master´s thesis

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Declaration

I hereby declare that I have authored this master's thesis carrying the name „ The effect of rutin on the stool metabolome "independently under the guidance of my supervisor. Furthermore, I confirm that I have used only professional literature and other information sources that have been indicated in the thesis and listed in the bibliography at the end of the thesis. As the author of the master's thesis, I futher state that I have not infringed the copyrights of third parties in connection with its creation.

In Prague on 14th April _____

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The effect of rutin on the stool metabolome

Summary:

Rutin is the glycoside combining with the flavonol quercetin and it is found in a number of edible plants (buckwheat, black tea, martyrs and apples). It is a low toxicity flavonol that is easily absorbed and metabolized in the human body. Rutin has anti–inflammatory, anti–allergenic, antioxidant, anti–inflammatory and anti–cancer effects. Due to its properties, it is widely used in therapy and the cosmetic industry.

The practical part of the diploma thesis was focused on the catabolism of rutin by faecal microbiota of large intestine. Rutin was fermented in an *in vitro* fecal fermentation system using fresh stool samples from 20 different donors. Spectral changes and metabolite formation were monitored at fermentation times 0, 2, 4, 8 and 24 hours. The samples were subjected to NMR analysis, the spectra were analyzed in XCMS and Chenomx 8.5, and then the results were statistically evaluated. The aim of the practical part was to determine the main catabolites of rutin and stool metabolom in the colon model.

Rutin was metabolically transformed by a colon microbiota. Differences in the microbial transformation of rutin and its metabolites have been shown. The resulting metabolites were quercetin, quercetin-3-galactoside, 3-hydroxyphenylacetic acid, phloroglucinol and taxifolin. Differences in conversion rate and metabolic rate were found between stool samples obtained from individual donors. The second objective was to evaluate how the addition of rutin affects the stool metabolome. Based on the paired t-test performed between the results of the treated variants and the control, it can be said that there is no statistically significant difference between them. Thus, the metabolome was not affected by the addition of rutin. Furthermore, the effect of age on the metabolome composition after rutin treatment (difference observed for leucine and ethanol) was also found for the control variant regardless of rutin. For ethanol, fructose and glucose, a statistically significant difference was found in the intestinal metabolome concentration of the control variant. Ethanol at hour 0 was found to be 1.7 % more concentrated in the younger group than in the older group. At the same time, fructose was also present at a 1.1 % higher concentration in the younger group at hour 0, while at hour 2 the concentration was 0.8% higher in the younger group. The glucose concentration was observed to be 4.1 % higher in the younger group.

The hypothesis of this work that rutin will be microbially transformed was confirmed. The hypothesis that the addition would affect the composition of the intestinal metabolome was not proven. The composition of the intestinal metabolome differed between the two age groups studied only in the concentration of leucine, with a 4.7 % higher concentration observed in the younger group at hour 8, while this was not observed for the other substances.

Keywords: gastrointestinal tract, health effects, intestinal microbiota, flavonoids, NMR spectroscopy, rutin

Content

1 Introduction

Thus, the anti-inflammatory effects of rutin have been demonstrated, and much attention has been paid to its effect on cancer cells, where inhibition of cell proliferation and cytotoxicity have been observed. Another area of interest is the effect on certain metabolic related diseases. It has a positive effect in diabetes mellitus and also affects lipid levels in the body. It prevents lipid peroxidation, which is a common manifestation of oxidative stress and poses a threat to a variety of cells. At the level of the central nervous system, rutin has a protective effect on damage to certain neurons and has a positive effect in Alzheimer's disease or depression. The antioxidant effects are also related to the protective effect of rutin in tissue damage after reperfusion, which follows ischemia, when large amounts of free radicals are released into the body. The results suggest that rutin may act on a variety of health complications and positively influence the gut microbiome.

Rutin is still the subject of a number of experiments that aim to verify its other positive effects that could find application in therapy.

The human gut microbiome is a broad term encompassing all microorganism life inhabiting the human gut. Bacteria found in the human gut represent the largest part of the human microbiome, make up a significant percentage of the human cellular make up and their genomes represent a significant part of the human genome. The gut microbiome has a major impact on human health and changes over the course of a lifetime, for example, depending on changes in diet and the use of medicines.

This work focuses on the study of rutin metabolism and the formation of metabolites in a colonic model. Newly developed 96-well deep-well plates *in vitro* colon model were used for the research and their efficiency was tested and compared with other fermentation methods.

2 Scientific hypothesis and aims of the thesis

The thesis' objective of the theoretical part was the literature review, which focused on metabolomics and the fate of rutin in the human gastrointestinal tract. The objective of the practical part was to determine catabolites of rutin that originated from microbial metabolism in an *in vitro* colon model that used stool samples from two age groups (up to 45 years and over 70 years). Another objective was to evaluate how the addition of rutin affected the intestinal metabolome.

Hypotheses:
1) Rutin is microbially transformed by human colon microbiota.
2) The addition of rutin affects the stool metabolome.
3) Stool metabolome differs in the two observed groups.

3 Literature research

3.1 Digestive system

The formation and renewal of tissues and all organism activity require an intake of food. Along with the excretion of their residues and products, this cycle is part of the metabolism, which is the life-sustaining chemical process. The digestive or gastrointestinal tract (GIT) provides the food intake, processing and absorption of nutrients containing the necessary building and controlling blocks of the organism. In addition to these three basic functions, the GIT performs several other activities necessary for life (Ogobuiro et al. 2021). The digestive tract begins with the oral cavity and ends with the anus (Smith & Morton 2010). The digestive system (see fig. 1) is divided into the following parts: the oral cavity with adjunct salivating glands, pharynx, esophagus, stomach, small intestine (consisting of the duodenum, jejunum, and ileum) and the large intestine (consisting of the ascending colon, descending colon, transverse colon and anus). There are two glands which are functionally connected to the digestive system – the pancreas and the liver (Cheng et al. 2010a).

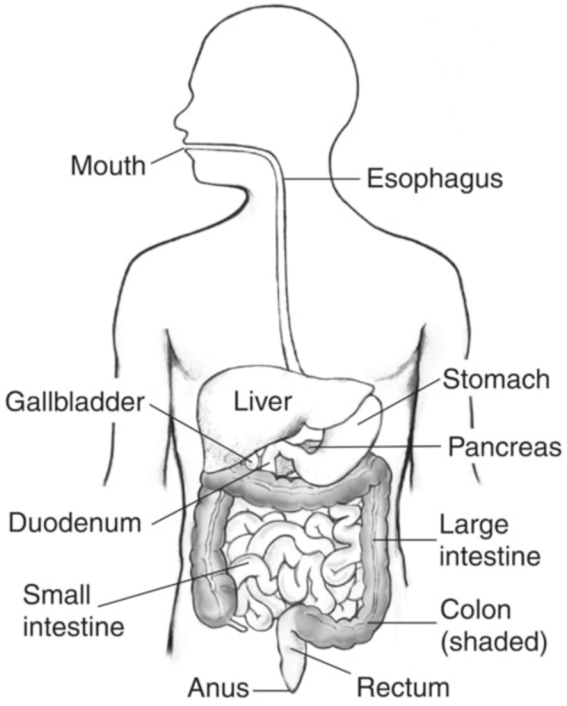

Figure 1: Digestive system ("Digestive tract within outline of male body, with labels pointing to small intestine, large intestine, and colon | Media Asset | NIDDK" n.d.)

The digestive system is divided into certain parts through which food must pass and which facilitates the excretion of residues from the body. This can be divided into three parts for simplification:

1. Upper GIT (oral cavity, esophagus, stomach)
2. Medium GIT (small intestine)

3. Lower GIT (colon) (Cheng et al. 2010b)

A fourth part is the action organs involved in the function of GIT but outside the digestive tube – sagittal glands, liver, and pancreas (Smith & Morton 2010).

The gastrointestinal tract has the following main tasks:
- mechanical and chemical processing of food – digestion
- transfer of selected substances by GIT wall – absorption – balancing of sudden food intake – storage
- disposal of microorganisms by their own immune system and digestion and agitation of the antigenic structure of substances – protection (Cheng et al. 2010a)
- removal of residue and certain harmful substances (Patricia & Dhamoon 2021).

3.1.1 Histology of small and large intestine

The entire gastrointestinal tract has certain identical structural features. The digestive tract is a hollow tube composed of a lumen of variable diameter and a wall formed by four base layers. These layers in particular sections show individual characteristics:

- mucous membrane (*tunica mucosa*)
- submucous (*tunica submucosa*)
- muscular layer (*tunica muscularis externa*)
  - inner circular layer
  - outer longitudinal layer
- serous membrane (*tunica serosa*) (Strobel et al. 2015)

The mucos membrane consists of the epithelium (*lamina epithelialis*), which forms the lining of the digestive tract and forms a barrier between the external environment (the lumen of the digestive tract) and the organism. It is also functionally involved in mechanical or chemical processing and resorption of nutrients. The character of the epithelium is adapted to the function in individual sections. A single-layered cylindrical epithelium is present in the stomach and intestine. It has a protective function in the stomach, so we find mucus-producing here, while resorption predominates in the intestine (Azzouz & Sharma 2021). The most prevalent cells in the gut, enterocytes, have a brush border typical of resorption epithelium. Nevertheless, mucous (goblet) cells are still found there. They increase in the aboral direction and in the large intestine they already predominate over resorption cells (referred to here as colonocytes). Furthermore, the mucous consists of a layer of lamina propria (*lamina propria mucosae*) is rich in blood and lymphatic vessels, smooth muscle cells and nerve cells, and contains glands and lymphoid tissue. The other part is the muscularis mucous (*lamina muscularis mucosae*), which usually consists of a thin inner circular and an outer longitudinal layer of smooth muscle cells separating the mucous from the submucosa (Strobel et al. 2015)

The submucosal ligament layer is composed of an areolar tissue permeated by numerous blood and lymphatic vessels, which occupies the nerve plexus (*plexus submucosus Meissneri*). It may also contain glands and lymphoid tissue (Liang et al. 2006).

The outer muscle layer consists of smooth muscle cells, which are arranged in a helix and divided into two layers according to the predominant direction of muscle fibres. In the inner layer (closer to the lumen), the orientation of smooth muscle cells is predominantly circular, in the outer layer the longitudinal arrangement prevails. Between these layers of muscle are deposited nerve plexuses and vegetative nerve ganglia (*plexus myentericus Auerbachi*), as well as blood and lymphatic vessels that run in the ligament interspersed between these muscle layers (Seeras et al. 2021).

Serous membrane is a thin layer of connective tissue, rich in blood and lymphatic vessels and adipose tissue, which is covered by the single-layered epithelium (*mesothelium*). Serous membrane is found on organs located in the peritoneal cavity. Only in a small part (on the esophagus and in the final part of the large intestine) we find the site of tunica externa (*tunica adventitia*), which connects the organs it surrounds to other tissues of the thoracic cavity and the pelvic floor (Michailova 1996).

The small intestine (*intestinum tenue*) is the site of the final digestion of food, the absorption of metabolites and endocrine secretion. It is approximately 5m in length and consists of three segments: duodenum, jejunum and ileum. The mucous membrane is formed by a single-layered cylindrical epithelium. The mucous membrane, together with submucosa, forms numerous permanent folds (*plicae circulares (Kerckringi*)), which have a semi-lunar, circular or spiral shape. They are most developed in the jejunum. Intestinal villi (*villi intestinales*) are protrusions of the mucous membrane (*epithelium and lamina propria*) 0.5 - 1.5 mm in length, protruding into the lumen of the small intestine. In the duodenum they are leaf-shaped and towards the ileum gradually take the shape of fingers. Between the villi there are small mouths of simple tubular glands, which are referred to as intestinal glands (*Lieberkühn's glands or crypts*). In these glands we find undifferentiated cells, absorption cells and goblet cells, Paneth cells and enteroendocrine cells (Collins et al. 2021).

The large intestine *(intestinum crassum)* measures 1.3 - 1.4 m in length with a width of 5-8 cm at its start and decreasing in the aboral direction. Its main function is the absorption of water and the formation of fecal matter, associated with the production of mucus. The main sections of the large intestine are the appendix (*caecum*), from which the worm-like protrusion (*appendix vermiformis*) is connected, colon ascending, transverse, descending and sigmoid (loop), rectum and rectal canal (*canalis analis*) (Azzouz & Sharma 2021).

The large intestine is provided with mucous membranes which, with the exception of the distal (*rectal*) section, are without folds. There are also no villi in this part of the digestive tract. Lieberkühn's crypts are long and characterized by the presence of a large number of goblet cells, absorbent elements and a small number of enteroendocrine cells.

The epithelial lining is made of cylindrical cells, equipped with short microvilli, which indicate the absorption function. Lamina propria is rich in lymphoid cells and follicles, which often invade the submucosa. The large amount of lymphoid tissue is probably the result of an extremely abundant colonization of the colon with bacteria (Sulegaon et al. 2015).

The lamina muscularis externa is composed of longitudinal and circular stripes. It differs from the small intestine in that the longitudinally oriented smooth muscle layer is substantially reduced and is well formed only in three strips evenly spaced apart on the circumference of the intestine, which run longitudinally. These are called colonic strips (*taeniae coli*) (Azzouz & Sharma 2021).

3.2 Microbiota in the gastrointestinal tract

The human body provides the ideal environment for the dynamic colonisation of microbiota, which is made up of trillions of microorganisms that include bacteria, archaeons, single-celled eukaryotes and viruses. These microorganisms colonize skin as well as cavities – the respiratory, genitourinary or the gastrointestinal tract (Sekirov et al. 2010). The most detailed studied domain of microorganisms includes gut bacteria that consists of more than one thousand taxonomic units (Baquero & Nombela 2012). The intestinal microbiota is reported to contain, on average, approximately 10^{14} bacterial cells (Ley et al. 2006) that have more than five million genes (Huttenhower et al. 2012). The combined weight of the intestinal microbiota is approximately 1-2 kg (Qin et al. 2010a).

