# Metabolism of the Challenged Intestinal Epithelial Cell

Metabolism of the Challenged Intestinal Epithelial Cell

*Anna F. Bekebrede*

Propositions Anna F. Bekebrede

1. Luminal nutrients are crucial for energy production in small intestinal epithelial cells *(this thesis).*

2. Enterocyte metabolic function is a target for nutritional interventions to improve intestinal health *(this thesis).*

3. Drawing conclusions about underlying molecular mechanisms based on analysis of only a few genes in a pathway is misleading.

4. Pregnancy in the late-PhD trajectory will result in a stress-resistant child (Zhang, Wang et al. 2021).

5. Churchill was right when he said: “first we make the buildings, then the buildings make us”.

6. Reading aloud to children and young adults is a social exercise rather than an educational one.

7. The addictive nature of smart wearables warrants similar legal treatment as psychoactive drugs.

Propositions belonging to the thesis, entitled

“Metabolism of the Challenged Intestinal Epithelial Cell”

Anna F. Bekebrede

Wageningen, 10th of February 2023

# Metabolism of the Challenged Intestinal Epithelial Cell

Anna F. Bekebrede

## Thesis committee

### Promotors

Prof. Dr Jaap Keijer
Professor of Human and Animal Physiology
Wageningen University & Research

Prof. Dr Walter J.J. Gerrits
Professor of Animal Nutrition
Wageningen University & Research

### Co-promotors

Dr Vincent C.J. de Boer
Assistant professor, Human and Animal Physiology
Wageningen University & Research

### Other members

Prof. Dr Sander Kersten, Wageningen University & Research
Prof. Dr Joris Michiels, Ghent University, Belgium
Dr Martine Morrison, TNO, Leiden, The Netherlands
Dr Guido Hooiveld, Wageningen University & Research

This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences

# Metabolism of the Challenged Intestinal Epithelial Cell

Anna F. Bekebrede

**Thesis**

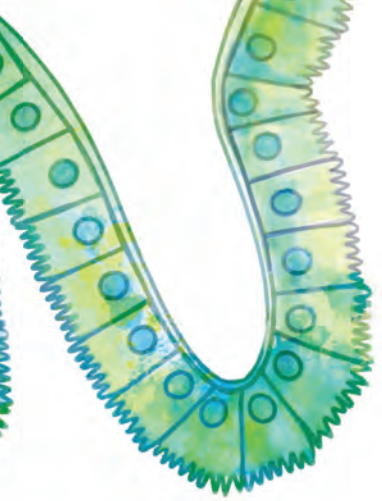
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on 10 February 2023
at 4 p.m. in the Omnia Auditorium

Anna F. Bekebrede
Metabolism of the Challenged Intestinal Epithelial Cell
244 pages

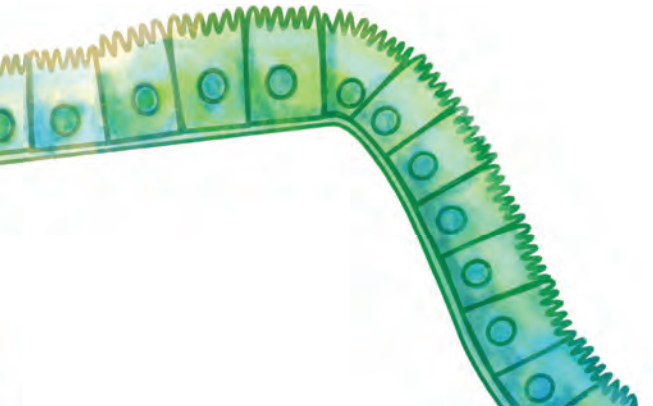
PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)
With references, with summary in English and Dutch

ISBN 978-94-6447-433-6
DOI 10.18174/578215

## Table of contents



# Chapter 1
General Introduction



## Gut health

The intestine as an individual organ is recognized for its vital importance in ensuring optimal nutrient uptake and overall health [1]. There is a need to understand what exactly it means to have a well-functioning intestine, and how this organ sustains overall health and well-being. In this thesis, I aim to further our knowledge of the fundamental qualitative aspects that underly intestinal health, both for humans and for animals.

The gastrointestinal tract in its most basic description is a tube, running from stomach to anus, in which food is processed. But this description does not do justice to the complexity of this organ, nor its vital importance in maintaining overall health. The intestine absorbs essential nutrients and minerals that the body requires to maintain all other functions, while it keeps unwanted environmental agents out [2]. Bischoff et al. [1] have posed five useful criteria for what constitutes gut health, these being: 1) effective digestion and absorption of food, 2) absence of gastro-intestinal illness, 3) normal and stable intestinal microbiome, 4) effective immune status, and 5) status of well-being. Conversely, these criteria inform what it means to have sub-optimal gut health, i.e. not meeting these criteria increases the likelihood of developing diseases.

In humans, gastrointestinal diseases are prevalent in infants, especially in low-income countries [3]. With regard to animals, pigs suffer from gastrointestinal diseases frequently, amounting to major losses of lives and decreased animal welfare [4, 5]. Pigs, apart from being an important target species in their own right, are also a good model for humans, particularly for large organ systems like the intestine [6, 7]. This thesis will therefore focus on pigs to improve our knowledge of what makes the intestine vulnerable to the onset of diseases.

An important feature of the intestine is that it is a metabolically highly active organ. Although it only accounts for ~5% of body weight, it consumes ~23% of energy [8, 9]. Especially after a meal, when the intestine is most actively digesting and absorbing nutrients, energy consumption increases. This is reflected by a whole-body increased resting metabolic rate of 15-20% in pigs [10], and 25% in humans [11]. Failure to produce sufficient energy in the intestine hampers gut functions, and can thereby increase susceptibility to disease. As such, intestinal energy production will be a main focus of this thesis. The manner in which insufficient energy production in the gut exacerbates susceptibility to intestinal disease is illustrated as follows. Firstly, patients suffering from intestinal bowel disease were reported to have decreased energy production in the colon [12-14]. Although it is still unclear whether this decreased energy production is cause or effect, the co-occurrence with intestinal diseases renders it part of the disease aetiology. Secondly, certain classes of drugs, e.g. the commonly used non-steroidal anti-inflammatory drugs (NSAIDs) and the anti-inflammatory drug

tacrolimus, were shown to impair the ability of the gut to maintain a strong barrier against the outside world, potentiating onset of disease [15, 16]. This decreased barrier function is likely caused by decreased cellular energy production in the gut after exposure to the drugs [15-17]. Decreased energy production is probably the result of mitochondrial uncoupling, during which mitochondrial electron transport is not used to drive ATP-production [15-17]. Thirdly, aging was found to decrease metabolic function of intestinal cells, possibly underlying the increased sensitivity to diseases in old age [18]. Although metabolic function of the intestine is thus clearly important in maintaining a healthy gut, there are still many unknowns as to how intestinal metabolic function supports intestinal health, and how the intestinal metabolic function responds to challenges.

## Morphology and cell types of the intestine

To perform all its functions, the human intestine harbors a large surface area of about 30 $m^2$ in the small intestine and 2 $m^2$ in the large intestine [19]. To generate this large surface within a relatively short tube, the small intestine contains macroscopic protrusions, called villi, that extend into the lumen (fig. 1.1). In addition, small-intestinal epithelial cells have microvilli structures that enlarge the surface area even more. This large area in the small intestine is used to facilitate nutrient and mineral uptake via both passive and active transporters, while brush-border enzymes that are present throughout the small intestine hydrolyze larger food components into absorbable fragments. While the epithelial cells in the large intestine also exhibit microvilli, the large intestine lacks a microvilli structure and instead only contains crypts. This difference in morphology between the small and large intestine may be because of the higher dry matter content and more abrasive composition of the luminal content in the colon. The colon is mainly responsible for water and mineral absorption, but also contains a large community of microbes. These microbes ferment the food which escaped digestion in the small intestine. The colonic microbiome consists of a large population of bacteria, viruses and fungi. Their composition varies both between individuals and within an individual, on a day-to-day basis [20]. These microbes form a complex and metabolically active ecosystem, that also helps to satisfy the host's nutrient demands [21, 22]. Later, I will expound in what manner this process leads to the production of fermentation metabolites, and how these potentially impact the intestinal mucosa

