Carbohydrate-boosted control of intestinal health

The intestine plays a crucial role in the overall health and well-being of humans and animals and contains a large number of microbial cells, known as the microbiota. The microbiota influences many biological processes such as digestion, nutrient absorption, metabolism, and immune function. Colonization of the gut begins at birth and at hatch, in humans and respectively in broiler chickens. The development of the gut microbiota composition is influenced by various factors, including genetics and the environment. During this early phase, the gut microbiota undergoes dynamic changes, resulting in a diverse and stable microbiota after 2-3 years in humans and 3-5 weeks in broiler chicks.

The composition of the microbiota can be influenced continuously during the development but also after a stable state is formed, by factors such as diet, the presence of pathogens, or antibiotic use. Generally, the microbiota can recover, but if the balance is severely disrupted, it can lead to dysbiosis, resulting in health issues such as intestinal infections or systemic inflammation. The disrupted balance between good and bad bacteria in broiler chickens is associated with intestinal inflammation and the shortening of the small intestinal villi, leading to reduced nutrient absorption and less efficient animal growth. A common intestinal disease in broilers is coccidiosis, which can lead to necrotic enteritis as a consequence. Necrotic enteritis is associated with overgrowth of the pathogenic bacterium *Clostridium perfringens* and has a significant impact on the entire flock. In premature babies, a gut disease is associated with the same bacterium, called necrotizing enterocolitis. This condition causes severe intestinal inflammation, leading to reduced blood flow to the intestine, which can result in parts of the intestine dying, and eventually death.

Considering the diverse and critical role of the gut microbiota in human and animal health, extensive research is dedicated to maintaining and improving the intestine's microbial composition and barrier function. Non-digestible oligosaccharides, also known as prebiotics, are used for this purpose, providing health benefits. For example, to prevent necrotizing enterocolitis, breastfeeding is recommended, since breastmilk contains natural prebiotics. But, additional research is needed to find alternatives next to breastmilk. This thesis focuses on two proven prebiotics, namely 2'-fucosyllactose (2'-FL), also present in breast milk, and Galacto-oligosaccharides (GOS), one of the most well-known oligosaccharides that are already added to formula milk due to their prebiotic effects. Furthermore, research is ongoing to find other prebiotics that can maintain and improve gut health. To test new potential prebiotics, *in-vitro* techniques are beneficial. Therefore, this thesis pays attention to an *in-vitro* pipeline that combines fermentation models and gut models, for both chickens and humans. This allows the screening of potential prebiotic effects of oligosaccharides. In this thesis this was done for Isomalto/malto-polysaccharide (IMMP) and 3 citrus pectins.

Chapter 2 focuses on the optimization and comparison with original cecal samples of the CALIMERO-2 system, which mimics the chicken cecum at the microbial level. The system is based on the validated TIM-2 system, designed to mimic colon fermentation in humans. For CALIMERO-2, temperature (41 °C) and pH (6.6) were adjusted to match the situation in the chicken cecum. Different medium compositions were tested and added to the system to mimic the nutrients passing to the chicken cecum. Samples obtained from fermentations with SIEM, the medium that is also used for TIM-2 experiments for the microbiota of humans, showed the most similarities with the microbial composition of the original chicken samples.

Chapter 3 describes the optimization of another *in-vitro* system, where chicken mini-intestines, also known as intestinal organoids, were created. These chicken intestinal organoids contain various types of cells present in the chicken intestine. To grow the cells and maintain this culture successfully for an extended period, growth factors of chicken origin were added since commonly used mouse growth factors differ biologically. In addition to the three-dimensional structures, chicken intestinal organoids can also be cultures two-dimensionally, making them more accessible for exposure studies with test-products, which otherwise would have to be injected inside the three-dimensional organoids.

In **Chapter 4** we determined the composition of four oligosaccharides, namely three pectins and Isomalto/malto-polysaccharide (IMMP), and investigated how these oligosaccharides can influence the gut microbiota in the *in-vitro* system described in Chapter 2, CALIMERO-2. This was done in comparison with the standard medium (SIEM) and the control Mannan-oligosaccharides (MOS). Structural analysis revealed slight differences in monosaccharide composition and molecular weight between the pectins. Particularly, pectin 1 showed a low degree of methyl esterification, while pectins 2 and 3 had high methyl esterification. These similarities between pectins 2 and 3 were also observed in beta-diversity analysis of the microbiota-modulation. The phylum *Bacteroidetes* was the most dominant, except in the two controls SIEM and MOS, where *Firmicutes* prevailed. Beneficial bacteria, especially *Lactobacillus*, remained stable in all samples. Pectin 1 showed a significantly lower total production of short-chain fatty acids compared to SIEM, IMMP, and pectin 3, while IMMP closely resembled the positive control MOS, especially regarding butyrate content. This study highlights the potential of these oligosaccharides to promote gut health in poultry, via the gut microbiota.

In **Chapter 5** the fermentation products of the prebiotics 2'-FL and GOS, obtained from the *in-vitro* large intestine formation model TIM-2 inoculated with a microbiota of 3-month old babies, were studied on the epithelial barrier-integrity and immune activation in a Caco-2/Peripheral blood mononuclear cells co-culture cell-model. These fermentation products include several metabolites produced during fermentation of the prebiotics. The intestinal epithelial cells were exposed to the fermentation products of 2'-FL and GOS, and also to the control i-SIEM, a variant of SIEM for babies. GOS increased epithelial permeability, associated with increased release of interferon-gamma by underlying immune cells. Both the fermentation products of the control as well as those of 2'-FL and GOS further increased the inflammatory release of interleukin-1 beta from activated immune cells. The fermentation products all contained substantial but comparable amounts of lipopolysaccharides (LPS). However, filtering out LPS or adding a TLR4 antibody, which blocks the binding of LPS to its receptor, did not result in a more favorable effect on barrier integrity or immune responses. These results emphasize the complexity of interaction between *in-vitro* fermented products and intestinal epithelial cells and immune cells.

Overall, the work of this thesis presents an *in-vitro* pipeline combining sophisticated fermentation models for chicken and man with intestinal models (organoids [chicken] or co-culture cell model [man) to investigate the potential prebiotic effect of carbohydrates. This *in-vitro* test pipeline for intestinal health is compliant to the principle of the "Three Rs": replacement, reduction and refinement; is more predictive than e.g. *in-vivo* mouse models, and can also be implemented for other mammals.