With its large surface and abundance of nutrients, the intestine is a perfectly adapted environment for microorganisms. The gut microbiota digests nutrients from food taken by the host organism, transforming them into multiple metabolic products that are involved in a large number of biochemical pathways. It can, therefore, be considered as a separate body with extensive metabolic capacity and substantial functional plasticity. Through its genetic equipment, the microbiota maintains a symbiotic relationship with intestinal mucosal cells and participates in important metabolic and immunological processes (Sekirov et al. 2010).These findings have provided a different view of gut microorganisms, and research that had focused primarily on detecting the quantity and diversity of microorganisms is now focusing more on the functional aspects of the microbiota (Jandhyala et al. 2015; Rowland et al. 2018). The study of the composition of the microbiota of the intestine encounters several problems that make it difficult to define what composition is physiological and what characterizes the rather pathological condition. The problem of determining the physiological microbiota of the intestine is mainly due to its wide variability.

Thanks to modern research techniques that are no longer reliant on the low denunciation of the cultivation of microorganisms, it has been shown that the composition of the intestinal microbiota is unique for each person and is influenced by a variety of factors. Not only does it change over the course of a lifetime with age or the development of diseases, but the composition is also determined by the nature of lifestyle and eating habits (Thursby & Juge 2017)(Thursby & Juge 2017). In addition to intra-individual variability, the composition of the intestinal microbiota is also affected by inter-individually (Lozupone et al. 2012).

3.2.1 Micriobiom of oral cavity

The oral microbiome is made up of many microorganisms. In addition to bacteria, there are also representatives of the Archaea domain, viruses, micromycetes or protozoa (Wade 2013). There are six main bacterial strains in the oral cavity: *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes* and *Fusobacteria*, as well as strains of *Euryarchaeota, Chlamydia, Chloroflexi, Synergistetes and Tenericutes*. Specifically, the following classes of *Firmicutes* are represented: *Clostridia, Bacilli and Erysipelotrichia.*

The order *Bifidobacteriales* includes the class *Bifidobacterium*, *Gardnerella, Scardovia* and *Parascardovia* (Dewhirst et al. 2010). These orders are found primarily in dental caries and

dental plaque (Mantzourani et al. 2009), with the exception of the genus *Gardnerella*, which is primarily isolated from the vagina. (Dewhirst et al. 2001).

3.2.2 Microbiom of stomach

The stomach environment is very acidic (pH = 1-2) (Rehfeld et al. 2017). The occurrence of microorganisms is minimal, yet some species are found in the stomach. The dominant bacterium is Helicobacter pylori with the ability to survive acidic conditions. The enzyme urease serves this purpose by breaking down urea into ammonia and thus maintaining the correct pH for survival. However, H. pylori has been found to contain chemoreceptors that detect hydrochloric acid and can probably guide the bacterium to the gastric epithelium where the pH is nearly neutral (Huang et al. 2016).

Jandhyala et al. (2015) reported that the representation of other microorganisms in the stomach depends on whether H. pylori is present as a commensal or as a pathogen. *Streptococcus* spp, *Prevotella* spp, *Veillonella* spp and *Rothia* spp are the most abundant.

3.2.3 Micriobiom of intestine

The gut microbiome is important for the proper functioning of the human body. Some of its most important roles include maintaining the integrity of the mucosal barrier, providing nutrients (e.g. vitamins), protecting the body against pathogens, and aiding in the proper function of the immune system (Thursby & Juge 2017). The amount of bacteria in the gastrointestinal tract increases distally. At the beginning of the small intestine, the number is around 10^3 CFU/g and in the large intestine it reaches up to 10^{12} CFU/g (Minalyan et al. 2017).

The small intestine has a low density of microorganisms mainly due to the fact that the outlet with bile acids that prevent their growth is located here (Tuohy & Scott 2015). Gram-positive aerobes are present in the first part of the small intestine, while only gram-positive anaerobes and facultative anaerobes are present at the end of the small intestine (Minalyan et al. 2017). Aidy et al. (2015) mention the genera *Clostridium* spp, *Escherichia* spp and *Turicibacter* spp, which occur in variable amounts in the small intestine. *Streptococcus* spp. and *Veillonela* spp. are reported to be the most abundant genera.

The large intestine contains a very rich mikrobiota (Mohn & Johnson 2015). The large intestine of a healthy adult contains hundreds of different bacterial species, mainly from the *Bacteroidetes, Firmicutes* and *Proteobacteria* strains (Ishiguro et al. 2018). The bacteria present in the colon produce enzymes that break down fiber, starch, and non-saccharide material. The most well-known polysaccharide-degrading bacteria include *Bacteroides* spp. (starch, cellulose), *Bifidobacterium* spp. (fructooligosaccharides, inulin, starch), Clostridium spp. (cellulose), *Enterococcus* spp. (cellulose), *Roseburia* spp. (inulin, starch) and *Ruminococcus* spp. (starch, cellulose, xylan) (Hoyles & Swann 2018). Degradation of polysaccharides during the fermentation process alters intestinal pH. At the beginning of the appendix, the pH is around 6, but slowly increases distally to 7.5 (Huttenhower et al. 2012). This metabolic reaction produces short-chain fatty acids, 95 % of which are used as an energy source for intestinal cells and the remainder is excreted (Ishiguro et al. 2018). Butyrate, propionate, and acetate are thought to be the main substances released in this process. Butyrate is a source of energy for colonocytes and is involved in the activation of intestinal

gluconeogenesis (Valdes et al. 2018). Butyrate induces apoptosis of colorectal cancer cells through hyperacetylation of histones (H3 and H4), resulting in DNA being in a more open form (Wong et al. 2006). Propionate is diverted to the liver where it controls the progress of gluconeogenesis, and acetate, which is an essential metabolite for bacterial growth, is involved in cholesterol metabolism and lipogenesis in peripheral tissues (Valdes et al. 2018). The gut microbiota is also capable of producing vitamins (thiamine, riboflavin, niacin, biotin, pantothenic acid, folic acid, vitamin K) and secondary bile salts. The aforementioned salts can be absorbed in the intestine or excreted in the faeces (Ishiguro et al. 2018). The metabolic activity and overall composition of the microbiota depends on the pH of the environment. The ascending colon is acidic in nature, but distally the pH slowly increases (Tuohy & Scott 2015).

3.2.4 Influences throughout life

The populations of bacteria required to colonize an individual's large intestine is decided by the bacteria type to which they are exposed during the first hours of life. The primary indicator of the composition of the microbiota is the method of childbirth (Murphy et al. 2010).

It is believed that the developing fetus comes into contact with bacteria through the placenta already established in the uterus. The placenta contains smaller amounts of bacteria mainly from non-pathogenic species such as *Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes* and *Fusobacteria* (Orrhage & Nord 1999). The composition of microorganisms is influenced by whether the baby is delivered naturally or by caesarean section (Dominguez-Bello et al. 2010). In those born by caesarean section, the absence of *Bifidobacteria* sp. is significant, while in children born through natural means Bifidobacterium *longum* and *Bifidobacterium catenulatum* are predominant (Biasucci et al. 2010). This difference in the composition of the intestinal microbiota could be due to the fact that during natural childbirth there is initial contact with bacteria from the vaginal and anal environment of the mother. Caesarean section was dominated by *Bacteroides, Bifidobacterium*, with *Streptococcus, Clostridium, Enterococcus, Blautia, Veillonella, Lactobacillus, Staphylococcus, Planococcus*, and others. Cesarean birth results in a gut microbiome that is less similar to that of the mother compared to vaginal birth, and is more likely to include skin and oral microbes, and bacteria from the operating room (Bäckhed et al. 2012).

The composition of the intestinal microbiota of aborted babies also differs from that of premature babies. In premature babies, *Proteobacteria* initially dominates with less representation of *Bifidobacterium* and *Lactobacillus* (Murphy et al. 2010). However, the individual is not only exposed to these bacteria, but also to bacteria occurring freely in the surrounding environment, including those of medical staff present, other newborns, air, apparatus and equipment. At this time, the intestinal microbiota is less complex and stable compared to that of an adult.

Milk through breast-feeding contributes to the formation of microbiota because it contains a diverse mixture of oligosaccharides, including cytokines, lysozymes and lactoferrin, which are essential for the correct development and ripening of the intestine. Milk oligosaccharides in human breast milk act as prebiotic substrates. Their task is to stimulate the growth of beneficial bacteria, such as *Bifidobacteria*, and can increase their action in the

intestine. After breastfeeding is completed and the switch to solid food, the composition of the intestinal microbiota changes and there is an increase in the number of *Bacteroidetes* and *Firmicutes* to increasingly resemble the microbiota of adults (Murphy et al. 2010).

During the first two to three years of life, the composition of the most important bacterial groups is stabilized (Petnicki-Ocwieja et al. 2009). However, this does not mean that all have the same microbiota composition. While individuals have a relatively stable microbiome core, the numbers of occasional intestinal microbes vary greatly in individuals depending on a variety of factors including diseases, the administration of drugs (especially antibiotics) and changes in diet (Marques et al., 2014). In most cases, however, at the end of the disrupting factor the original gut microbiota is restored to its level from early childhood

(Dinan & Cryan 2012). At the same time, the composition of gut microbiota has been shown to be influenced by age: for example, the number of bifidobacteria has been found to decrease with age, which in turn adversely affects the health of the senior host (Dinan & Cryan 2012). The change in the gut microbiota is associated with various diseases, namely obesity, diabetes, atherosclerosis or inflammatory bowel disease (Murphy et al. 2010).

When the gut microbiota is balanced, this condition is referred to as symbiosis. If there is an overpopulation of pathological organisms, a state of dysbiosis occurs and the risk of an inflammatory reaction increases that can disrupt the functions of the central nervous system (CNS). Mutual communication is realized mainly by vagus nerve, production of signaling molecules, for example gamma-aminobutyric acid (GABA), dopamine (DA), norepinefrina (NE), acetylcholin (Ach), short-chain fatty acids (SCFAs) and more.

3.2.5 Diet

The effect of diet on the composition of the intestinal microbiome is evident in early childhood. Studies show a difference in the composition of the microbiome in breastfed and fed infants. In breastfed infants, *Staphylococcus, Streptococcus*, lactic acid bacteria and *Bifidobacteria*, which predominated by 60-90 %, were the most common. In formula-fed children, the composition of the intestinal microbiota was closer to that of an adult. Facultative anaerobic *Bacteroides, Clostridium, Staphylococcus, Streptococcus* and *Enterobacteriaceae* were the most represented (Collado et al. 2012). The type of diet at a later age has an effect. A diet rich in fruits, vegetables and fibre is generally understood to lead to greater microbial diversity and better health. Many bacteria metabolizing plant polysaccharides from the Firmicutes strain (*Roseburia, Eubacterium rectale* and *Ruminococcus bromii*) have been reported in these individuals. While a diet high in meat, sugar, fat and low in fiber results in less bacterial diversity, it worsens health and can lead to obesity, coronary heart disease and malignancies. People eating in this way have a higher number of bile tolerant bacteria (*Alistipes, Bilophila* and *Bacteroides*) (Albenberg & Wu 2014; David et al. 2014)

3.2.6 Nutrient metabolism

Carbohydrate fermentation is one of the main metabolic functions of bacteria that colonizes the human gut. Under anaerobic conditions, short-chain fatty acids are formed, especially acetate, butyrate, and propionate, which are energy sources for colonic epithelial cells. They also produce various gases, mainly hydrogen, methane, and carbon dioxide, and

generate the energy that bacteria requires to grow and maintain cellular functions (Cummings et al. 1987; Sartor 2008). These fermenting bacteria include species from the genus *Bacteroides* and the families *Clostridiaceae* and *Lactobacillaceae* (Belizário & Napolitano 2015). Gastrointestinal bacteria are also involved in the breakdown of proteins into amino acids and the conversion of these amino acids to biogenic amines, immunomodulatory substances and other signaling molecules (Hollister et al. 2014). Using the bacterial enzyme histamine decarboxylase, which is found in fermentation bacteria such as *lactobacilli*, the bacteria decarboxylate L-histidine to histamine and convert glutamate to gamma-aminobutyric acid thanks to the enzyme glutamate decarboxylase (Thomas et al. 2012; Damiano et al. 2015). Another important function is the synthesis of vitamins K and cholines. Choline is an essential component of acetylcholine and is involved in fat metabolism in the liver (Arslan 2014). Some intestinal microorganisms can use it to form trimethylamine, ethyl acetate and ethanol (Krishnan et al. 2015). In 2015, Martínez-del Campo et al. analyzed the sequenced bacterial genomes and found that choline utilization gene clusters were present in four bacterial strains, *Proteobacteria, Firmicutes, Actinobacteria* and *Fusobacteria*. However, they were missing from the Bacteroides strain. Vitamin K2, menaquinone, is a fat-soluble vitamin and is important for bone growth and blood clotting. It can be ingested in food, but bacteria from the genus *Bacteroides* are also involved in its formation (Flore et al. 2013).

3.3 Mutual communication between the intestinal microbiome and the brain

The gut not only receives regulatory signals from the central nervous system (CNS), but it can send signals to the brain to be received. In literature, the term gut-brain axis generally occurs, and this includes afferent and efferent nerve connections, endocrine, nutritional, and immune signals (Wang & Kasper 2014). This concept of the entero-encephalic axis can be extended to the microbiota-gut-brain axis because even intestinal microbiota can affect the brain. The abilities of the intestinal microbiota and their metabolites include modulation of both the peripheral nervous system and the CNS, as well as influencing the development of the brain and brain functions. This two-way communication system affects the body's normal homeostasis and its possible changes and the resulting dysregulation of the gastrointestinal, central, autonomic or immune system, which may be one of the causes of various diseases (Foster & McVey Neufeld 2013).