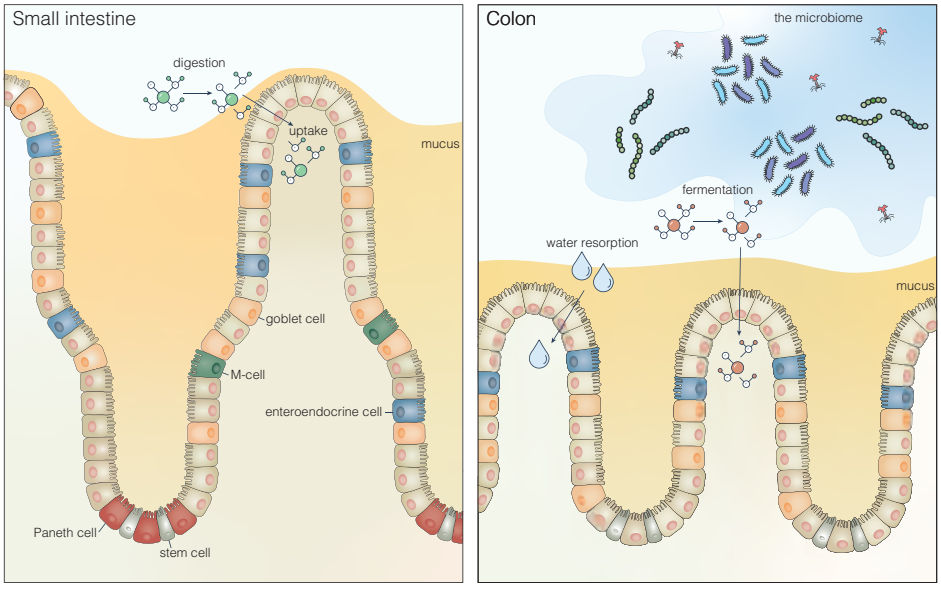



Figure 1.1 **Intestinal architecture and functions.** The small intestine primarily digests and absorbs dietary nutrients, and contains macro- and microvilli. The colon has crypts, and is mainly responsible for water and mineral absorption. The colon also contains a microbiome that ferments undigested carbohydrates and proteins.

The intestines are responsible for breaking down food, absorbing its nutrients and solidifying its waste, while simultaneously forming an active barrier against the external environment. At the core of performing these tasks are the intestinal epithelial cells (IECs), which are therefore an integral part of this research. IECs are all the terminally differentiated cells that form a firmly connected, single layer of cells in the intestine, as depicted in Figure 1.1. All IECs are generated from intestinal epithelial stem cells, that reside at the bottom of intestinal crypts, the most protected place in the intestine. As the stem cells divide, they result in to a population of transient, rapidly proliferating cells that migrate to the villus tip as they mature and differentiate, forming the distinct crypt-villus structure of the intestine (fig. 1.1). Finally, the IECs at the villus tip are shed into the lumen, by a form of programmed cell death called anoikis, that is induced by loss of cell contact to adjacent extracellular matrixes [23, 24]. Due to the abrasive contents of the intestinal lumen, IECs at the tip of the villi are replaced about every 5-7 days [25]. The diverse types of IECs each have their own task. Presence of various IEC types vary in the different regions of the intestinal tract, in accordance with the distinct function of that segment of the intestine. Enterocytes, also termed colonocytes in the colon, are the most abundant cell type, which is responsible for nutrient, mineral and water absorption [26, 27]. Goblet cells produce mucins to form a protective layer on top of the epithelial lining, and are more prominent in the large intestine than in the small intestine. Enteroendocrine cells secrete hormones, and while they make up

only about 1% of the cells in the gastrointestinal tract, this still makes the intestine the largest endocrine system in humans [26]. Paneth cells reside in close proximity to stem cells, produce antimicrobial factors, and supply nutrients and growth signals to the stem cells [28]. Although there is clear evidence for the existence of Paneth cells in the porcine intestine [29, 30], some studies have disputed this claim due to absence of the acidic lysozyme marker phloxine-tartazine [31]. Tuft cells only constitute about 0.5% of the intestinal epithelium and have chemo-sensing and immune functions [32, 33]. Finally, microfold (M)-cells sample the luminal environment and mainly play a role in the intestinal immune response [34]. Of these cell types, Paneth and M-cells are only present in the small intestine, while the other epithelial cell types can be found throughout the intestinal tract. Metabolism in IECs

### *The role of cytosolic glycolysis and mitochondrial OXPHOS in IECs in energy generation*

To maintain all their functions, IECs need to produce cellular energy in the form of adenosine-tri-phosphate (ATP). There are two major metabolic pathways to generate ATP. Firstly, ATP can be generated in the cytosol by glycolysis, which is a series of consecutive metabolic steps that convert glucose into pyruvate, simultaneously generating ATP. The newly formed pyruvate can then be excreted from the cell as lactate, but it can also be imported into mitochondria, where it is further metabolized to generate additional ATP. Mitochondria are cellular organelles that perform a number of essential metabolic reactions. They are specialized in ATP production, using not only pyruvate, but a variety of metabolic substrates. In addition to pyruvate and lactate, fatty acids, but also ketones and amino acids are major substrates for mitochondrial energy production. Mitochondrial energy production is highly efficient compared to glycolysis. Per mole of glucose, two moles of ATP are generated in glycolysis, while mitochondria generate another 30 moles of ATP from the 2 moles of pyruvate that are formed per mole of glucose. Pyruvate and amino acids are metabolized in the tricarboxylic acid (TCA) cycle, while fatty acids are metabolized via -oxidation (and TCA cycle). During these processes, the energy sources are oxidized to $CO_2$, simultaneously reducing the electron carriers nicotinamide-adenine dinucleotide ($NAD^+$, which is reduced to NADH) and flavin adenine dinucleotide (FAD, which is reduced $FADH_2$). The reduced forms of these carriers donate their electrons to the electron transport complexes that are located in the mitochondrial inner membrane. The subsequent flow of electrons through the electron transport complexes is, in Complex I, III and IV, coupled to proton transfer from the mitochondrial matrix to the mitochondrial inter membrane space. At complex IV, oxygen is the terminal electron acceptor, which is converted into water. The proton transfer by complex I, III and IV generates a proton gradient over the mitochondrial inner membrane. This so-called proton-motive force drives protons through Complex

V ($F_0F_1$-ATPase) into the matrix, simultaneously providing energy for ATP synthesis, i.e. for converting ADP into ATP. The use of oxygen is directly and tightly coupled to ATP production, lending this process the name 'oxidative phosphorylation' (OXPHOS). Over 90% of cellular oxygen use is in oxidative phosphorylation [11]. Therefore, oxygen consumption is a proxy for, and is often used to measure, mitochondrial ATP synthesis. Besides their role in ATP production, mitochondria have vital roles in cell physiology, which I will now turn to.

### *Other functions of mitochondria: mitochondria as central cellular communication hubs*

Cell metabolism is a complex phenomenon that varies between cell types. For instance, metabolism is not identical in different types of IECs, while the development of IECs from stem cells is also accompanied by changes in metabolism [35, 36]. As the intestinal progenitor cells divide and mature, they require more energy to perform their functions, reflected by an increased metabolic rate [37, 38]. Mitochondrial function is emerging as an active participant in these phenotypic transformations. It is useful to summarize our understanding of the role mitochondria play in cell physiology, in order to understand the importance of mitochondria in maintaining cell function.