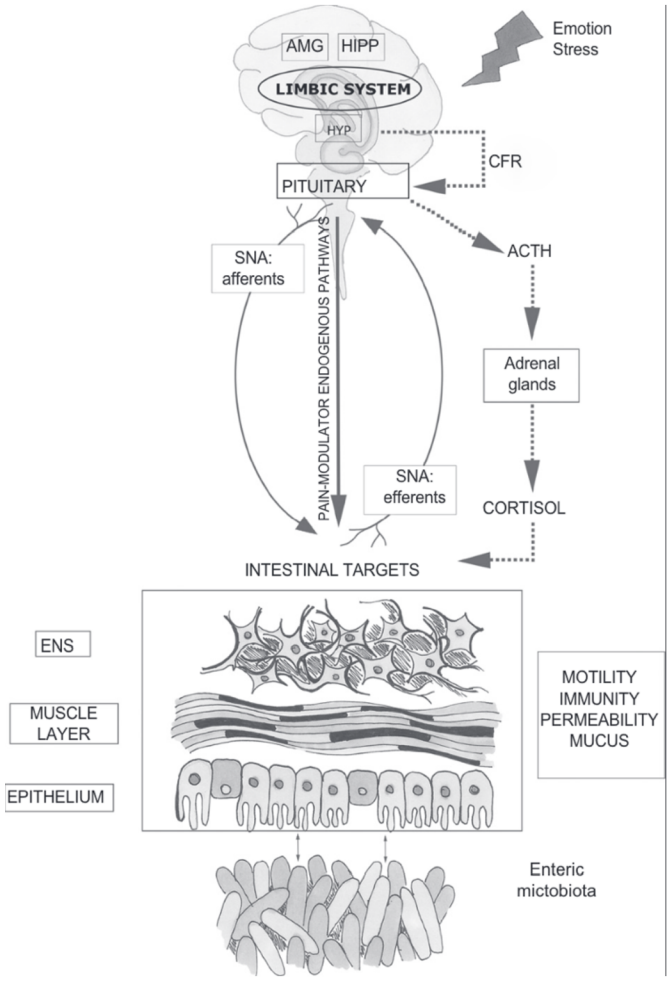

Figure 2: Microbiome gut–brain axis structure (available from: Carabotti et al. 2015)

Figure 2 shows the mechanisms by which the gut microbiota affects the brain and vice versa. This communication goes both ways. The figure on the left shows, how the brain influences the gut microbiota. Disrupting the normal gut population through stress will result in changes in physiology, epithelial function, mucin production, enteroendocrine (EE) cell function and motility. Neurotransmitters are released. On the right is shown how the gut microbiota affects the brain. Via activation of neural afferent connections, as well as activation of the mucosal immune response and the production of metabolites.

3.3.1 How the brain affects the microbiome

One of the ways the brain acts on the microbiome is through the use of pathways and mediators for the central system of hunger and satiety. Thanks to this control, it is possible, for example, to induce a change in the availability of nutrients for the intestinal microbiota and thus affect the composition of the microbiota. For this control to be possible, satiety-inducing peptides are required, and these are transported by the blood to the brain during food intake. Although they are primarily produced in the GIT, the majority are formed in the brain (Wang & Kasper 2014). Another possibility is influence through neural and endocrine pathways, either directly or indirectly. The autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis can modulate intestinal physiology through motility, epithelial permeability, and

secretion (Wang & Kasper 2014). The HPA axis, the main stress axis, allows hormones to communicate within the gut-brain axis. The ontogenetic development of this axis is important because if the axis is sufficiently functional it will provide the individual with the ability to cope and adapt to mental and physical stressors. If an individual is exposed to a stressor that is able to activate the HPA axis soon after birth, it can also affect the developing microbiota towards its imbalance and an inappropriate stress response (O'Mahony et al. 2015). Research conducted on offspring exposed to maternal separation showed a systemic cytokine response, increased gut wall permeability including changes in microbiota composition. This condition has contributed to a greater susceptibility of the GIT to both infectious and chemical inflammatory stimuli (Collins & Bercik 2009). Studies performed in macaques that were separated from the mother showed a reduction in fecal bacteria, mainly *lactobacilli*, three days after separation, together with the occurrence of stress-like behavior. In this vulnerable period, stress leads to a deepening of the dysfunction of the HPA axis and consequently to a significant reduction in resistance to various diseases (Tappenden & Deutsch 2007; O'Mahony et al. 2015). The intestinal microbiota is also immediately affected by the release of signaling molecules, cytokines, and antimicrobial peptides (AMPs) into the intestinal lumen via neurons, immune and endocrine cells by direct or indirect stimulation from the CNS (Wang & Kasper 2014).

3.3.2 How the microbiome affects the brain

The microbiome affects the brain through several mechanisms, namely the neural, endocrine, metabolic and immunological pathways.

3.3.2.1 Nerve mechanism

The nerve mechanism through which information is transmitted from the gut to the brain runs through the enteric nervous system (ENS). It is in charge of all GIT functions (Wang & Kasper 2014). Bacterial products such as endotoxins or inflammatory cytokines such as interleukin 1β (IL–1β) and tumor necrosis factor α (TNFα) can stimulate the vagus nerve. Following this stimulation, the release of proinflammatory cytokines by the intestinal macrophages is suppressed (Collins & Bercik 2009). In a study of rats by Dinan & Cryan 2012 corticoliberin (CRF) in the hypothalamus as well as plasm levels of adrenocorticotropic hormone (ACTH) and corticosterone after vagal stimulation were determined. Two hours after this stimulation, CRF-mRNA in the hypothalamus was increased and plasm ACTH levels were also significantly increased. Elevated plasm corticosterone levels have also been observed. Thus, the effect of the vagus nerve on the activity of the HPA axis has been demonstrated (Dinan et al. 2013).

3.3.2.2 Endocrine mechanism

In regard to the endocrine mechanism of action, the intestinal microbiome has an important role in the development and regulation of the HPA axis. Enteroendocrine cells are scattered in the intestinal epithelium, and these cells secrete hormones and other signal peptides. This secretion occurs in response to luminal stimulus (Wang & Kasper 2014). Bacterial by-products have the ability to stimulate enteroendocrine cells which produce several different

peptides (peptide YY, neuropeptide Y, cholecystokinin, glucagon-like peptides 1 and 2, and substance P). After secretion, neuropeptides are released through the *lamina propria*. They either enter the bloodstream or act locally on sensitive nerve endings. It is not known whether neuropeptides act in a direct mechanism in microbiota–CNS communication. Direct communication between enteroendocrine cells and neurons innervating the small and large intestine has been described by (Kelly et al. 2016) using the so-called neuroepithelial circuit. This circuit can act as a communication channel that transmits messages from the lumen to the ENS and CNS in the opposite direction. It has been found that when the composition of the intestinal microbiota is changed, changes in neuropeptides and neurotransmitters occur. An example is antibiotic induced microbial dysbiosis (ATB). Elevated levels of substance P in the colon have been found in this dysbiosis (El Aidy et al. 2015). Commensals have the ability to produce a large number of neuroactive molecules, such as serotonin, gamma-aminobutyric acid (GABA), catecholamines, melatonin, acetylcholine or histamine (Wang & Kasper 2014). These signaling molecules can reduce inflammation, alleviate the stress response and improve mood. Histamine and catecholamines can stimulate the ENS and CNS through afferent vagus nerve fibers. Dopamine plays a role in controlling GIT functions, such as reducing motility. In the CNS, it is used in learning and creating memory tracks, while it plays an important role especially in the management of emotional states and moods. GABA affects GIT functions (motility, transport, secretion). In the CNS, it plays an important role in the emotional modulation of fear and anxiety (anxiolytic effect). GABA receptors are associated with this effect (Pokorný & Mourek 2014). GABA is a major inhibitory neurotransmitter in the CNS, and dysfunctions in GABA signaling are associated with anxiety and depression (Foster & McVey Neufeld 2013). Cholecystokinin is another neuroactive molecule that is an important hormone of the GIT. Cholecystokinin secretion increases after a meal in relation to the onset of satiety. It is usually part of the response to stress, during which it is released, and also during periods of anxiety and fear (Pokorný & Mourek 2014).

3.3.2.3 Metabolic mechanism

This mechanism is involved in microbiome–gut CNS signaling. The main function of the microbiome is to facilitate host metabolism. The intestinal microbiota produces a large number of substances that can modulate CNS functions. We can include short chain fatty acids (SCFAs) from acetate, propionate and butyrate. These acids are an energy source for colonic epithelial cells and are a by-product of the fermentation of plant polysaccharides by intestinal microbes. SCFAs have anti-inflammatory functions that they induce by binding to a specific G-protein-coupled receptor. These beneficial effects are not limited to the gut, but SCFAs also have a positive effect on diseases that are distant from the gut, such as asthma or arthritis. Beneficial functions also occur through the inhibition of histone deacetylases, which induce regulatory T cells (Treg cells, formerly also suppressor T cells) or suppress the production of proinflammatory cytokines by antigen presenting cells. Furthermore, adenosine triphosphate (ATP) derived from commensal bacteria has been shown to promote the induction of pro-inflammatory Th17 cells in the gut. Th17 cells can gradually colonize peripheral immune organs and thus affect the CNS – specific autoimmunity (Berer & Krishnamoorthy 2014). *In vivo* studies in mice have shown that butyrate regulates energy homeostasis, stimulates leptin

production in adipocytes and induces the secretion of several neuropeptides (e.g., glucagon-like peptide 1). Insulin secretion, glucose and lipid metabolism and (Icaza-Chávez 2013) food intake could be affected by this process. Butyrate also has anti-inflammatory effects and has been found to impact on behavior and mood. Due to these effects, SCFAs increase serotonin release, as confirmed by *in vitro* studies. Wang and Kasper (2014) described changes in fecal concentrations of SCFAs in children with autism spectrum disorders. Altered production of these neuroactive peptides of microbial origin may represent a mechanism by which bacteria may affect brain function (El Aidy et al. 2015).

3.3.2.4 Immune mechanism

The final way to implement gut–brain signaling is the immune mechanism. This pathway is CNS–independent (Wang & Kasper 2014). Hygiene theory states that excessive purity and reduced exposure to bacteria will cause immunoregulatory mechanisms to malfunction. These mechanisms prevent inadequate T–cell responses and the subsequent occurrence of autoimmune and inflammatory diseases. Icaza–Chávez 2013 found that the improvement in hygienic conditions will reduce the microbiological load, and this can cause an increased incidence of autoimmune diseases. In a study of germ-free mice (GF mice), at three weeks of age the mice were colonized by a microbiota of normal mice and the result was a permanently adjusted composition of the intestinal microbiota as well as the development of an anti-inflammatory immune response. The conclusion is that the germ-free period shortly after birth has adverse effects on the immune system. However, if GF mice are colonized by a conventional microbiota in the first weeks of life, these effects are not observed (Icaza-Chávez 2013). The host's immune system is able to recognize and eliminate pathogens (not the body's own substances) and at the same time tolerate its own molecules, which serve to maintain the body's homeostasis. The immune system is dependent on colonization by the intestinal microbiota. The microbiota is important for normal functioning (El Aidy et al. 2015). An important role of the intestinal mucous is to mediate adaptive immunity, as it comes into primary contact with many antigens. It is also involved in phylogenetically inherited immunity and response to specific antigens. These specific antigens are pathogen-associated molecular patterns (PAMPs). This means that the germ-free period shortly after birth has adverse effects on the immune system. Some PAMPs ligands for these receptors contain lipids that are required for their agonist activity. Another example is bacterial LPS (bacterial endotoxins), which are ligands for TLR4s. LPS are an important component of the bacterial cell wall and have the ability to induce an intense innate immune response. TLRs are localized in cells that are part of innate immunity (macrophages, epithelial cells, adipocytes). However, we can also find them in acquired immune cells, namely in B–lymphocytes, T - lymphocytes and in dendritic cells. Dendritic cells are located in the *lamina propria* and play an important role in inducing acquired immunity. TLR–mediated signalling leads to the induction of dendritic cells and the subsequent production of cytokines (Icaza-Chávez 2013). GF mice are characterized by reduced reactivity of Toll–like receptors 2, 4, 5, but their activity increases during colonization. From this, the interrelationships between these receptors and the microbiota can be deduced (Collins & Bercik 2009). During initial microbial colonization, changes in mucosal and systemic immunity occur.