Mitochondria are recognized to play an essential role as cellular biosynthetic and signalling hubs [39]. In addition to ATP generation, mitochondria also harbour essential biosynthesis pathways, including the biosynthesis of heme and iron-sulphur clusters. Mitochondria also have a key role in $Ca^{2+}$ homeostasis, programmed cell death, immune signalling and metabolic signalling. Furthermore, intermediates of substate oxidation can serve as precursors for the biosynthesis of essential cellular components, such and DNA, RNA, proteins and a variety of lipids [39-41]. Proliferating cells, such as the intestinal epithelium, are especially dependent on mitochondrial biosynthetic precursors, as these are required to support cell division. Conversely, aberrant mitochondrial function in intestinal stem cells impairs their proliferative and differentiation potential [35, 42, 43]. As signalling hubs, mitochondria adapt their metabolism to cellular needs and signal their metabolic status to various cellular energy sensors. These signals include $Ca^{2+}$ concentration, the $NAD^+$/NADH ratio, the ADP/ATP ratio and reactive oxygen species (ROS) [44]. ROS are especially interesting, because they have been shown to modulate metabolism through e.g. activation of the metabolic sensor adenosine monophosphate-activated protein kinase (AMKP) [45]. In addition, ROS can modulate critical intestinal cell functions like stem cell proliferation [46, 47] and IEC apoptosis [36, 48]. ROS are the result of an escape of electrons from the electron transport system. Since mitochondria are the main site of electron transfer, mitochondria are also the major site of ROS production under non-inflamed conditions. In mitochondria, ROS is primarily produced at Complex I and Complex III

of the electron transport system [49], but it can also be generated by other electron transferring metabolic enzymes. For two major members of the ROS family the origin is as follows: first, the highly reactive free radical superoxide ($O_2$-) emerges from electron leakage. It is then rapidly converted to hydrogen peroxide ($H_2O_2$), a different ROS, by superoxide dismutases. $H_2O_2$ has the properties of a signalling molecule, in particular because of its ideal life-time and also because it is a non-charged, non-polar small molecule that can diffuse over the mitochondrial membranes without the need for a transporter [50].

Apart from ROS, major mitochondrial signals include mitochondrial metabolites. These are generated through e.g. TCA cycle and -oxidation. In a process called retrograde signalling, these metabolites communicate the mitochondrial metabolic status to the nucleus, to align nuclear and mitochondrial activities. For example, succinate was found to stabilise HIF1, a transcription factor that is often activated as a result of hypoxia to facilitate, among others, increased glycolysis [51]. All in all, although the role of mitochondria as bioenergetic, biosynthetic and signalling hubs is well recognised, it is less established how these mitochondrial functions change upon altered metabolism of intestinal cells and upon nutrient availability.

### *Preferential metabolic substrate use of IECs*

Cells preferentially use certain metabolic substrates, but they can adapt in response to altered availability of substrates. This is exemplified by the whole-body shift away from glucose oxidation and towards lipid oxidation upon fasting [52]. It is useful to investigate whether such a metabolic substrate shift occurs in the gut, and under what circumstances, be this due to a change in substrate availability or to changes in the metabolic function of the cell. In order to investigate this, it is important to know what the preferential energetic substrates are, and by which substrates they might be (partially) substituted. For most healthy tissues, glucose, lactate, ketones and fatty acids constitute the preferred metabolic substrates. This does not hold up for the gut. In the small intestine, in particular glutamate is thought to constitute a major energy source. In fed piglets, it was found that enteral glutamate contributed most to $CO_2$ production in the portal drained viscera [53], which implies this to be the dominant metabolic substrate. Likewise, a series of experiments showed that glutamate, but also glutamine and asparagine, were the most important metabolic substrates of the small intestine in fed rats (summarized by [54]). In the large intestine, the short chain fatty acids (SCFAs) acetate, propionate, and butyrate are major end-products of microbial fibre fermentation. These SCFAs are important energy substrates for the body [55]. For the large intestine, especially butyrate is thought to be an important energy source [56]. This is further underscored by the finding that unavailability of butyrate leads to energy-deprived colonocytes [57].

Substrate utilization is influenced by overall metabolic substrates available in the environment of a cell. For example, colonocyte oxidation rate was shown to be impacted by the mixture of available metabolic substrates [56]. Furthermore, butyrate was found to impact whether glucose is oxidized in cultured cells, with some studies reporting an increase in glucose oxidation [58-61] and others a decrease [62]. Together, it is well established that cells preferentially use certain types of metabolic substrates, and that the availability of different substrates influences the type of substrates used. However, the exact influence of different nutritional environments on substrate oxidation is still largely unknown. Given that the impact of specific substrates is often studied in only one specific nutritional condition and culturing environment, this is crucial to understand. Ultimately, more insight is needed into how nutritional environment affects metabolism of cells, and how this finally impacts intestinal physiology.

## Nutritional interventions applied in this thesis to challenge IEC metabolism

In the above section, I explained that mitochondria are bioenergetic and biosynthetic hubs, essential to maintain cellular and consequently intestinal homeostasis. However, this notion is largely inferred from *in vitro* studies or from transcriptome data. Because gene expression does not necessarily accurately reflect altered mitochondrial oxidative capacity and metabolic flux, functional measurements of mitochondrial metabolism in the intestine are therefore needed to complement these studies. In addition, the importance of IEC metabolic function for maintaining intestinal homeostasis needs to be further established. Various methods can be applied to study this. For example, IEC metabolic function can by abrogated using genetic knock-out of key mitochondrial genes. Another method is to manipulate the IEC nutritional environment using nutritional interventions. In my research, we have applied two nutritional interventions, constituting challenges to the intestine, that are relevant for both human and animal nutritional: diet and fasting.

### *First intervention: fibre and protein fermentation metabolites*

The luminal environment provides essential nutrients, which can be used by IECs for their energetic needs. Fermentation of undigested food components by the extensive colonic microbiota provides energy and metabolites for both the microbes and the host. Host and microbiota are thus in a symbiotic relationship; the host providing a nutritious environment for the microbiota, and the microbiota degrading food components that cannot be digested by the host's enzymes [63]. Examples of food components that end up in the colon for fermentation are (poorly digestible) proteins and carbohydrates, including slowly digestible starch and fibres.

Fibres have drawn interest as positive regulators of gastrointestinal health [64], and it is useful to first explain their role in physiology. There are many types of fibres, differing in functional and physico-chemical properties. These properties ultimately determine how fibres influence gastrointestinal health [65]. Fibres have an important role in maintaining stool consistency, and are the primary substrate for the production of the SCFAs acetate, propionate and butyrate. These SCFAs are produced in molar ratios of approximately 60:25:15, respectively, and they are metabolic substrates for the body, being extensively oxidized and assimilated into the host's carbohydrates and lipids [55, 66]. Apart from being a nutrient source, the SCFAs were also found to decrease systemic inflammation and insulin resistance [67-69]. As mentioned above, these SCFAs are also an energy source for colonic IECs, and especially butyrate is considered the main metabolic substrate for colonocytes [56]. However, butyrate is also endowed with other functions. For example, it was found to selectively kill cancer cells, by increasing its own oxidation and simultaneously limiting glycolysis in cancer cells [70]. Butyrate was additionally found to increase intestinal barrier integrity [71-73], and promote an anti-inflammatory immune response [74, 75]. Likely, butyrate exerts these effects at least in part through interactions with G-protein coupled receptors and by functioning as a histone deacetylase inhibitor, thereby steering metabolic and transcriptional regulation. Despite the positive effects of fibre fermentation metabolites, colonic fermentation may also result in metabolites that are harmful for the host. Fibre fermentation is generally thought beneficial for gut health, as described above. However, some studies in rats showed that a high level of the non-digestible fructose oligo-saccharides (FOS), decreased intestinal barrier function [76, 77], further evidenced by increased salmonella translocation [78]. Fibre fermentation can thus potentially produce adverse metabolites, and high concentrations of usually beneficial metabolites can become detrimental for gut health.

Protein fermentation metabolites, more so than fibre fermentation metabolites, are particularly regarded as being harmful. Feeding high protein diets to promote muscle growth in pigs has been associated with decreased fecal consistency and fecal dry matter content [79, 80]. These negative effects are possibly caused by the formation of harmful protein fermentation metabolites, such as ammonia, hydrogen sulfide and polyamines. Regarding ammonia, *in vitro* studies have shown that it decreased the ability of colonocytes to oxidize butyrate [81-83]. Next, hydrogen sulfide is detoxified within the electron transport system, thus limiting the ability of cells to produce ATP [84, 85]. Finally, polyamines are essential for maintaining cellular protein synthesis, while their excretion is facilitated via attachment of an acetyl-CoA group to the polyamines, which possibly depletes intracellular acetyl-CoA availability [86-88]. In addition, polyamine catabolism can lead to the formation of toxic by-products such as aldehydes and $H_2O_2$ [89], and thereby lead to cell damage. Currently, it is not well

understood what the *in vivo* physiological effects are of increased concentrations of protein-fermentation derived metabolites in the colon.