Maturation of the immune system commences in the fetal period. Dysfunctions of many components of the immune system have been reported in the neonatal period and early childhood. Decreased dendritic cell differentiation, impaired phagocytosis, and erroneous interactions between dendritic cells, T cells, and Treg cells have been observed in neonates. Insufficient response to acquired humoral activity, including IgA production in human neonates, is caused by the suppressed activity of transplacentally transferred IgA antibodies. Immune immaturity was also evident in GF mice, where in the first days of colonization, reduced Peyer's patches and reduced helper (CD4+) and CD8+ lymphocytes were identified. Plasma cell and intraepithelial lymphocyte counts were also reduced, as were secretory IgA (sIgA) levels. SIgA aims to prevent bacteria from moving across the intestinal barrier (El Aidy et al. 2015). CD 4+ lymphocytes have an auxiliary and inductive role in the secretion of immunoglobulins, other T lymphocytes play the role of immunoregulators. B lymphocytes make up about 40% of lymphoid cells, producing mainly IgA immunoglobulins. These immunoglobulins are secreted in the form of dimers, and their synthesis and secretion are among the important immune defense mechanisms in the GIT. Their main function is to prevent bacteria from adhering to epithelial cells and to prevent colonization and proliferation of these pathogens. Other functions of IgA include neutralization of toxins, inhibition of viruses and blocking of their transport through the intestinal epithelium. They also affect the balance of metabolic and immune pathways in intestinal epithelial cells. The intestinal microbiota changes the expression of genes involved in fat metabolism and its storage in the presence of IgA. As a result, it alters the homeostatic microbial-immune-metabolic response (El Aidy et al. 2015). Sudden changes in childhood that elicit disease-associated gene responses (metabolic disorders, behavioral changes) can have lasting consequences for human health (El Aidy et al. 2015). The intestinal microbiota in the homeostatic state stimulates the chronic low-level state of activation of the innate immune system. Bacteria stimulate intestinal macrophages and T-lymphocytes. These cells produce pro-inflammatory cytokines and mediate basal activation of the immune system on the surface of the intestinal mucous, which may eventually affect the entire body (El Aidy et al. 2015).

3.4 Polyphenols

Polyphenolic compounds also known as polyphenols are substances which contain two or more hydroxyl groups attached to the aromatic nucleus in their molecule. They are represented in almost all plants, where they perform a variety of functions. These include the protection of plants from oxidative stress, UV radiation and pathogens. Tannins protect plants from being eaten by herbivores, lignans form the mechanical reinforcement of the plant body and other polyphenols can function as signaling molecules. In the human body, polyphenolic compounds have a wide range of biological effects (Rechner et al 2002).

Plant polyphenols are the most common compounds in our diet that enter the redox processes. Their daily intake has been estimated at up to 1 g and is significantly higher than the intake of antioxidant vitamins such as tocopherols, carotenes or ascorbic acid. One third of the daily intake is made up of phenolic acids and the remaining two thirds are flavonoids (Scalbert & Williamson 2000). The intake of polyphenols depends on eating habits and personal preferences when choosing food. This applies not only to the total intake of polyphenols,

different classes of polyphenols, but also to individual phenolic compounds (Hertog et al. 1993). The most accurate research on daily polyphenol intake has been conducted for flavonols, flavones (Hertog et al. 1993) and isoflavones (Reinli & Block 1996). The intake of flavonols (namely quercetin) and flavones in the Dutch population was set at 2-21mg per day (Hertog et al. 1993). For the Japanese, the daily intake of isoflavonoids is 30-40mg (Kimira et al. 1998; Wakai et al. 1999). Consumption in Western countries is significantly lower in comparison due to limited consumption of soy products (Kirk et al. 1999).

3.4.1 Rutin

Rutin (fig. 3) is a flavonol derived from quercetin, more specifically 3,3´,4´,5,7-pentahydroxyflavone-3-rhamnoglucoside (Hosseinzadeh & Nassiri-Asl 2014). Rutin is also known as vitamin P (Khan et al. 2009).

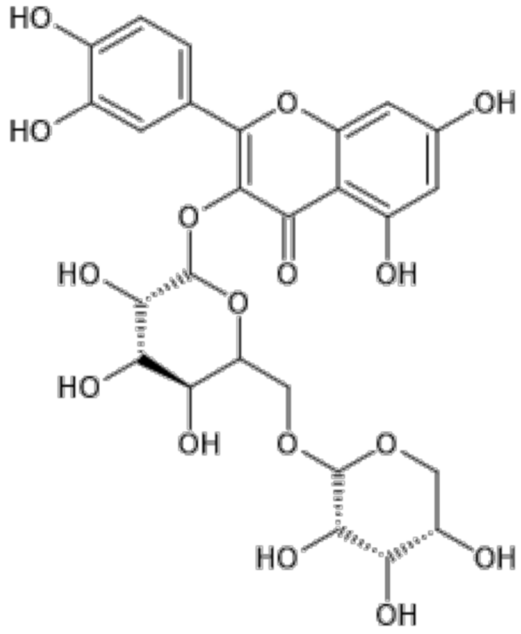

Figure 3: Rutin structure

Rutin is found in a number of edible plants such as black tea, buckwheat, martyrs and apples (Hosseinzadeh & Nassiri-Asl 2014). In metabolism, rutin has anti-inflammatory, anti-allergenic, antioxidant, and anti-cancer effects (La Casa et al. 2000). It is often used as a part of commonly available therapeutics for strengthening the venous wall (Český lékopis, 2009).

3.5 Metabolism of polyphenols

To achieve the overall biological effect of the substance, if it is not a local effect on structures in the digestive tract, it is necessary to cross the enterocyte barrier into the plasma and further distribute the substance to the target structures. The absorption of substances into the body can take place from different parts of the gastrointestinal tract (GIT), which differ in function, structure, pH, enzymatic equipment and the presence of specific transporter systems.

The part in which the substance can be resorbed has a significant effect on the bioavailability of the substance.

3.5.1 Oral cavity

The oral cavity is the first site of absorption of polyphenols from the gastrointestinal tract into the body. Some stilbenes are absorbed by the mucous (Asensi et al. 2002) and it has been shown that saliva levels of epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) in saliva appear when the green tea extract is retained in the mouth for several minutes later also EGC in urine. EGC–3–gallate was thought to be converted to EGC by salivary catechin esterase, and EGC was absorbed through the mucous into the bloodstream and subsequently excreted in the urine. The experiment shows that slow drinking of tea is a suitable method for distributing higher concentrations of catechins in the oral cavity and esophagus (Yang et al. 1999).

Polyphenols ingested with food are exposed to the salivary α–amylase ptyalin in the oral cavity before it is inactivated by an acidic gastric environment. Natural flavonoid glycosides have a β-glycosidic bound sugar component and are therefore not subject to ptyalin degradation.

3.5.2 Stomach

The food proceeds from the oral cavity to the stomach, where it is exposed to low pH caused by the secretion of hydrochloric acid, to create a suitable environment for pepsin protease function. It also forms a barrier against the penetration of microorganisms further into the gastrointestinal tract. Studies show that under normal conditions flavonoids are stable in the gastric environment (Gee et al. 1998; Olthof et al. 2001), although polymeric proanthocyanidins decompose into catechin dimers and free catechins after 3.5 hours in the gastric environment (Heim et al. 2002). However, food remains in the stomach under normal conditions for thirty to ninety minutes and the polyphenols are then transferred with it to other parts of the digestive tract.

3.5.3 Small intestine

The structure of polyphenols is probably too hydrophilic to enter enterocytes by passive diffusion, and specific transporters for polyphenols have not yet been demonstrated. An exception is the Na+ dependent system in the small intestine of rats transporting cinnamic acid (Ader et al. 1996).

The determinant of the fate of flavonoids is their chemical structure. As mentioned earlier, most flavonoids occur in nature as glycosides with β-glycosidic sugar. Glucosides, with a sugar component consisting of glucose, utilize a glucose transporter for transport from the intestinal lumen (Hollman et al. 1995; Hollman & Katan 1997; Gee et al. 1998). Thus, it is possible to explain the rapid absorption of quercetin glucoside from onions versus the slow absorption of rutin (quercetin rutinoside) from tea, as described by Hollman (Hollman & Katan 1997). Experiments with flavonoid aglycones (luteolin, campferol, quercetin) show that they can also pass into the body in the small intestine (Gee et al. 1998). The rate and extent of

absorption of these substances are inconsistent and more aglycones are found in the gut only after artificial oral administration due to the natural β-glycosidic form, which cannot be cleaved by pancreatic enzymes.

The presence of three types of β glucosidases has been demonstrated in the human body (Day et al. 1998). Two of them, glucocerebrosidase (EC 3.2.1.62) and lactase-florizine hydrolase (EC 3.2.1.108) are membrane bound and have been shown to be substrate specific. The third is a cytosolic enzyme found in large amounts in the liver, kidneys and small intestine of mammals. Glucocerebrosidase is a lysosomal enzyme that hydrolyzes glucoceramide from endogenous membrane glycolipids. Lactase-florizine hydrolase is found in the brush border of the small intestine and its primary function is the hydrolysis of lactose. Its hydrophobic part catalyzes the hydrolysis of phlorizine to phloretine and glucose. Hydrolysis of glycosides facilitates their entry into the enterocyte, where they are a substrate for the enzym UDP– glucuronyltransferase, which catalyzes the formation of glucuronic acid conjugates.

Flavonoids of polymeric structures and those that cannot be absorbed in the small intestine enter the large intestine, where they are metabolised by bacterial enzymes, as demonstrated in a study in ileostomy patients whose plasm metabolites cannot be found after rutin administration in contrast to people with undamaged colon (Hollman et al. 1995). Glycosides can be hydrolyzed and the aglycones resorbed or cleaved at the bond between the C3–C4 pyran ring. For example, 3,4–dihydroxyacetic acid and phloroglucinol are formed from quercetin (Winter et al. 1991). The most common products of these reactions are phenylvaleric, phenylpropionic, phenylacetic and benzoic acids.

3.5.4 Large intestine

Only a small portion of the rutin ingested through food is absorbed by the gut. Most of it is metabolized by the intestinal microbiota to a number of substances that are also absorbed. Quercetin and monophenols (3,4-dihydroxytoluene, (3,4-dihydroxyphenyl) acetic acid, (3- hydroxyphenyl) acetic acid and homovanillin were identified in the blood of animals orally administered rutin) (Cervantes-Laurean et al. 2006).

Monoglucosides of quercetin are enzymatically hydrolyzed in the small intestine, thus facilitating the absorption of individual metabolites. Glycosides that do not contain glucose in the sugar moiety are not substrates for these hydrolytic enzymes. Their hydrolysis takes place only by the action of the microbiota of the large intestine. It is possible, therefore, that the absorption of rutin is lower compared to other glycosides and is delayed due to transportation to the colon (Jaganath et al. 2009).

During the colonic process, the antioxidant effects of rutin may occur. Its main metabolites (3,4-dihydroxyphenyl) acetic acid and 3,4-dihydroxybenzoic acid, which also exhibit these properties, can then be concentrated in the colorectal lumen. These substances thus contribute to the maintenance of a healthy intestinal microbita and mucous. The presumed progression of the rutin to the colon was verified when administered to human volunteers. Plasma concentrations of selected metabolites (quercetin and isorhamnetin conjugates) were significantly lower than when glucose–containing glycosides were administered. These can already be hydrolyzed in the small intestine, and therefore their metabolites are previously

detectable (Murota et al. 2010). The colonic environment was simulated in an *in vitro* experiment in which the conversion of rutin was monitored in the presence of a microphore contained in human stool. The amount of β-rhamnosidase-producing bacteria affected the efficiency of hydrolysis to quercetin. The addition of glucose to the reaction mixture facilitated the growth of these bacteria and the hydrolysis was more efficient. Metabolism continues with the conversion of quercetin.

After incubation under the same conditions, the following substances were identified: (3–hydroxyphenyl) acetic acid, (3,4–dihydroxyphenyl) acetic acid, 3,4–dihydroxybenzoic acid, 4–hydroxybenzoic acid and 3–(3–hydroxyphenyl) propionic acid, 1,3,5–trihydroxybenzene, alphitonin and taxifolin (Fig.4). The proportion of individual compounds varied between samples and at the same time depended on the presence of glucose during the incubation. As a result, dietary composition and glucose release in the colon may affect rutin metabolism (Jaganath et al. 2009).