In this thesis, I will examine the interactive effects of fibres and poorly digestible protein on the metabolic function of colonic IECs. Diets high in fibre and/or (poorly digestible) protein could lead to increased fibre and/or protein flow towards the colon. Consequently, the colonic microbiota can ferment these fibres and proteins, leading to the production of fermentation metabolites that potentially impact IECs. Exactly how such diets impact colonic IECs is hardly known, and their impact on functional colonic metabolism has not been investigated. It is imperative to investigate this, because there are multiple developments in both animal and human diets that lead to various combinations of fibre and protein availability in the colonic environment. For example, high protein diets are common in pig nutrition to promote muscle growth, and are also popular in human nutrition as a means to lose weight or build muscle [90]. On the other hand, there is an increasing interest in high levels of fibre as a way to promote health in both animal and human nutrition [91-93]. Finally, plant-based diets are regarded as a sustainable and environmentally friendly food choice for humans, but such diets contain more fibres, while plant proteins have poorer protein digestibility, potentially increasing protein flux towards the colon [94]. Possibly, combinations of high fibre and (poorly digestible) protein impact the type and amount of fermentation metabolites produced by the colonic microbiota, thereby impacting IEC function, as will be elaborated on below.

To summarise, fibres and poorly digestible proteins can impact colon health by giving rise to both beneficial and harmful metabolites. Given the increased interest in diets high in fibre and (poorly digestible) protein, it is important to understand how fibres and proteins interact in the colon, and in what way this may impact energy metabolism of colonic enterocytes.

### *Second intervention: fasting*

The second nutritional intervention that will be studied is fasting. In pig husbandry, fasting is common at transitionary time-periods, such as upon weaning [95-98], and is associated with increased disease incidence [95, 99, 100]. (Forced) fasting, or periods of very low feed intake, is also relevant for the human population, because nearly one-third of the world population did not have adequate access to food in 2020, predominantly in low-income countries [101, 102]. Malnutrition is identified as one of the underlying causes potentiating diarrhea development [103, 104], and childhood diarrhea accounts for nearly 1.7 billion cases every year, leading to over half a million deaths in children under 5 years of age [104]. Despite its obvious relevance, it is poorly studied whether fasting decreases functional mitochondrial metabolism of

IECs. Furthermore, it is unknown how if decreased mitochondrial function upon fasting underlies decreased intestinal function, and thereby contributes to intestinal disease development.

While metabolic substrates for ATP production in IECs can be obtained from the arterial blood supply, luminal nutrients are major energy substrates in both the small and the large intestine [53, 56, 57], as described above. During periods of fasting, luminal nutrient supply is completely abolished for a certain amount of time. As a result, fasting was found to reduce intestinal gene expression of TCA cycle and electron transport system genes [105]. Older studies also showed decreased activity of electron transport system complexes in fasted rat muscle [106], and decreased substrate oxidation in isolated rat colonocytes [107]. However, it is still not understood what the functional metabolic and molecular consequences of fasting on piglet IECs entail. For instance, we know from that cells are often able to switch to a different metabolic substrate and metabolic pathway upon unavailability of their preferred substrate. The most prominent example is how the brain switches to ketone oxidation when glucose becomes limiting during fasting [108]. Yet, it is largely unknown whether the gut can likewise alter its nutrient source and metabolic pathway, i.e. to switch from oxidative metabolism to glycolysis, and thereby maintain sufficient ATP production to perform critical gut functions. In addition, although evidence suggests that there is decreased substrate oxidation and mitochondrial function upon fasting, the functional effects and associated pathway changes are not known.

Fasting is thus a relevant intervention, especially at the time of weaning, when it most often occurs in pig husbandry. Furthermore, because it is a highly controlled intervention, it allows us to further investigate how altered IEC metabolism influences gut physiology. There are several complications to determining the exact response to fasting at the time of weaning. Weaning is associated with many additional stressors, such as maternal separation and a new environment [95, 109], while the intestine is almost always not yet fully developed. It can therefore be difficult to separate the fasting response from the effects of these additional stressors, but this separation is needed to isolate the impact of fasting at the time of weaning. A way to distinguish the fasting response from other weaning associated stressors is to wean and habituate piglets to solid feed. Exposure to a solid diet induces structural changes in the intestine of piglets, that stimulate intestinal maturation to ensure proper nutrient digestion, absorption and barrier integrity [110-113]. Comparing the response to fasting at weaning and after solid feed habituation can thus help to distinguish the fasting response per-se from the response to additional weaning associated stressors like maternal separation, a new environment and an underdeveloped gut. This constitutes the starting point for the second intervention, which concerns fasting piglets at two time-points: four-week-old

suckling piglets at the time of weaning, and six-week-old solid feed habituated piglets.

## Aims and thesis outline

Generating energy to support cellular functionality is a fundamental criterium for all living organisms. While the crucial role of energy production in IECs is recognized, information on how energy production is affected by nutritional challenges is not functionally measured, but inferred from other data, such as gene transcriptomics. Furthermore, it is poorly known how IEC metabolism is altered upon challenges imposed in the form of nutritional interventions under different conditions, and how this may be related to other IEC functions. The **overall aim** of this thesis is therefore to increase our knowledge of how IEC metabolism adapts to changes in nutrient environment under various conditions. A better understanding of the metabolic function of challenged IECs may translate into strategies to boost intestinal metabolic function in order to prevent disease onset.

The specific aims of this thesis are:

1. To study how IEC metabolic function is influenced by fermentation metabolites, by:
    a. providing an overview of the protein fermentation-derived **polyamines** on *in vivo* IEC function
    b. assessing the effect of the fibre fermentation metabolite **butyrate** on metabolism of cultured colonocytes
    c. optimizing a method to isolate and functionally measure metabolic function of primary pig IECs
    d. assessing the *in vivo* effects of **fibres** and **poorly digestible protein** on colonocyte metabolic function in pigs
2. To establish the functional metabolic and molecular consequences of fasting on intestinal epithelial cells, by:
    a. functional metabolic and molecular profiling of IECs from fasted, solid food habituated piglets

    assessing the molecular response to fasting in jejunum of just weaned and solid food habituated piglets

An overview of the challenges imposed, and the chapters describing the results, is depicted in Figure 1.2.

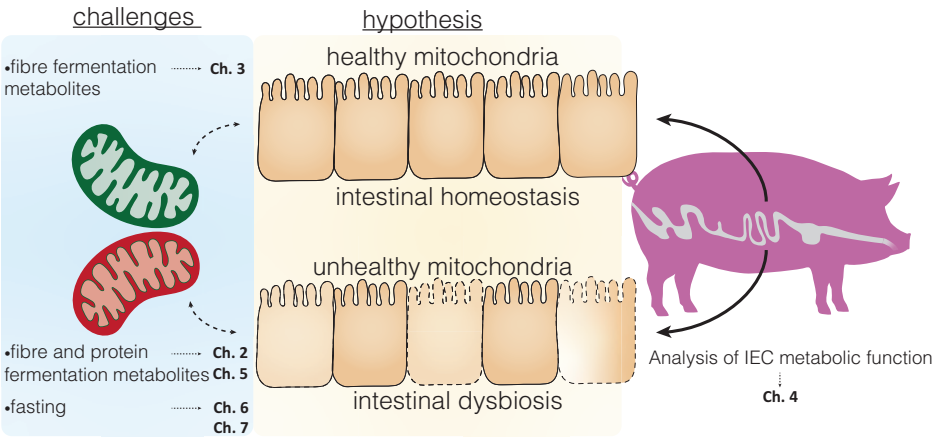



Figure 1.2 **Overview of interventions, which constitute challenges to the intestine, that were applied in this thesis, the hypothesis for their impact on IEC metabolism and function, and the thesis chapters.** Two main types of interventions, or challenges, were applied in this thesis. The first challenge was diets containing fermentable fibre and poorly digestible protein, which were expected to increase both fibre and protein fermentation metabolites. These metabolites were expected to both increase (fibre metabolites, **chapter 3**) and decrease (fibre and protein metabolites, **chapters 2 and 5**) mitochondrial function, thereby affecting IEC function. The second challenge was a fasting intervention, that was expected to decrease mitochondrial function, thereby decreasing IEC function (**chapters 6 and 7**). To investigate the impact of the imposed challenges, a method was optimised to isolate primary pig IECs and measure their metabolic function (**chapter 4**).

In **Chapter 2,** I reviewed the current knowledge of the biochemical, cellular and physiological functions polyamines in the intestine. Although polyamines are known to be important regulators of cell proliferation, as they are especially important highly proliferative tissues like the intestine, they can also negatively impact intestinal health by interfering with cellular metabolic functions. An overview of the role of polyamines in gut health and metabolic function is lacking, and will be provided in this chapter.