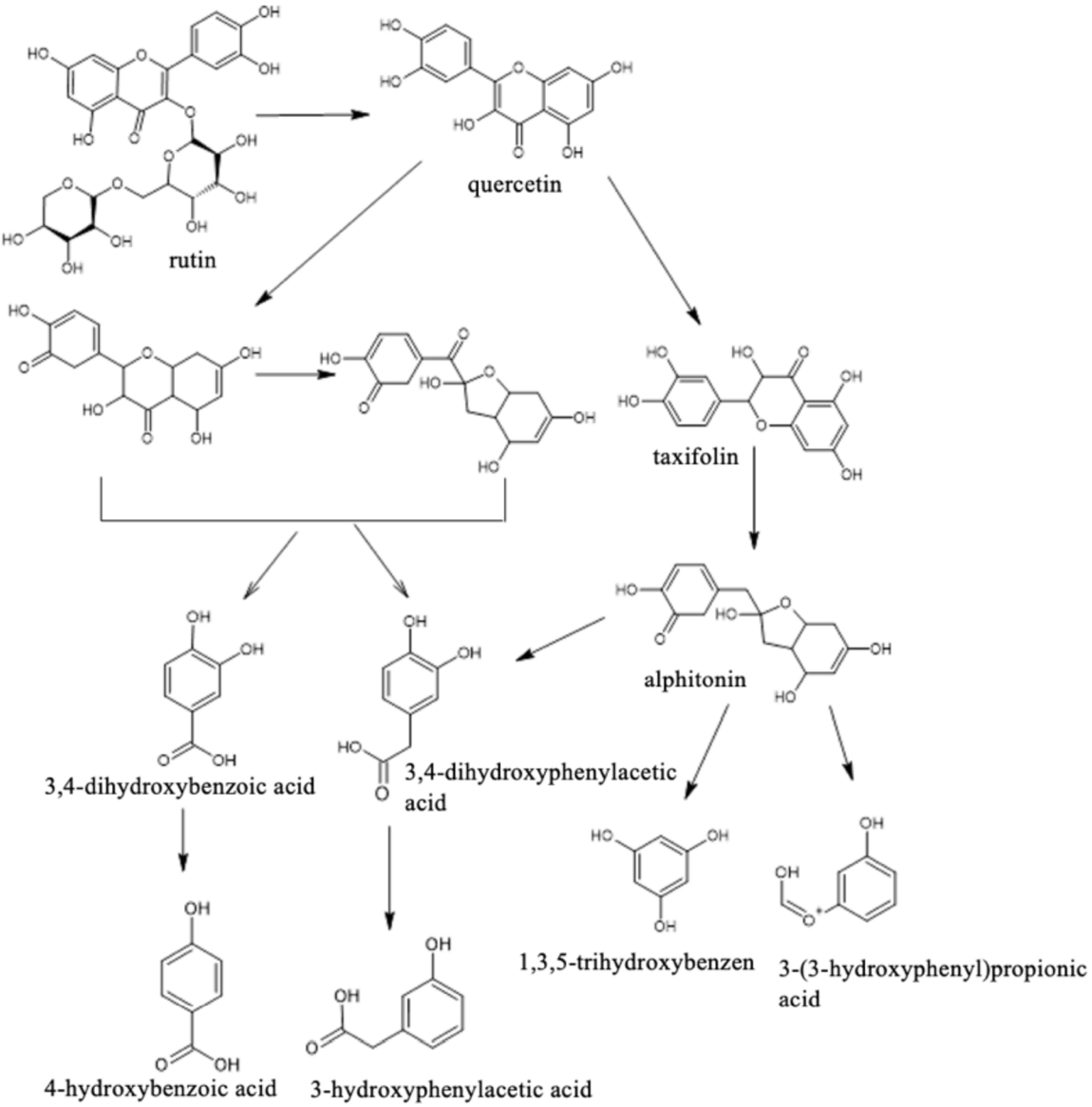

Figure 4: Scheme of metabolism of rutin

The effect of food composition on rutin absorption was investigated in a study in which rats were fed a rutin-containing diet with either pectin or cellulose. The mixture with pectin significantly affected the composition of the intestinal microbita. Plasm concentrations of rutin metabolites (quercetin and isorhamnetin) were significantly higher in this group, indicating better absorption of rutin (Tamura et al. 2007). In an earlier study, the urinary content of individual metabolites was monitored after administration of rutin to humans. 3,4- dihydroxyphenylacetic and 3-hydroxyphenylacetic acids as well as 3-methoxy-4- hydroxyphenylacetic acid were again detected. This was not found after the *in vitro* action of intestinal bacteria, so it is likely to be the product of the next phase of 3,4- dihydroxyphenylacetic acid metabolism, which probably takes place in the liver. These substances were not found in the urine of people after ileostomy (after removal of the colon). This is probably due to the absence of colon bacteria that hydrolyze rutin (Jaganath et al. 2006). Rutin in unchanged form was detected in the blood and lymph of rats given this glycoside directly into the duodenum (Chen et al. 2010) It is possible that hydrolysis by the intestinal microbita, which is generally considered necessary for the absorption of rutin, did not result from dissolution in polyethylene glycol 400 (PEG)/ethanol (4:1). PEG can generally improve the solubility of a substance and facilitate its passage across biological barriers; in this case apparently across the intestinal wall (Cermak & Wolffram 2010).

3.6 Health effects of rutin

3.6.1 Antioxidant effect

Reactive oxygen radicals play an important role in kidney damage caused by ischemia and subsequent reperfusion. Rutin has protective properties, probably by radical inhibition and antioxidant activity. Rats (*Wistar albino*), which had their kidneys removed unilaterally, were ingested for testing and were subjected to a 45 minute closure of the left renal pelvis for 45 weeks, followed by reperfusion for 3 hours. One hour before induction of ischemia, they were given either rutin (1 g/kg) or saline solution. At the end of the reperfusion period, kidney samples were taken to determine malodialdehyde and glutathione levels, manganase superoxide dismutase (MnSOD) activity, and histological examination. Concentrations of serum creatinine, blood urea nitrogen and lactate dehydrogenase were measured to determine renal function.

The ischemia/reperfusion process caused a significant decrease in glutathione levels and manganase-superoxide dismutase activity, which in turn caused a significant increase in malondialdehyde levels in the renal tissue. Similarly, serum creatinine, nitrogen and lactate dehydrogenase levels increased compared to the control group. Pre-procedure rutin administration significantly alleviated renal dysfunction, suppressed elevated malondialdehyde levels, and restored depleted MnSOD activity and glutathione levels. These beneficial changes in biochemical parameters have also been associated with changes in histopathological findings (Korkmaz & Kolankaya 2010).

The processing of rutin into an aqueous nanostructured dispersion has resulted in improved photostability and prolonged *in vitro* antioxidant activity. Antioxidant activity was evaluated by the formation of a hydroxide radical formed after exposure to hydrogen peroxide

to UV radiation. Rutin nanostructures decomposed less compared to rutin ethanol solution. The presence of a polymer layer in the nanoparticles is important to prolong the antioxidant activity (Almeida et al. 2010).

3.6.2 Anti–inflammatory effects

Rutin can be averted by the negative effects of septic arthritis caused by the yeast *Candida albicans*, the main pathogen causing fungal arthritis. Rutin (1 mg) was administered intraperitoneally in three daily doses 24 hours after infection. Edema was measured for 17 days. The results showed that rutin reduced edema by 45 % on day 11. After another 6 days, there was a further reduction of 35 %. The anti–artitic activity of rutin has been found to be due to its ability to inhibit nitric oxide production from proliferating macrophages and T–lymphocytes. This flavonoid also inhibits the growth of *Candida albicans* yeast cells and does not cause hemolysis. These data suggest that rutin, which has both antiarthritic and antifungal properties, can be safely administered into the bloodstream to treat septic arthritis (Han 2009).

With age, leucine, an amino acid that stimulates protein synthesis, decreases in the muscles. Rutin and other antioxidants alleviate inflammation and improve the anabolic response in muscles. A study in 20 rats (*Wistar*) given rutin, vitamin E, vitamin A, zinc and selenium for 7 weeks found that the antioxidants administered did not directly affect muscle tissue, but increased antioxidant and anti–inflammatory protection in other organs, e.g. liver and spleen, thereby improving the oxidative state in the muscles (Mosoni et al. 2010).

3.6.3 Cytoprotective effects

Bifentrin, a synthetic pyrethroid with a broad spectrum of insecticidal activity, induces oxidative stress in human erythrocytes. In an *in vitro* study, the antioxidant effect of quercetin and rutin was demonstrated. Incubation of erythrocytes with bifenthrin (42.2; 211 and 1055 ppm) increased malondialdehyde and lipid peroxidation and decreased superoxide dismutase and catalase activity. Rutin (80 μM) administered before bifenthrin suppressed elevated malondialdehyde levels. Together with quercetin (80 μM), they reversed the inhibitory effect on catalase activity. Quercetin alone had a greater effect on superoxide dismutase activity (Sadowska-Woda et al. 2010).

The effect of rutin was also studied on erythrocytes exposed to another frequently used pyrethroid, β-cyfluthrin. Erythrocytes were incubated with three concentrations of pyrethroid (0.43; 215 and 1075 ppm) or first with flavonoids and then with β-cyfluthrin. All concentrations of β-cyfluthrin suppressed the activity of catalase, the activity of superoxide dismutase was significantly reduced only by the highest concentration of the substance, which also caused hemolysis of 36 % of erythrocytes. Rutin and quercetin suppressed hemolysis and increased antioxidant enzyme levels (Sadowska-Woda et al. 2010).

When testing the effect of rutin on peroxide-induced apoptosis in umbilical vein endothelial cells, it was found that preventive administration of rutin significantly alleviated peroxide-induced apoptosis. Rutin pretreatment has been shown to significantly reduce the formation of reactive oxygen species in endothelial cells exposed to hydrogen peroxide. In addition, rutin prevents the increasing formation of DNA fragments, glutathione depletion, and inhibition of mitochondrial membrane collapse, which protects the umbilical vein from

oxidative damage and helps restore its integrity more rapidly. Rutin (50 μM) blocks apoptosis by reducing reactive oxygen radicals, increasing glutathione, restoring mitochondrial membrane tension and thus preventing DNA damage (Gong et al. 2010).

Launaea procumbens (*Asteracea*) is used to treat renal disease, rheumatism and painful urination. The methanol, chloroform, ethyl acetate and n-hexane fractions of the extract were tested against carbon tetrachloride nephrotoxicity in rats.

Rats divided into 16 groups were given carbon tetrachloride at a dose of 3 ml / kg body weight (30 % in olive oil, i.p.) twice a week for 4 weeks. The other groups were orally administered an extract (100, 200 mg/kg body weight) or rutin (50 mg/kg) 48 hours after carbon tetrachloride application or 200 mg/kg carbon tetrachloride-free extract again twice a week for 4 weeks. The action of carbon tetrachloride significantly created oxidative stress in the kidneys. Extract and rutin and suppressed the effects of oxidative stress and increased levels of enzyme antioxidants (catalase, superoxide dismutase and glutathione). Positive effects of extract and rutin fractions have also been reported on renal function and decreased urobilinogen, urea, albumin, urinary creatinine, and histopathological findings by reducing glomerular atrophy, tubular degeneration, capillary blockage, epithelial necrosis, and edema (Khan et al. 2009).

3.6.4 Antitumor effects

Reactive oxygen species are potent inducers of vascular endothelial growth factor (VEGF) angiogenic hormone. Although rutin in combination with vitamin E has a synergistic effect in inhibiting oxidative damage, it is unclear whether the combination of the two substances inhibits VEGF secretion in tumor cells.

Using the human promyelocytic leukemia (HL-60) cell line, rutin in combination with vitamin E has been shown to have anticancer effects by suppressing tumor cell vascularization, synergistically reducing VEGF protein and mRNA expression. Furthermore, these substances have been shown to significantly reduce the binding capacity of nuclear factor activator protein-1 (AP-1(to the VEGF gene promoter and also to reduce cJun protein expression. It also acts synergistically to reduce insulin receptor substrate-1 IRS-1 protein expression in HL-60 cells. These results demonstrate that rutin alone and in combination with vitamin E has anticancer effects (Chuang et al. 2010).

Rutin induces apoptosis in WEHI-3 leukemia cancer cells in BALB/c mice. Spleen enlargement is typical for this type of cancer. Rutin reduces the percentage of the Mac-3 marker, indicating that differentiation of macrophage precursors and immune system T-cells has been inhibited, and reduces spleen weight. Rutin promotes macrophage phagocytosis activity (Chen et al. 2010).

3.6.5 Effects on the cardiovascular system

The effect of rutin on lysosomal enzymes was studied in rats with isoproterenol-induced cardiotoxicity. Male Wistar rats were pre-administered with rutin 80 mg / kg once daily for 42 days. After this time, they were injected subcutaneously with isoproterenol at a dose of 100 mg /kg every 24 hours for two consecutive days. Serum creatine kinase-MB activity and serum troponin T levels and serum and cardiac lysosomal enzyme activities (β-glucuronidase, β-N-acetylglucosaminidase, β-galactosidase, cathepsin-B and D) were monitored, all of which

had significantly increased activity and levels in rats with cardiotoxicity. Experimentally induced cardiotoxicity in rats also led to a decrease in membrane stability, which was reflected in a decrease in β-glucuronidase and cathepsin-D activity in the mitochondrial, nuclear, lysosomal and microsomal fractions.

Rutin administration prior to isoproterenol administration to rats prevents changes in the activity of these enzymes. The mechanism of action of rutin is to protect the lysosomal membrane against isoproterenol-induced heart damage and results from the ability of rutin to scavenge free radicals, antioxidants and stabilize membranes (Stanely Mainzen Prince & Priya 2010).

3.6.6 Effects on the nervous system

The ability of rutin to inhibit neurodegeneration and affect memory was tested in rats. The Morris Water Labyrinth, a round pool 145 cm in diameter and 45 cm high, filled with water to a height of 32 cm and divided into 4 quadrants, was used to assess the effect of the rutin. The spatial orientation and the ability to remember the route were monitored. Spatial memory loss and CA3 loss of pyramidal neurons in the hippocampus were induced in rats by a single dose of trimethyltin (8.5 mg/kg) orally. Rutin administration significantly reversed memory impairment and pyramidal neuron destruction in the CA3b region of the hippocampus (Koda et al. 2008).

During ischemia-reperfusion injury to the brain, nerves are damaged by free radicals. This condition was induced in rats by closure of the central cerebral artery for 2 hours and subsequent reperfusion for 22 hours. Rutin was administered at a dose of 25 mg/kg once daily for 21 days prior to artery occlusion. In rats, rutin administration was found to reduce ischemic nerve apoptosis by reducing p53 protein expression. Prevention of morphological changes and increased endogenous antioxidant enzymatic activity were observed. According to the authors of the thesis, rutin is a promising substance in reducing the risk or improving brain function in difficulties caused by ischemia and reperfusion (Khan et al. 2009).

The anticonvulsant effect of rutin was studied in pentylenetetrazole-treated rats (90 mg / kg, i.p.). During testing, the animals were given an aqueous solution, rutin and diazepam. The effect of rutin is dose-dependent (it was administered at concentrations of 25, 50, 150 μM), rutin had the best results at a concentration of 150 μM, it delayed the onset of seizures the most compared to the aqueous solution. Administration of flumazenil (5 μM) abolished the anticonvulsant effect of rutin. These results demonstrate that rutin has an anticonvulsant effect through the GABAA-benzodiazepine receptor complex. It is possible that rutin is a ligand for the benzodiazepine receptor (Korkmaz & Kolankaya 2010).