**Chapter 3** is dedicated to the fibre-fermentation metabolite butyrate. Mechanistic studies on the effects of butyrate are principally performed using cell lines, and these differ from the *in vivo* environment in crucial aspects like nutrient availability and metabolic phenotype. **Chapter 3** therefore aimed to investigate how different metabolic phenotypes, i.e., glycolytic or oxidative, and nutritional environments influence the metabolic fate of butyrate in cultured cells, as well as in colon-derived primary cells.

In **Chapter 4**, I optimized an IEC isolation technique to harvest viable primary IECs from the pig colon to measure mitochondrial respiration and glycolytic flux. This technique was applied in **chapters 5 and 6**, and enabled me to measure functional metabolism of IECs after various *in vivo* interventions.

While there is increasing interest in fibre in pig feeds due to the beneficial effects

ascribed to it, it is still largely unknown how this interacts with various protein sources and impacts intestinal physiology. Therefore, **Chapter 5** describes a study where pigs were fed diets differing in protein sources and fibre level, to create contrasts in the amount of protein- and fibre-fermentation occurring in the large intestine. Protein- and fibre-fermentation metabolites and functional metabolism of isolated colonocytes were analyzed to obtain insight in effects of the diets on colonic IECs

**Chapter 6** describes the effects of fasting on function metabolism of isolated small-intestinal IECs of six-week-old, feed-habituated piglets. These results were substantiated with transcriptome analysis, to identify the underlying molecular alterations. **Chapter 7** aimed to investigate whether the fasting response of the jejunum is different between suckling piglets and piglets habituated to solid feed. The main aim of the study was to identify which molecular pathways are differentially regulated to better understand the increased vulnerability to fasting in suckling piglets.

Finally, in **chapter 8** I summarize and combine the main findings of this thesis, discuss the implications and give directions for future research.

## References


1. Bischoff, S.C., *'Gut health': a new objective in medicine?* BMC Medicine, 2011. **9**(1): p. 24.
2. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nature Reviews Immunology, 2014. **14**(3): p. 141-153.
3. GBD 2019 Diseases and Injuries Collaborators *Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019.* The Lancet, 2020. **396**(10258): p. 1204-1222.
4. Alvarez, J., et al., *Impact of Porcine Epidemic Diarrhea on Performance of Growing Pigs.* PLOS ONE, 2015. **10**(3): p. e0120532.
5. Heo, J.M., et al., *Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds.* Journal of Animal Physiology and Animal Nutrition, 2013. **97**(2): p. 207-237.
6. Heinritz, S.N., R. Mosenthin, and E. Weiss, *Use of pigs as a potential model for research into dietary modulation of the human gut microbiota.* Nutrition Research Reviews, 2013. **26**(2): p. 191-209.
7. Ziegler, A., L. Gonzalez, and A. Blikslager, *Large Animal Models: The Key to Translational Discovery in Digestive Disease Research.* Cellular and Molecular Gastroenterology and Hepatology, 2016. **2**(6): p. 716-724.
8. Burrin, D.G., et al., *Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough?* The American Journal of Clinical Nutrition, 2000. **71**(6): p. 1603-10.
9. Eldelstone, D.I. and I.R. Holzman, *Gastrointestinal tract O2 uptake and regional blood flows during digestion in conscious newborn lambs.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1981. **241**(4): p. G289-G293.
10. van Erp, R.J.J., et al., *Reduced Feed Intake, Rather than Increased Energy Losses, Explains Variation in Growth Rates of Normal-Birth-Weight Piglets.* The Journal of Nutrition, 2018. **148**(11): p. 1794-1803.
11. Rolfe, D.F. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals.* Physiological Reviews, 1997. **77**(3): p. 731-758.
12. Roediger, W.E.W., *The colonic epithelium in Ulcerative Colitis: an energy-deficiency disease?* The Lancet, 1980. **316**(8197): p. 712-715.
13. Kameyama, J.-I., et al., *Energy Level in Large Intestinal Mucosa in Patients with Ulcerative Colitis.* The Tohoku Journal of Experimental Medicine, 1984. **143**(2): p. 253-254.
14. Den Hond, E., et al., *In vivo butyrate metabolism and colonic permeability in extensive ulcerative colitis.* Gastroenterology, 1998. **115**(3): p. 584-590.
15. Gabe, S.M., et al., *The effect of tacrolimus (FK506) on intestinal barrier function and cellular energy production in humans.* Gastroenterology, 1998. **115**(1): p. 67-74.
16. Bjarnason, I. and K. Takeuchi, *Intestinal permeability in the pathogenesis of NSAID-induced enteropathy.* Journal of Gastroenterology, 2009. **44**(19): p. 23-29.
17. Somasundaram, S., et al., *Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat.* Alimentary Pharmacology & Therapeutics, 2000. **14**(5): p. 639-650.
18. Funk, M.C., J. Zhou, and M. Boutros, *Ageing, metabolism and the intestine.* EMBO reports, 2020. **21**(7): p. e50047-e50047.
19. Helander, H.F. and L. Fändriks, *Surface area of the digestive tract – revisited.* Scandinavian Journal of Gastroenterology, 2014. **49**(6): p. 681-689.
20. Vandeputte, D., et al., *Temporal variability in quantitative human gut microbiome profiles and implications for clinical research.* Nature Communications, 2021. **12**(1): p. 6740.
21. Isaacson, R. and H.B. Kim, *The intestinal microbiome of the pig.* Animal Health

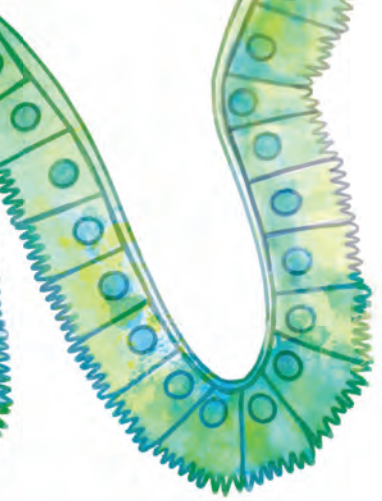
Research Reviews, 2012. **13**(1): p. 100-109.
22. Visconti, A., et al., *Interplay between the human gut microbiome and host metabolism.* Nature Communications, 2019. **10**(1): p. 4505-4505.
23. Frisch, S.M. and R.A. Screaton, *Anoikis mechanisms.* Current Opinion in Cell Biology, 2001. **13**(5): p. 555-562.
24. Wang, M., et al., *The developmental changes in intestinal epithelial cell proliferation, differentiation, and shedding in weaning piglets.* Animal Nutrition, 2022. **9**: p. 214-222.
25. Potten, C.S., C. Booth, and D.M. Pritchard, *The intestinal epithelial stem cell: the mucosal governor.* International Journal of Experimental Pathology, 1997. **78**(4): p. 219-243.
26. Busslinger, G.A., et al., *Human gastrointestinal epithelia of the esophagus, stomach, and duodenum resolved at single-cell resolution.* Cell Reports, 2021. **34**(10): p. 108819.
27. Elmentaite, R., et al., *Cells of the human intestinal tract mapped across space and time.* Nature, 2021. **597**(7875): p. 250-255.
28. Sato, T., et al., *Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts.* Nature, 2011. **469**(7330): p. 415-418.
29. van der Hee, B., et al., *Optimized procedures for generating an enhanced, near physiological 2D culture system from porcine intestinal organoids.* Stem Cell Research, 2018. **28**: p. 165-171.
30. Myer, M.S., *The presence of Paneth cells confirmed in the pig.* Onderstepoort Journal of Veterinary Research, 1982. **49**(2): p. 131-2.
31. Verdile, N., et al., *Evolution of pig intestinal stem cells from birth to weaning.* Animal, 2019. **13**(12): p. 2830-2839.
32. Banerjee, A., et al., *Interpreting heterogeneity in intestinal tuft cell structure and function.* The Journal of Clinical Investigation, 2018. **128**(5): p. 1711-1719.
33. Kalita, A., et al., *Small intestinal mucosal cells in piglets fed with probiotic and zinc: a qualitative and quantitative microanatomical study.* Folia Morphologica, 2021. **80**: p. 605-617.
34. Corr, S.C., C.C.G.M. Gahan, and C. Hill, *M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis.* FEMS Immunology & Medical Microbiology, 2008. **52**(1): p. 2-12.
35. Rodriguez-Colman, M.J., et al., *Interplay between metabolic identities in the intestinal crypt supports stem cell function.* Nature, 2017. **543**(7645): p. 424-427.
36. D'Errico, I., et al., *Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1alpha) is a metabolic regulator of intestinal epithelial cell fate.* Proceedings of the National Academy of Sciences (PNAS), 2011. **108**(16): p. 6603-8.
37. Xiong, X., et al., *Differential expression of proteins involved in energy production along the crypt-villus axis in early-weaning pig small intestine.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2015. **309**(4): p. G229-G237.
38. Yang, H., et al., *Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis.* Scientific Reports, 2016. **6**: p. 31917.
39. Spinelli, J.B. and M.C. Haigis, *The multifaceted contributions of mitochondria to cellular metabolism.* Nature Cell Biology, 2018. **20**(7): p. 745-754.
40. Sullivan, Lucas B., et al., *Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells.* Cell, 2015. **162**(3): p. 552-563.
41. Ducker, G.S. and J.D. Rabinowitz, *One-Carbon Metabolism in Health and Disease.* Cell Metabolism, 2017. **25**(1): p. 27-42.
42. Zhang, F., M. Pirooznia, and H. Xu, *Mitochondria regulate intestinal stem cell proliferation and epithelial homeostasis through FOXO.* Molecular Biology of the Cell, 2020. **31**(14): p. 1538-1549.
43. Schell, J.C., et al., *Control of intestinal stem cell function and proliferation by mitochondrial pyruvate metabolism.* Nature Cell Biology, 2017. **19**(9): p. 1027-1036.
44. Quirós, P.M., A. Mottis, and J. Auwerx, *Mitonuclear communication in homeo-*