Demographic aging is causing an increase in the population with cognitive deficits and age-related behavioral disorders, which may be associated with an increasing prevalence of neurodegenerative disorders such as Alzheimer's disease. In this disease, a decrease in the cholinergic system has been observed, which plays a major role in memory formation. A protective role for quercetin and rutin was observed in a *Danio rerio* (Danio striped) fish model with scopolamine-induced amnesia or disruption of cholinergic neurotransmission. Flavonoid administration prevented scopolamine-induced memory impairment. None of these substances

affected the mobility of the tested fish. The results suggest that these substances could have promising uses in medically neurodegenerative diseases (Richetti et al. 2011).

3.6.7 Hypoglycaemic effects

The antidiabetic action of rutin was tested in adult rats. Diabetes was induced by intraperitoneal administration of streptozotocin (STZ) at a dose of 60 mg/kg body weight dissolved in 0.01 M citrate buffer. After 48 hours of STZ administration, glycaemia was measured with a glucometer and only subjects with glycaemia above 250 mg/dl were included in the study. After 7 days, rutin in propylene glycol solution was administered intraperitoneally in a single weekly dose of 50 mg/kg, for 45 days. Rutin was administered to non-diabetic and diabetic rats. rutin administration significantly reduced hyperglycemia in diabetic rats. The level of total cholesterol, LDL triacylglycerides was reduced to the original value, the level of HDL cholesterol increased (Fernandes et al. 2010).

In another study in rats (Wistar), in which diabetes mellitus was caused by streptozocin administration, rutin was administered to control and diabetic rats for 45 days at doses of 25, 50 and 100 mg/kg. Levels of fasting plasma glucose, glycated hemoglobin, thiobarbituric acid and its reactive substances, lipid peroxidation, levels of insulin, C-peptide, total hemoglobin, proteins, non-enzymatic antioxidants were monitored.

Following the administration of rutin to diabetic rats, there was a significant increase in body weight. It did not have this effect on healthy rutin individuals. Compared to the behavior of the control diabetic group, the rutin group showed reduced food and water intake, again no significant changes were observed in normal individuals with rutin. Rutin administered at the three concentrations mentioned caused a decrease in plasma glucose of 44.36 %, 50.92 %, 62.73 % to sick individuals. Administration of the same doses to healthy individuals had no significant result. These findings indicate that rutin at 100 mg/kg produced the greatest effect. Furthermore, rutin increased the levels of plasma insulin, C–peptide, total proteins, total hemoglobin and decreased the level of glycated hemoglobin compared to diabetic control rats.

Rutin, with its hypoglycemic and antioxidant effects, plays a significant role in delaying the late complications of diabetes, resulting from elevated blood glucose levels and the subsequent formation of oxidative and glycosylation products (Kamalakkannan & Prince 2006).

3.7 *In vitro* model of gastrointestinal tract

An *in vitro* system is defined as an environment outside a living organism that provides a simple and controlled system aimed at studying ecological interactions. A major problem in studying the metabolism of the gut microbiome *in vitro* is the lack of interactions with the host. The ability to model gut-like environments *in vitro*, especially for high-throughput analysis, makes *in vitro* studies a good starting point for *in vivo* studies (Saeidnia et al. 2015). If the human gut is the particular focus, several types of modelling systems are used.

The most prominent systems mainly include conventional in-line fermenters with various modifications as well as multistage continuous fermenters with or without the addition of components to simulate interactions with the human host, for example by adding human cells to normal culture systems (Paul et al. 2018).

The in-line fermenters are the simplest and most widely used models. Although they are closed systems where we have to add all the important components to initiate microbial growth at the beginning, we can then adjust the pH of the system and gradually add important nutrients. We can then monitor the growth of the strain by conventional methods such as flow cytometry, optical density measurements or plate counts (Falony et al. 2009). The main disadvantage of this method is the inability to quantify steady states. Examples of standard in-vessel fermentation systems also include in-vessel reactors, tubes, microtiter plates.

Continuous culture systems, in contrast to batch fermenters, allow the inflow and outflow of the medium to be balanced, thus maintaining bacterial cultures in a specific growth rate and physiological state. By combining several continuous reactors, several different environmental conditions can be modulated in a stepwise manner. For example, pH, residence time in the medium can be varied, and additionally, the addition of specific components at different stages can modulate the conditions of different compartments of the intestinal tract (Paul et al. 2018). It is clear that continuous culture models inoculated with human fecal samples are unable to reproduce the exact composition of the microbial state observed in the human gut. The limitation, of course, is that, notwithstanding the fact that some kinetic aspects such as the transit time of the gastrointestinal tract are almost exactly preserved in such models, the presence of the host is quite ignored.

However, continuous culture models are a rapid screening tool for xenobiotic transformations to investigate the effect of nutraceuticals. Examples of standard continuous culture systems include chemostats and minibioreactors (Auchtung et al. 2015).

The use of a microtiter plate is a specific type of inoculated fermentation system that offers a low-cost, high-throughput method of investigating a large number of bacterial interactions at once. Because the volume on microtiter plates is small, it is not as easy to control pH and add new substrates as it is with a full-scale implantable system (Duetz 2007).

Clearly, no culture system has yet been able to completely mimic the microbiome *in vivo*. However, the best results are shown by the gut-on-chip method (Kim et al. 2016), it is one of the most advanced methods to investigate the gut microbiome, allowing the study of the interaction between live mucus-producing intestinal epithelial cells and bacteria in an environment with controlled oxygen levels (Jalili-Firoozinezhad et al. 2019). (The authors of the paper described the design of the modeling device, epithelial and endothelial cells of human cell lines were placed in the anaerobic chamber, and sensors for oxygen determination were placed above and below. A porous membrane matrix separated the two cell layers (Marzorati & Van De Wiele 2016).

Combinatorial culture of strains in microtiter plates with the aim of subsequent optical density measurement and sequencing of 16S ribosomal RNA to parameterize the mathematical model have also been used (Venturelli et al. 2018).

It can be argued that given the complexity of interactions and the multiplicity of microbial strains in the gastrointestinal tract, the best way to reveal this complexity is through controlled, repeatable *in vitro* experiments. However, as mentioned many times, we cannot do without *in vivo* experiments.

An alternative technique for studying the gut microbiota is the use of bioreaction systems that allow the study of gut bacterial metabolism *ex vivo*. These bioreactors allow the study of metabolic events over time in conditions very similar to the actual environment of the

human gut. Regardless of the fact that the bioreactor systems are completely artificial (therefore we have to be very careful in interpreting the results obtained), the approach to studying the human microbiome includes a key advantage, namely the possibility to control the conditions of the ecosystem over time. Because of this, the technique has seen a sharp increase in popularity and so is now widely used in studies of gut metabolism (Purohit 2018).

The study of metabolism and internal relationships between different bacterial species is challenging mainly because individual cultures have unique nutritional and environmental requirements for their development and life functions that are not entirely achievable using conventional culture methods. Bioreactors provide a solution to overcome the many limitations of conventional techniques described above. The establishment and propagation of microbial cultures is enabled by the tunability of conditions within the bioreactor that can mimic those of the gastrointestinal tract. Bioreactors facilitate the study of complex microbial interactions and disorders *in vitro* in a controlled environment without confounding biotic and abiotic variables (Wissenbach et al. 2016).

Metagenomic studies of the human gut microbiome have provided a rough overview of metabolic reactions within the ecosystem (Wang & Achkar 2015), but according to the authors of this paper, the next step to understanding the functioning of the microbiome-host system must be a more detailed understanding of the individual metabolic pathways. Potential metabolism can be predicted based on metagenomic data, however, there is a significant difference between gene information and the metabolomic phenotype of a particular microbiome (Wang & Achkar 2015).

In metabolomic studies, the aim is to obtain a list of metabolites of a cell or tissue, and usually a combination of two methods is used: nuclear magnetic resonance (NMR) and mass spektrometry (MS) (Dumas et al. 2014). Each of these methods has its strengths and weaknesses, so NMR is quantitative in nature but not as sensitive. Mass spectrometry has higher sensitivity but needs standards for quantification. Given the complexity of intestinal metabolism, the best method of investigating it is to screen the overall profile of substances and then modulate and confirm metabolic pathways by these narrowly focused techniques. Dual mass spectrophotometry associated with liquid chromatography (LC-MS/MS) is used. Finally, MS/MS spectra of all metabolites are available, which are then quantified using tools such as XCMS online (Smith et al. 2005). Using XCMS, we are able to normalize the spectral data and obtain quantitative data from fullscan (Ivanisevic et al. 2013). The information obtained is then used in databases such as Metlin (Smith et al. 2005) or MassBank (Horai et al. 2010), and finally substances can be identified. The identification of metabolic events and reactions themselves can also be automated using other tools, which are modern databases. One example is Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000). It is desirable to complement the data obtained from mass spectrometry results with targeted analyses aimed at confirming the existence of certain key substrates.

There is much debate in metabolomics about the relationship between global and targeted analysis in the study of gut microbiome metabolites, so we need to figure out how we can combine the two methods to obtain the most meaningful biological results.

As mentioned, one-dimensional proton resonance, NMR, is also used to study intestinal metabolism, but these are small molecules. The methods are complementary because they extend the spectrum of detectable substances.

The most dramatic disadvantage of analyzing isolated metabolomic data is that it is not possible to predict which bacterial species have activity that would be responsible for the production of certain detected metabolites in a phylogenetically highly diverse gut microbiome. Groups of scientists have already attempted to bioinformatically assess the metabolic functions of the microbiome in relation to metagenomic data. Thus, other researchers have attempted to compare the activity of a single isolated bacterial culture with global metagenomic data. Regardless, while both methods were beneficial and researchers came to a greater understanding of the correlation between experimental and model data, it is important to understand that bacterial cultures that grow in isolation in an artificial bioreactor environment have a different metabolic profile than those found in natural conditions. A partial solution to this problem lies in the use of the stable isotope probing (SIP) method, the principle of which is based on the introduction into a given ecosystem of isotopically labeled substrates that are incorporated into metabolites in the form of fatty acids, proteins, DNA and RNA (Horai et al. 2010). Thus, the combination of metagenomic approach with metabolomics and SIP (Eyice et al. 2015) is becoming a central and fundamental method to investigate metabolic activity within a microbial ecosystem. Although the use of the SIP method has its drawbacks when investigating human and animal metabolism, this method still remains the best in studying the ex vivo metabolism of the gut microbiota, where we can arbitrarily adjust the conditions and control the amount and composition of substrates entering the system.

4 Methodology

4.1 Chemicals

NH4HCO3 (amomonium bicarbonate), NaHCO3 (sodium bicarbonate), Na2HPO4 (monosodium phosphate), KH2PO4 (Monopotassium phosphate), MgSO4 (Magnesium sulfate), CaCl2 (chlorid vápenatý), MnCl2*4H2O (Manganese(II) chloride), CoCl2*6 H2O (Cobalt(II) chloride), FeCl3 (Iron(III) chloride), tryptone, glucose, maltose, yeast extract, vitamin K1, hemin, resazurin, cystein hydrochloride, 1M NaOH (sodium hydroxide), Na2S*9 H2O (sodium sulphate nonahydrate), 6M HCl (hydrochloric acid), NaN3 (sodium azide), (CH3)2SO (dimethyl sulfoxide), rutin stock solution and nitrogen gas.

4.2 Fermentation medium

The fermentation medium was prepared from 2,25 g of tryptone, 2,25 g of glucose, 1,125 g of maltose, 2,25 g of yeast extract, 50,7 µl vitamin K1 (0,5 mg/l of metanol), 5,07 mg of hemin (these compounds were dissolved in 450 ml distilled water), 225 ml of CO3 buffer solution (4 g of NH_4HCO_3, 35 g $NaHCO_3$, 1 l of distilled water), 112,5 µl of macromineral solution (2,5 g of $CaCl_2$, 2,5 g of $MnCl_2{\cdot}4H_2O$, 0,25 g of $CoCl_2{\cdot}6\ H_2O$, 1,25 g of $FeCl_3$, 25 ml of distilled water), 225 ml of macromineral solution (5,7 g of Na_2HPO_4, 6,2 g of KH_2PO_4, 0,3g of $MgSO_4$, 1 l of distilled water) and 1,125 ml of 0,1% resazurin solution.

4.3 Sodium Phosphate buffer and Reducing solution

Phosphate buffer, which is used for preparation of a solution from stool samples, was created of 390 ml 1/15M KH_2PO_4 and 610 ml of 1/15M Na_2PO_4 (pH 7). The reducing solution was prepared from 312,5 mg of cystein hydrochloride, 2 ml of 1M NaOH, 101,5 mg of $Na_2S \cdot 9\ H_2O$ and 50 ml o distilled water.

4.4 Rutin Preparation

Stock solution of rutin was prepared by dissolution in DMSO (dimethylsulfoxid). The working solution was created by dissolving 500µl of stock solution of rutin in v 2000 µl of fermentation medium. The concentration of rutin in the working solution corresponded to 2 mg/ml.

4.5 Faecal Slurry Preparation

Stool samples were collected from 20 healthy volunteers of two age groups (up to 45 years and over 70 years). The volunteers had no dietary restrictions and indigestion. Sampling was performed in disposable kits and stool samples were sealed in an anaerogen bag (Biomérieux, Lyon, France). Subsequently, they were processed within 2 hours of collection. A sample of fresh stool (24 g) from the donor was co-homogenized with sodium phosphate buffer (75 ml) in a stomacher (Laboratory Blender, Stomacher® 400 Circulator, EU) and then filtered through a nylon filter. The resulting fecal mixture had a concentration of 24 %.

4.6 Preparation of 96 deep-well plates

The sodium phosphate buffer and fermentation medium were boiled for 7 minutes and then cooled to 37 ° C with constant nitrogen access. The pH was adjusted to 7 with 6M HCl. 835 µl of fermentation medium and 40 µl of reducing solution were pipetted into 96-deep-well plates. Subsequently, the plates were sealed in vacuum bags together with the anaerogen. The plates thus prepared were stored at 4 °C until the next day.

4.7 Preparation of the rutin solution

The 10 mg/ml rutin stock solution was prepared by dissolution in DMSO. The working solution was created by dissolving a 500 µl stock solution rutin in 2000 µl fermentation medium. The concentration of rutin in the working solution was 2 mg/ml.

4.8 Fermentation process

To the plates prepared the previous day, 100 µl of fecal suspension or sodium phosphate buffer for the control sample and 25 µl of rutin solution (final concentration 50 µg/ml) or DMSO as control were added. Again, the plates were sealed in vacuum bags together with the anaerogen and placed in an incubator (37 °C, 100 rpm). Samples were taken sequentially from the plates at times of 0, 2, 4, 8 and 24 hours. At these times, 950 µl of the sample were taken

from each well within 1.5 ml of Eppendorf microtubes and mixed with 50 µl of sodium azide (the final concentration of NaN_3 in the samples was 1.5 mg/ml). The samples prepared in this way were stored at -80 °C until the final analysis and stored in a thermostat at 37 °C in an anaerobic environment.

4.9 Preparation of samples for NMR analysis

Before measurement, the samples were thawed at room temperature, vortexed and centrifuged (4 ° C, 15,000 rpm, 10 min) to separate the solid and liquid phases. Then 600 µl was collected, to which 66.66 µl of NMR buffer (1.5M phosphate buffer, pH 7.4 0.2 % sodium azide and 5mM TSP in D2O) was added. The solution was centrifuged again (4 ° C, 5 min, 15,000 rpm) and 600 µl of the supernatant was pipetted into NMR cuvettes.

4.10 NMR analysis

The spectra were measured on the Bruker Avance III spectrometer equipped with the SmartProbe broadband observation probe (BBFO) with Z-axis gradients (Bruker BioSpin GmbH, Rheinstetten, Germany), which operates at a proton frequency of 500.23 MHz. The measurement temperature was 298 K (25 °C). 1H NMR spectra were obtained and processed under the same conditions. To suppress the water signal, the pulse sequence noesy1d at 4,704 ppm was used. A one-dimensional 1H experiment with the following parameters was used for each sample: number of NS scans 64, number of data points 64k at a spectra width of 16 ppm, relaxation delay 1 s, acquisition time 4 s, mixing time 0,1 s. Instrument tuning, 90o pulse calibration and chiming have been optimized automatically using standard automatic routines (atma, lock, rga, pulsecal and topshim). The free precession signal (FID) was processed by zero filling, line broadening 0.3 Hz and exponential multiplication prior to the Fourier transform. Spectra were manually phased and referenced to TSP 0.00 ppm in Topspin. Alignment and export of spectra was carried out in the Mestrenova program, annotation of substances and quantification in the program Chenomx 8.5. and XCMS.

4.11 UHPLC/Q-TOF analysis

The analysis was performed on a UHPLC/MS system consisting of a Dionex Ultimate 5000 liquid chromatograph (Thermo Fisher Scientific, USA) and a Q-TOF ultra-high resolution, high precision mass spectrometer (HRAM) IMPACT II (Bruker Daltonik, Germany). For chromatographic separation by gradient elution, an Acclaim RSLC 120 C18 column (2.2 µm, 2.1 × 100 mm, Thermo Fisher Scientific, USA) and a combination of mobile phases of 0.1% formic acid (solution A) and methanol (solution B) were used. The gradient elution started at 2% phase B (0-1 min) and continued at 100% B at 18 min followed by a wash (up to 22.5 min) and a 10-min equilibration of 2% A. The column was tempered at 35 °C and the mobile phase flow rate was set at 250 µl/min. The sample injection volume was 5 µL. The samples were measured by ESI ionization in positive mode, and spectra were collected at a mass resolution of more than 60,000 and a scan rate of 1 Hz over a mass range of 60 - 1,500 m/z.

4.12 Statistical analysis

Statistical analysis was performed in Statistica and IBM SPSS Statistics 27. With a total of 20 samples, a two-sample t-test between group A and group B for each timepoint separately for treated and control samples and a paired t-test between treated and control samples for each timepoint were performed.

5 Results

Outputs from *in vitro* fermentation in 96-well deep-well plates analyzed by NMR and ultra-high resolution UHPLC/Q-TOF were evaluated in Chenomx 8.5 and XCMS software. The data obtained were used to determine the major catabolites of rutin and to evaluate the effect of rutin on the fecal metabolome in 20 donors of two age groups.

5.1 UHPLC/Q-TOF

The formation and development of rutin metabolites in 20 donors of two age groups was studied. The first group (A) consisted of donors under 45 years of age, the second group (B) consisted of donors over 70 years of age. Rutin was degraded into individual metabolites during fermentation by the gut microbiota. Five metabolites were identified in the analyzed samples using XCMS software. Among the metabolites identified were, taxifolin, quercetin-3- galactoside, phloroglucinol, quercetin, 3-hydroxyphenylacetic acid. Figure 5 shows the structural formulas of the individual rutin metabolites.

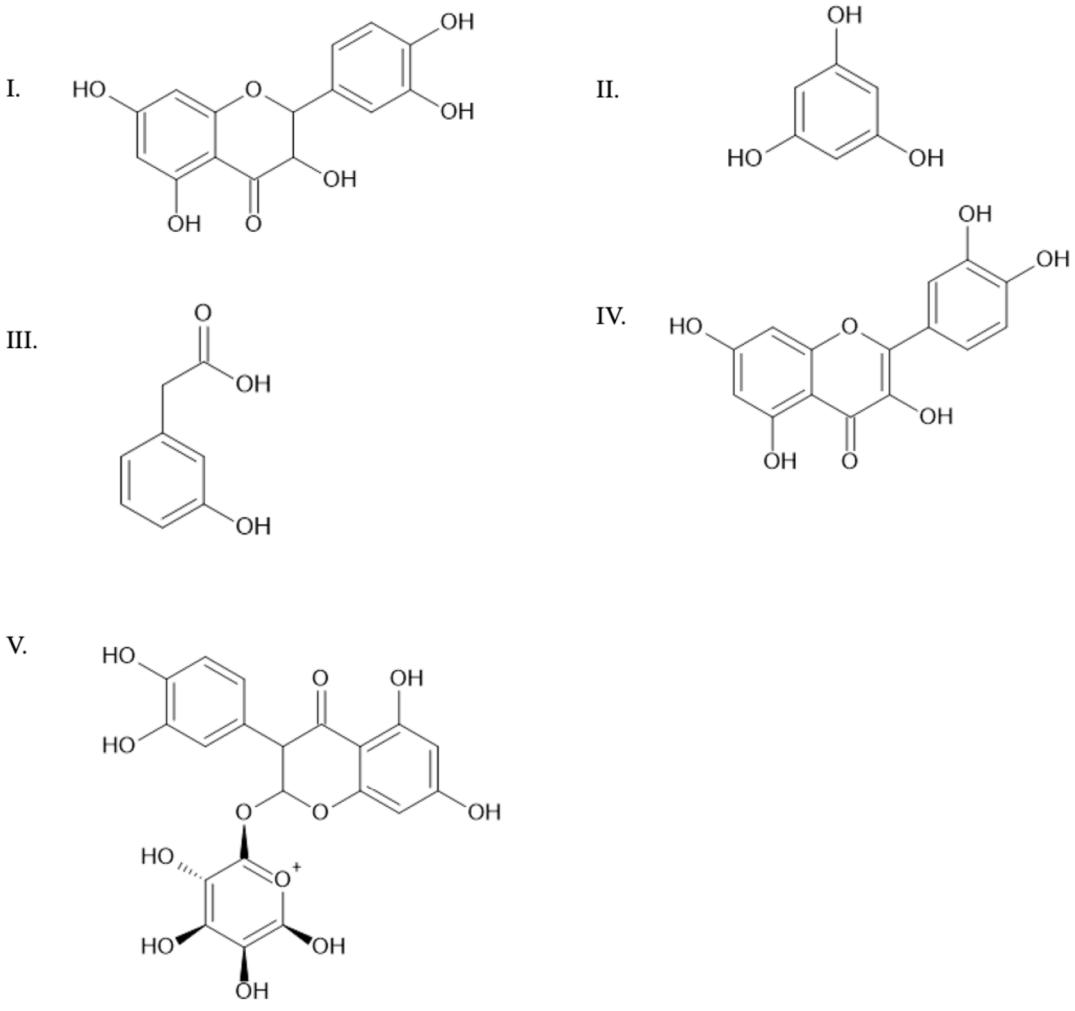

Figure 5: Structural formulas of individual rutin metabolites; I. taxifolin, II. phloroglucinol, III. 3-hydroxyphenylacetic acid, IV. quercetin, V. quercetin-3-galactoside,

Figure 6 shows graphically the difference in rutin concentration as a function of time. The intensity of rutin metabolism varied between donors. In donors, the amount gradually decreased from time 0 to 24 hours.

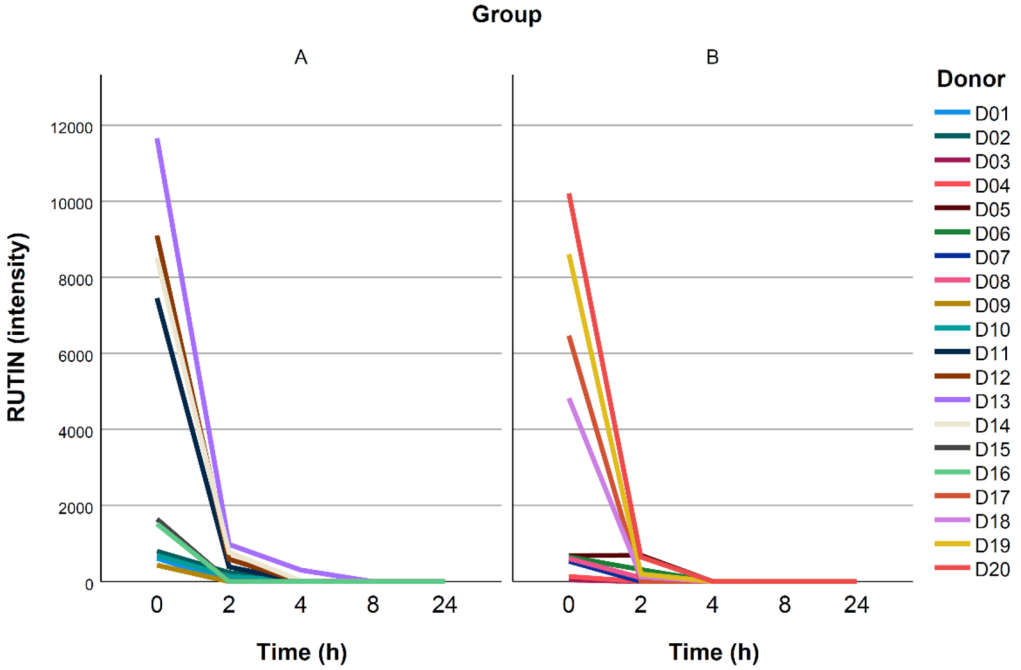

Figure 6 Graph of rutin intensity measured by UHPLC/Q-TOF versus time by individual donors from age group A and B, N = 20; age group A includes donors up to 45 years, age group B includes donors over 70 years

Table 1 shows the dependence of individual compounds on time from all donors in the treated variant and control. A statistically significant difference was observed in the intensity of metabolism of phloroglucinol, quercetin, quercetin-3-galactoside, rutin, taxifolin and 3- hydroxyphenylacetic acid.

Paired sample T-test					
Hour Compound	0h	2h	4h	8h	24h
Phloroglucinol	0,01	x	x	x	x
Quercetin	0,01	0,01	x	x	x
Quercetin-3-galactoside	0,01	0,01	x	x	x
Rutin	0,01	0,01	x	x	x
Taxifolin	0,03	x	x	x	x
3- hydroxyphenylacetic acid	x	x	x	x	0,01

Table 1: Identified compounds by UHPLC/Q-TOF, paired t-test between control and treatment in timepoints; p-values; x: $p > 0,05$

Figure 7 shows graphically the dependence of rutin and quercetin concentration on time in the treated variant. A statistically significant difference was observed in the intensity of metabolism at timepoint 0 h and 2 h.