*stasis and stress.* Nature Reviews Molecular Cell Biology, 2016. **17**(4): p. 213-226.
45. Zmijewski, J.W., et al., *Exposure to Hydrogen Peroxide Induces Oxidation and Activation of AMP-activated Protein Kinase.* Journal of Biological Chemistry, 2010. **285**(43): p. 33154-33164.
46. Myant, Kevin B., et al., *ROS Production and NF-κB Activation Triggered by RAC1 Facilitate WNT-Driven Intestinal Stem Cell Proliferation and Colorectal Cancer Initiation.* Cell Stem Cell, 2013. **12**(6): p. 761-773.
47. Formentini, L., et al., *The Mitochondrial ATPase Inhibitory Factor 1 Triggers a ROS-Mediated Retrograde Prosurvival and Proliferative Response.* Molecular Cell, 2012. **45**(6): p. 731-742.
48. Mori, Y., et al., *Extracellular ATP facilitates cell extrusion from epithelial layers mediated by cell competition or apoptosis.* Current Biology, 2022. **32**(10): p. 2144-2159. e5.
49. Muller, F.L., Y. Liu, and H. Van Remmen, *Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane* Journal of Biological Chemistry, 2004. **279**(47): p. 49064-49073.
50. Winterbourn, C.C., *Chapter One - The Biological Chemistry of Hydrogen Peroxide*, in *Methods in Enzymology*, E. Cadenas and L. Packer, Editors. 2013, Academic Press. p. 3-25.
51. Tannahill, G.M., et al., *Succinate is an inflammatory signal that induces IL-1ß through HIF-1α*. Nature, 2013. **496**(7444): p. 238-42.
52. Secor, S.M. and H.V. Carey, *Integrative Physiology of Fasting.* Comprehensive Physiology, 2016. **6**(2): p. 773-825.
53. Stoll, B., et al., *Substrate oxidation by the portal drained viscera of fed piglets.* American Journal of Physiology, 1999. **277**(1 Pt 1): p. E168-75.
54. Windmueller, H.G. and A.E. Spaeth, *Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate.* Journal of Biological Chemistry, 1980. **255**(1): p. 107-112.
55. Boets, E., et al., *Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study.* The Journal of Physiology, 2017. **595**(2): p. 541-555.
56. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-429.
57. Donohoe, D.R., et al., *The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon.* Cell Metabolism, 2011. **13**(5): p. 517-526.
58. Tan, H.T., et al., *Quantitative and Temporal Proteome Analysis of Butyrate-treated Colorectal Cancer Cells.* Molecular & Cellular Proteomics, 2008. **7**(6): p. 1174-1185.
59. Ghorbaniaghdam, A., O. Henry, and M. Jolicoeur, *A kinetic-metabolic model based on cell energetic state: study of CHO cell behavior under Na-butyrate stimulation.* Bioprocess and Biosystems Engineering, 2013. **36**(4): p. 469-487.
60. Matthews, G.M., G.S. Howarth, and R.N. Butler, *Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism.* Chemotherapy, 2012. **58**(2): p. 102-109.
61. Tabuchi, Y., et al., *Identification of genes responsive to sodium butyrate in colonic epithelial cells.* Biochemical and Biophysical Research Communications, 2002. **293**(4): p. 1287-1294.
62. Butler, R.N., et al., *Pyruvate sparing by butyrate and propionate in proliferating colonic epithelium.* Comparative Biochemistry and Physiology Part B, 1990. **97**(2): p. 333-337.
63. Gill Steven, R., et al., *Metagenomic Analysis of the Human Distal Gut Microbiome.* Science, 2006. **312**(5778): p. 1355-1359.
64. Reynolds, A., et al., *Carbohydrate quality and human health: a series of systematic reviews and meta-analyses.* The Lancet, 2019. **393**(10170): p. 434-445.
65. Gill, S.K., et al., *Dietary fibre in gastrointestinal health and disease.* Nature Reviews Gastroenterology & Hepatology, 2021. **18**(2): p. 101-116.

66. den Besten, G., et al., *Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2013. **305**(12): p. G900-10.
67. McLoughlin, R.F., et al., *Short-chain fatty acids, prebiotics, synbiotics, and systemic inflammation: a systematic review and meta-analysis.* The American Journal of Clinical Nutrition, 2017. **106**(3): p. 930-945.
68. den Besten, G., et al., *Short-Chain Fatty Acids Protect Against High-Fat Diet–Induced Obesity via a PPARγ-Dependent Switch From Lipogenesis to Fat Oxidation.* Diabetes, 2015. **64**(7): p. 2398-2408.
69. van der Beek, Christina M., et al., *Distal, not proximal, colonic acetate infusions promote fat oxidation and improve metabolic markers in overweight/obese men.* Clinical Science, 2016. **130**(22): p. 2073-2082.
70. Li, Q., et al., *Butyrate Suppresses the Proliferation of Colorectal Cancer Cells via Targeting Pyruvate Kinase M2 and Metabolic Reprogramming.* Molecular & Cellular Proteomics 2018. **17**(8): p. 1531-1545.
71. Miao, W., et al., *Sodium Butyrate Promotes Reassembly of Tight Junctions in Caco-2 Monolayers Involving Inhibition of MLCK/MLC2 Pathway and Phosphorylation of PKCβ2.* International Journal of Molecular Sciences, 2016. **17**(10): p. 1696.
72. Nielsen, D.S.G., et al., *Effect of butyrate and fermentation products on epithelial integrity in a mucus-secreting human colon cell line.* Journal of Functional Foods, 2018. **40**: p. 9-17.
73. Peng, L., et al., *Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers.* The Journal of Nutrition, 2009. **139**(9): p. 1619-1625.
74. Schulthess, J., et al., *The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages.* Immunity, 2019. **50**(2): p. 432-445.e7.
75. Segain, J.P., et al., *Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease.* Gut, 2000. **47**(3): p. 397.
76. Ten Bruggencate, S.J.M., et al., *Dietary Fructooligosaccharides Increase Intestinal Permeability in Rats.* The Journal of Nutrition, 2005. **135**(4): p. 837-842.
77. Rodenburg, W., et al., *Impaired barrier function by dietary fructo-oligosaccharides (FOS) in rats is accompanied by increased colonic mitochondrial gene expression.* BMC Genomics, 2008. **9**(1): p. 144.
78. Alam, M.A., F.I. Al-Jenoobi, and A.M. Al-mohizea, *Everted gut sac model as a tool in pharmaceutical research: limitations and applications.* Journal of Pharmacy and Pharmacology, 2012. **64**(3): p. 326-336.
79. Pieper, R., et al., *Fermentable Fiber Ameliorates Fermentable Protein-Induced Changes in Microbial Ecology, but Not the Mucosal Response, in the Colon of Piglets.* The Journal of Nutrition, 2012. **142**(4): p. 661-667.
80. Wellock, I.J., et al., *The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs.* Animal Science, 2006. **82**(3): p. 327-335.
81. Cremin, J.D., Jr., M.D. Fitch, and S.E. Fleming, *Glucose alleviates ammonia-induced inhibition of short-chain fatty acid metabolism in rat colonic epithelial cells.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2003. **285**(1): p. G105-14.
82. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-798.
83. Darcy-Vrillon, B., et al., *Short chain fatty acid and glucose metabolism in isolated pig colonocytes: modulation by NH4+.* Molecular and Cellular Biochemistry, 1996. **156**(2): p. 145-151.
84. Libiad, M., et al., *Hydrogen sulfide perturbs mitochondrial bioenergetics and triggers metabolic reprogramming in colon cells.* Journal of Biological Chemistry, 2019. **294**(32): p. 12077-12090.
85. Lagoutte, E., et al., *Oxidation of hydrogen sulfide remains a priority in mamma-*

*lian cells and causes reverse electron transfer in colonocytes.* Biochimica et Biophysica Acta (BBA), 2010. **1797**(8): p. 1500-11.
86. Kramer, D.L., et al., *Polyamine Acetylation Modulates Polyamine Metabolic Flux, a Prelude to Broader Metabolic Consequences.* Journal of Biological Chemistry, 2008. **283**(7): p. 4241-4251.
87. Kee, K., et al., *Activated polyamine catabolism depletes acetyl-CoA pools and suppresses prostate tumor growth in TRAMP mice.* Journal of Biological Chemistry, 2004. **279**(38): p. 40076-40083.
88. Jell, J., et al., *Genetically Altered Expression of Spermidine/Spermine N1-Acetyltransferase Affects Fat Metabolism in Mice via Acetyl-CoA.* Journal of Biological Chemistry, 2007. **282**(11): p. 8404-8413.
89. Pegg, A.E., *Toxicity of Polyamines and Their Metabolic Products.* Chemical Research in Toxicology, 2013. **26**(12): p. 1782-1800.
90. Leidy, H.J., et al., *The role of protein in weight loss and maintenance.* The American Journal of Clinical Nutrition, 2015. **101**(6): p. 1320S-1329S.
91. Anderson, J.W., et al., *Health benefits of dietary fiber.* Nutrition Reviews, 2009. **67**(4): p. 188-205.
92. Bikker, P., et al., *The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets.* Journal of Animal Science, 2006. **84**(12): p. 3337-3345.
93. Jha, R. and J.F.D. Berrocoso, *Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review.* Animal Feed Science and Technology, 2016. **212**: p. 18-26.
94. Agnoli, C., et al., *Position paper on vegetarian diets from the working group of the Italian Society of Human Nutrition.* Nutrition, Metabolism and Cardiovascular Diseases, 2017. **27**(12): p. 1037-1052.
95. Campbell, J.M., J.D. Crenshaw, and J. Polo, *The biological stress of early weaned piglets.* Journal of Animal Science and Biotechnology, 2013. **4**(1): p. 19-19.
96. Dybkjær, L., et al., *Eating and drinking activity of newly weaned piglets: Effects of individual characteristics, social mixing, and addition of extra zinc to the feed1.* Journal of Animal Science, 2006. **84**(3): p. 702-711.
97. Bruininx, E.M.A.M., et al., *Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs1.* Journal of Animal Science, 2002. **80**(6): p. 1413-1418.
98. Bruininx, E.M.A.M., et al., *Individually measured feed intake characteristics and growth performance of group-housed weanling pigs: effects of sex, initial body weight, and body weight distribution within groups.* Journal of Animal Science, 2001. **79**(2): p. 301-308.
99. Le Dividich, J. and B. Sève, *Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet.* Domestic Animal Endocrinology, 2000. **19**(2): p. 63-74.
100. Madec, F., et al., *Measurement of digestive disorders in the piglet at weaning and related risk factors.* Preventive Veterinary Medicine, 1998. **35**(1): p. 53-72.
101. FAO, I., UNICEF, WFP and WHO, *The State of Food Security and Nutrition in the World 2021: Transforming food systems for food security, improved nutrition and affordable healthy diets for all*. 2021, Food & Agriculture Organisation of the United Nations: Rome.
102. Fan, S. and J. Brzeska, *Sustainable food security and nutrition: Demystifying conventional beliefs.* Global Food Security, 2016. **11**: p. 11-16.
103. Pelletier, D.L., et al., *The effects of malnutrition on child mortality in developing countries.* Bulletin of the World Health Organization, 1995. **73**(4): p. 443-448.
104. World Health Organization *Diarrhoeal disease.* 2017; Available from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease.
105. Sokolovi, M., et al., *Fasting induces a biphasic adaptive metabolic response in murine small intestine.* BMC genomics, 2007. **8**: p. 361-361.

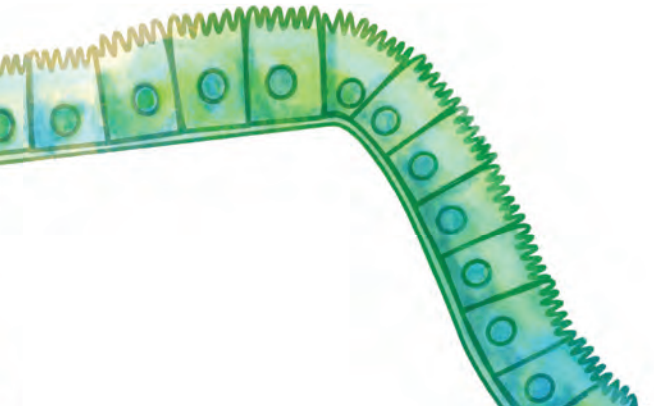
106. Madapallimattam, A.G., L. Law, and K.N. Jeejeebhoy, *Effect of hypoenergetic feeding on muscle oxidative phosphorylation and mitochondrial complex I–IV activities in rats.* The American Journal of Clinical Nutrition, 2002. **76**(5): p. 1031-1039.
107. Firmansyah, A., D. Penn, and E. Lebenthal, *Isolated colonocyte metabolism of glucose, glutamine, n-butyrate, and β-hydroxybutyrate in malnutrition.* Gastroenterology, 1989. **97**(3): p. 622-629.
108. Owen, O.E., et al., *Brain metabolism during fasting.* The Journal of Clinical Investigation, 1967. **46**(10): p. 1589-95.
109. Moeser, A.J., C.S. Pohl, and M. Rajput, *Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs.* Animal Nutrition, 2017. **3**(4): p. 313-321.
110. Montagne, L., et al., *Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning.* British Journal of Nutrition, 2007. **97**(1): p. 45-57.
111. Pluske, J.R., D.J. Hampson, and I.H. Williams, *Factors influencing the structure and function of the small intestine in the weaned pig: a review.* Livestock Production Science, 1997. **51**(1): p. 215-236.
112. Meng, Q., et al., *Weaning Alters Intestinal Gene Expression Involved in Nutrient Metabolism by Shaping Gut Microbiota in Pigs.* Frontiers in Microbiology, 2020. **11**: p. 694-694.
113. Boudry, G.l., et al., *Weaning Induces Both Transient and Long-Lasting Modifications of Absorptive, Secretory, and Barrier Properties of Piglet Intestine.* The Journal of Nutrition, 2004. **134**(9): p. 2256-2262.