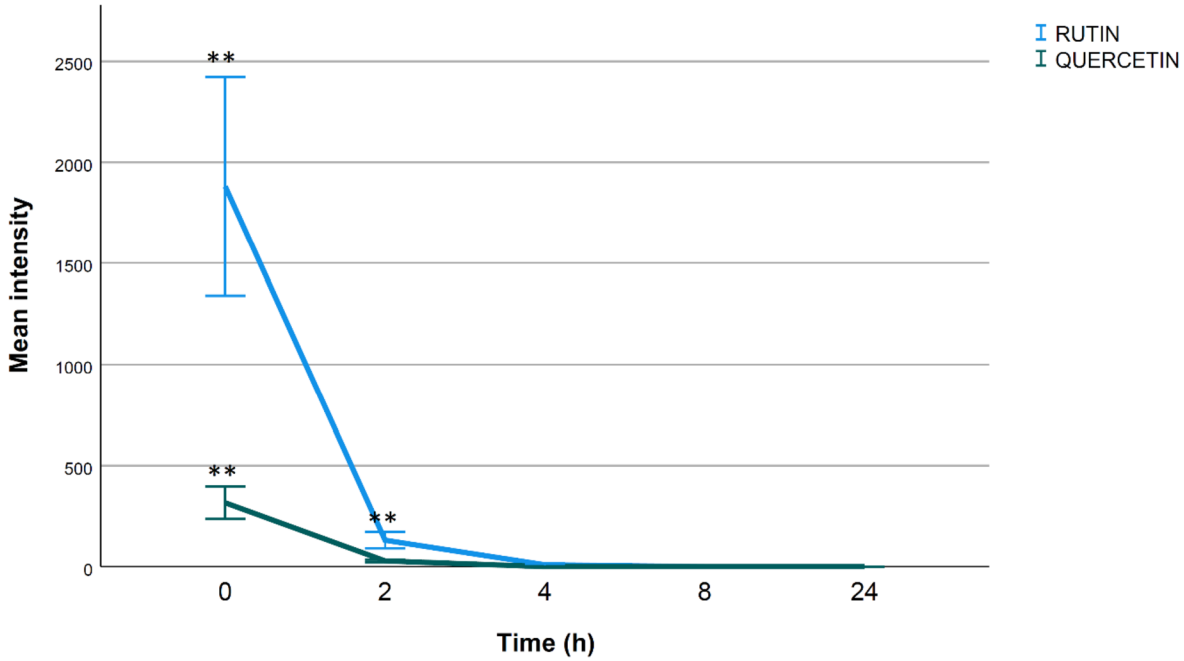

Figure 7: Graph of rutin and quercetin mean intesity measured by UHPLC/Q-TOF (± standard deviation) versus time in the treated variant, N=20; paired t-test between treated variant and control: * $p < 0.05$, ** $p < 0.01$

5.2 NMR

The measured data from the NMR analysis were evaluated using Chenomx 8.5 software. Twelve compounds were identified in the samples analyzed. The identified compounds included SCFAs, namely acetate, butyrate, propionate and isobutyrate, carbohydrates, namely fructose, glucose, trehalose, amino acid representatives – leucine and methionine, as well as alcohols – methanol, ethanol and one carboxylic acid – caffeic acid. Differences in the composition and spectrum of compounds were observed in different donors. A summary of all the compounds observed is given in Table 2. The concentration of each compound was compared between the treated sample and the control within a given hour. Average higher concentrations of acetate, butyrate, ethanol, fructose, methanol, methionine and propionate were observed in TRT regardless of age category. In contrast, lower concentrations within TRT (regardless of age category) were observed for caffeic acid, glucose, leucine and trehalose. Based on the paired t-test performed between the results of treated variants and control, it can be said that there is no statistically significant difference between them. Thus, the addition of rutin did not affect the metabolome.

An independent samples t-test was performed between the young and old groups for each time point separately for both treated and control samples. Statistical analysis revealed a statistically significant difference in ethanol concentration at time 0 between the control group of young and old subjects. The mean ethanol concentration at time 0 for group A was in 1,7 % higher than group B. A statistically significant difference between the control groups was also found in the case of fructose concentration at hour 0 and hour 2. The fructose concentration at hour 0 in group A was o 1,1 % vyšší než u skupiny B. At hour 2, the fructose concentration was found to be 0.8 % higher for group A than for group B. Statistically significant difference was

also observed in glucose concentration at hour 0, where the concentration of group A is 4.1 % higher than controled group B.

In the comparison of treated samples between different age groups, statistically significant difference was observed only in leucine concentration. Different concentrations were observed at 8th hour, when group B was 4,7 % higher than group A.

Compound	TREATMENT vs CONTROL TREATMENT					A vs B (CONTROL) A					A vs B (CONTROL) B					A vs B (TREATMENT) A					A vs B (TREATMENT) B				
	0	2	4	8	24	0	2	4	8	24	0	2	4	8	24	0	2	4	8	24	0	2	4	8	24
Acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Butyrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	↓*	-	-	-	-	↑*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Caffeic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	-	-	-	-	-	↓*	↓*	-	-	-	↑*	↑*	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	-	-	-	-	-	↑*		-	-	-	↓*		-	-	-	-	-	-	-	-	-	-	-	-	-
Isobutyrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑*	-	-	-	-	↓*	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propionate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2 Summary of monitored substances and results from statistical analyses – independent sample t-test, * p <0,05; N (B – old) = 10, N (A – young) = 10; ↑ ↓ arrows indicate decreased or increased mean concentration of the monitored substance compared to the comparison group

6 Discussion

The aim of this thesis was to determine the catabolites of rutin in a colon model using stool donors from two age groups (under 45 years and 70 years and older). The second aim was to determine whether the addition of rutin affects the intestinal metabolome, and if so, how. Rutin is metabolized by the colonic microbiota, its addition affects the intestinal metabolome, and the composition of the intestinal metabolome differs between age groups. Identifying and understanding differences in the human intestinal metabolome and rutin metabolism are important steps that may aid in further research and the clinical application of rutin.

However, the metabolism of flavonoids is not uniform due to their heterogeneous structure and wide distribution in the diet (Cook 1996). The colonic microbiota is a major site of flavonoid metabolism (Hollman & Katan 1997). Due to the size of polymeric flavonoid molecules, degradation to lower molecular weight compounds is required before flavonoids can be absorbed by the intestinal epithelium (Heim et al. 2002). Enterobacteriaceae appear to hydrolyse flavonoid glycosides in the appendix and colon. The resulting aglycones are more readily absorbed by epithelial cells, with their lipophilicity facilitating passage across the phospholipid bilayer of membranes. A large proportion of the metabolites can be excreted in bile, may still be hydrolysed and reabsorbed by intestinal cells or excreted in the faeces (Andlauer et al. 2001; Murota & Terao 2003).

This research focused on rutin and its catabolism. The course of rutin catabolism was influenced by individual differences in the bacterial composition of stool samples. According to Biagi et al. (2010), reduced intestinal motility results in slower intestinal passage and reduced excretion of intestinal bacteria alters intestinal fermentation processes in an adverse manner. This affects the homeostasis of the bacterial ecosystem in the intestinal tract.

In this research, a decrease in rutin concentration over time was observed in all donors. However, in some fecal culture samples, rutin yields were observed to be lower than the applied amount at 0 hours. This phenomenon may be due to the adsorption of rutin to other components of the faeces (e.g. sugars) or to the surface of microorganisms present. The magnitude of this effect was dependent on the stool samples used from individual donors. The results are consistent with those published in a study by (Jaganath et al. 2009).

Five metabolites were identified in the samples analysed. These are taxifolin, quercetin-3-galactoside, phloroglucinol and quercetin. Jaganath et al. (2009), described the conversion of rutin to taxifolin, phenypropionic acid, quercetin-3-galactoside, 3,4-dihydroxyphenylacetic acid, phloroglucinol and quercetin. Among other metabolites resulting from the conversion of rutin, they also mention 3- dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid 3-(3-hydroxyohenyl)propionic acid (Jaganath et al. 2009). This research confirmed that these metabolites are among the main and most common products of rutin metabolism produced by the gut microbiota.

The concentration of rutin during fermentation dropped to almost zero after 2 hours. In an experiment conducted by (Jaganath et al. 2009), donors also show differences in the breakdown of rutin. In some samples, rutin was completely degraded after only 30 minutes.

One of the metabolites, quercetin, was higher in concentration at time 0 h and then began to decline gradually. At 24 h, quercetin was already fully metabolized and the concentrations

were therefore low. Quercetin is known to undergo rapid oxidation leading to its conversion to semiquinone free radicals and a wide range of unidentified reaction products when present in high concentrations (Galati et al. 2001; Krishnamachari et al. 2002; Qin et al. 2010b). Consistent with these findings, similar observations were obtained in the present study in that quercetin was found to oxidize to polar compounds (Awad et al. 2002).

The hypothesis supporting the theory that rutin supplementation affects the stool metabolome was not confirmed. Due to the resulting statistical investigation performed on the basis of the concentrations of the individual substances in the control and treatment groups in this experiment, no statistically significant difference was found. A different result was reached by Vernocchi (2016), who in his study demonstrated a positive effect of rutin on the restoration of the gut microbiota and the associated production of short-chain fatty acids. They found this through analysis including gas chromatography, liquid chromatography, high-pressure chromatography, ultra pressure LC (UPLC), Fourier transform infrared spectroscopy (FTIR), ion cyclotron resonance-FT (ICR-FT), capillary electrophoresis (CE) coupled to mass spectrometry (MS), and nuclear and proton nuclear magnetic resonance spectroscopy (NMR-1H-NMR). These results are not consistent with my research results and it would be necessary to determine if there was a statistically significant effect of another substance on the microorganisms present in the samples.

The aim of the experiment was also to investigate and compare rutin metabolism between two age groups (age group A under 45 years, age group B over 70 years). Age was found to have a statistically significant effect on rutin metabolism only in the case of the amino acid leucine, which was observed in higher concentrations in donors younger than 45 years. Since aging causes disturbances in many metabolic pathways in the human body, I also focused on other substances of the intestinal metabolome (acetate, butyrate, ethanol, caffeic acid, fructose, glucose, isobutyrate, leucine, methanol, methionine, propionate, trehalose). Metabolomics of human stool samples has great potential to provide information on the metabolic changes that accompany aging (Cui et al. 2021).

In this study, a statistically significant difference in ethanol, fructose and glucose concentrations was found in control stool samples using a two-sample t-test. The results suggest that rutin played no role in this case. Ethanol and fructose were more produced by older donors, and glucose was produced by younger donors, but there was an intense increase in older donors after 24 hours. This may have been due to the effect of the age difference between donors. The gut microbiota in the elderly (age over 70 years) is strongly influenced by changes in dietary habits and nutrient absorption, but also by a decrease in physical activity. For example, there is a decrease in the number of anaerobic bacteria from the genus Bifidobacterium and the strain Bacteroidetes or an increase in the number of bacteria from the genus Clostridium, the strain Firmicutes and Proteobacteria (Monda et al. 2017; Rinninella et al. 2019).

In this research, there was no similar difference between age groups for acetate, butyrate, caffeic acid, isobutyrate, leucine, methanol, methionine, propionate and trehalose even without the addition of rutin. Cui et al. (2021) also investigated the effect of age on the metabolite profile of human feces. They found that the most important metabolites for age class distribution were short carbon chain fatty acids, including acetate, butyrate, isovalerate, and propionate, amino acids (alanine, phenylalanine, valine, isoleucine, tyrosine, and threonine), and alkaloids (methanol and ethanol). Short-chain fatty acids were predominant in the faecal

profiles of young subjects (age 18 years), whereas amino acids were more abundant in the older group (age 65-80 years). The results of my experiment regarding fatty acids, amino acids and sugars are not in agreement with the experiment conducted by (Cui et al. 2021).

Alcohols (including ethanol) are known to be produced by anaerobic gut bacteria and yeast as fermentation products of carbohydrates and play a key role in the prevention and treatment of some diseases (metabolic and intestinal disorders, some cancers, etc.) (Wissenbach et al. 2016; Oliphant & Allen-Vercoe 2019). This could suggest that rutin did not affect the presence of certain bacteria and yeasts responsible for the production of these substances.

7 Conclusion

The aim of this study was to determine the major catabolites of rutin in a colon model using stool from donors of two age groups (under 45 years and over 70 years).

Three hypotheses were established. The first hypothesis is that rutin will be microbially transformed by the colonic microbiota. The second hypothesis is that the catabolites of rutin will be different in the two age groups studied. The third hypothesis is that the addition of rutin will affect the stool metabolome.

Rutin was degraded by the gut microbiota into 5 identified metabolites during our study.

The catabolites of rutin differed between the two study groups and we were able to statistically demonstrate different concentrations for each metabolite. However, due to the small number of subjects studied (N=20) from different backgrounds and with different lifestyles, they cannot be compared. Therefore, we cannot confirm or refute the second hypothesis stated.

The second aim was to evaluate how the addition of rutin affects the stool metabolome. Based on the paired t-test performed between the results of the treated variants and the control, we can say that there is no statistically significant difference between them. Thus, the metabolome was not affected by the addition of rutin. Furthermore, the effect of age on the metabolome composition was found after rutin treatment (difference observed for leucine and ethanol) as well as in the control variant regardless of rutin (difference observed for acetate and trehalose). For ethanol, carbohydrates (fructose and glucose), there was a statistically significant difference in the gut metabolome concentrations in the control variants.

The hypothesis of this work that rutin would be microbially transformed was confirmed. The hypothesis that the addition would affect the composition of the intestinal metabolome was not proven. The composition of the intestinal metabolome differed between the two age groups studied only in terms of leucine concentration; this was not found for other substances.

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9 List of abbreviations and symbols

ACTH – adrenocorticotropic hormone
Ach – acetylcholine
AMP – antimicrobial peptides
ATB – antibiotic induced microbial dysbiosis
ATP – adenosine triphosphate
CNS – central nervous system
CRF – corticoliberin
DA – dopamine
DNA – deoxyrubinucleic acid
EE – enteroendocrine
EGC – epigallocatechin
EGCG – epigallocatechin-3-gallate
ENS – enteric nervous system
GABA – gamma-aminobutyric acid
GF – germ free
GIT – gastrointestinal tract
HL-60 – human promyelocytic leukemia
HPA – hypothalamic-pituitary-adrenal
IgA – immunoglobulin A
IL–1β – interleukin 1β
KEGG – Kyoto Encyclopedia of Genes and Genomes
LC–MS/MS – mass spectrophotometry associated with liquid chromatography
LDL – low density lipoproteins
LPS – lipopolysaccharide
MnSOD – manganase superoxide dismutase
MS – mass spectrophotometry
NMR – nuclear magnetic resonanc
PAMP – pathogen-associated molecular patterns
PEG – polyethylene glycol
RNA – ribonucleic acid
SCFA – short chain fatty acids
STZ – streptozotocin
TNF α – tumor necrosis factor α
UV – ultraviolet
VEGF – vascular endothelial growth factor

10 List of figures and tables