# Chapter 2

## The Molecular and Physiological Effects of Protein-Derived Polyamines in the Intestine

Anna F. Bekebrede
Jaap Keijer
Walter J.J. Gerrits
Vincent C.J. de Boer



**Nutrients 2020;**
doi:10.3390/nu12010197

## Abstract

Consumption of a high-protein diet increases protein entry into the colon. Colonic microbiota can ferment proteins, which results in the production of protein fermentation end-products, like polyamines. This review describes the effects of polyamines on biochemical, cellular and physiological processes, with a focus on the colon. Polyamines (mainly spermine, spermidine, putrescine and cadaverine) are involved in the regulation of protein translation and gene transcription. In this, the spermidine-derived hypusination modification of EIF5A plays an important role. In addition, polyamines regulate metabolic functions. Through hypusination of EIF5A, polyamines also regulate the translation of mitochondrial proteins, thereby increasing their expression. They can also induce mitophagy through various pathways, which helps to remove damaged organelles and improves cell survival. In addition, polyamines increase mitochondrial substrate oxidation by increasing mitochondrial $Ca^{2+}$-levels. Putrescine can even serve as an energy source for enterocytes in the small intestine. By regulating the formation of the mitochondrial permeability transition pore, polyamines help maintain mitochondrial membrane integrity. However, their catabolism may also reduce metabolic functions by depleting intracellular acetyl-CoA levels, or through the production of toxic by-products. Lastly, polyamines support gut physiology, by supporting barrier function, inducing gut maturation and increasing longevity. Polyamines thus play many roles, and their impact is strongly tissue- and dose-dependent. However, whether diet-derived increases in colonic luminal polyamine levels also impact intestinal physiology has not been resolved yet.

**Keywords**: protein; polyamines; intestine; metabolism; mitochondrial function; hypusine

## Introduction

Intake of protein when consuming a 'normal' Western diet contributes to around 15% of total energy intake [1], of which 85%–95% is digested in the small intestine [2]. The undigested protein from the small intestine, together with endogenously generated protein, moves towards the colon [3]. Here, the protein can be utilised by the microbiota to support their function and survival. Interestingly, by metabolizing the undigested protein, microbiota form and secrete metabolites that also impact the host [4]. Among these protein-derived metabolites, most are small molecule metabolites, like hydrogen sulfide, branched-chain fatty acids, phenolic compounds and polyamines [4, 5]. Increasing protein intake beyond 'normal' levels has been shown to be effective for weight-loss management as well as sports performance [6, 7]. Although these benefits of higher protein intake are well documented, it is not completely understood what the physiological effects are of increased concentrations of undigested proteins in the human colon. In pig studies, it has been shown that high protein intake leads to increased levels of the metabolites derived from protein fermentation in the gut, which have been associated with negative effects on gut health [8, 9]. Among the protein fermentation end-products, polyamines have attracted much interest, in part, because of their essential role in cell proliferation, as well as their roles in other cellular functions, like cell growth, mitochondrial metabolism and histone regulation [10-12]. Combined, it is clear that polyamines influence multiple aspects of normal physiology. In this review, we focus on the biochemical, cellular and physiological aspects of the role of polyamines in regulating metabolism and proliferation in cells, with a special focus on the effects within the colon.

## Polyamine Synthesis and Catabolism

### *Bacterial Polyamine Production in the Colon is Dictated by Microbiome, Diet and Host Factors*

Spermine, spermidine, putrescine and cadaverine (Figure 2.1) are the most common polyamines in the human body. Based on faecal sample analysis it appears that putrescine is the most abundant polyamine in the human colon, followed by spermine, spermidine and cadaverine successively [13, 14]. To produce polyamines, the microbiota in the colon need to break down proteins, in order to generate amino acids, which can serve as precursors for polyamine production [15]. Predominant bacterial species involved in proteolysis in the human gut are *Bacteroides* [16]. One resulting amino acid, arginine, can be converted, via citrulline, into L-ornithine, after which putrescine is produced [17]. Alternatively, arginine can also be converted into agmatine, which is then converted into putrescine, either directly by the enzyme agmatine ureaohydrolase, or indirectly through intermediate production of N-Carbamoyl-

Putrescine [17]. Putrescine can then be further converted into spermidine and spermine. Recently, it was shown that microbial production of putrescine is a complex process, in which different bacterial species exchange polyamine intermediates, to finally produce putrescine [17]. The microbiome of humans mainly consists of the phyla Firmicutes (60%–80%) and Bacteroidetes (20%–40%) [18]. In other species, such as pigs, Firmicutes and Bacteroidetes are also the most abundant, but comprise a lower percentage of the total microbiome (~30% and ~10%, respectively) [19]. Although the contribution of these phyla to the total population of microbiota is lower in pigs, the functional metabolic pathways are similar between pigs and humans, indicating that microbial polyamine metabolism could be comparable [19].

Since microbiota produce polyamines from sources provided through the diet, dietary changes influence microbial polyamine production in the gut lumen. Indeed, in rats, diets high in fat were shown to increase spermine and spermidine concentrations in the mucosa, as compared to a soy protein-based diet, which led to much lower mucosal concentrations of these polyamines [20]. Similarly, in pigs that were given a feed containing casein, higher polyamine levels were found in the lumen of the proximal colon, as compared to the lumen of pigs fed a soy diet [21]. Since diets can shape the microbiome composition [22], the influence of diet on polyamine levels could also be explained by dietary-induced changing of the microbiota composition. This was for example shown for the proteolytic species *Bacteroides*, which become more abundant under conditions of higher protein availability in *in vitro* fermentations using human fecal inoculates [23]. This rise in proteolytic bacteria can thus contribute to increased polyamine levels in the colon by increasing substrate availability for polyamine production. Taken together, the most prominent determinants of luminal polyamine levels in an individual are found to be diet, host species and microbiome composition.

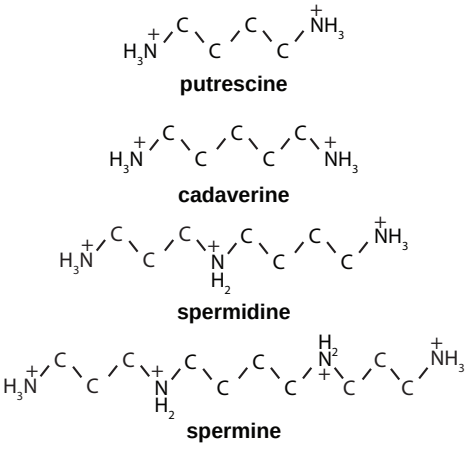



Figure 2.1**. Polyamine structures**. The two-dimensional structures of the diamines cadaverine and putrescine, the tri-amine spermidine and the tetra-amine spermine.

### *Mammalian Polyamines Synthesis Pathways*

In mammalian cells, the precursor for putrescine, spermidine and spermine is L-ornithine. Putrescine is synthesised via decarboxylation of the amino acid L-ornithine by the enzyme ornithine decarboxylase (ODC1) [12], which is one of the rate-limiting step in the formation of all downstream polyamines in this pathway. The diamine putrescine is converted to the triamine spermidine by spermidine synthase (SRM), and spermidine can be subsequently converted into spermine by spermine synthase (SMS) (Figure 2.2). SMS and SRM both use S-adenosylmethioninamine (dcAdoMET), the decarboxylation product of s-adenosyl methionine (SAM) as the aminopropyl-group source to produce spermidine and spermine. DcAdoMet is fully dedicated to the production of polyamines, since it is only used as an aminopropyl-donor for the formation of polyamines. Spermine can be converted back to spermidine through direct oxidation by spermine oxidase (SMOX) [24]. Alternatively, spermine can first undergo acetylation by spermidine/spermine-N1-acetyltransferases 1 and 2 (SSAT1, with a higher catalytic activity for spermidine; SSAT2, with equal catalytic activity for spermine and spermidine) before being oxidised by polyamine oxidase (PAOX) [12]. Acetylation by SSATs decreases the charge of polyamines, thereby making them less reactive and more easily degraded by PAOX [25]. Acetylspermine can also be oxidised by SMOX, but with a much lower $K_m$ than spermine itself [25]. Although spermidine cannot be directly oxidised, it can be acetylated by SSATs, followed by oxidation by PAOX [26].

Cadaverine, a diamine like putrescine, is formed via a different route than the other polyamines. Cadaverine is synthesised from the amino acid L-lysine instead of from L-ornithine as for the other polyamines [27]. It is known that in bacteria, cyanobacteria and plants, L-lysine is converted into cadaverine by lysine decarboxylase (cadA), but in mammalian and fungal cells, the *cadA* gene has not been identified [28]. Cadaverine is nevertheless present in mammalian cells [29]. Therefore, some have suggested that cadaverine is also synthesised from L-lysine through ODC1 in mammalian cells. However, these findings come from experiments performed when both polyamines and serum are depleted [30], or in isolated tissues with very high ODC1 activity [31], but not under physiological conditions. In addition, even when blocking ODC1 activity, increased cadaverine levels were observed in cultured cells [32], suggesting that there may be other pathways involved in the mammalian production of cadaverine. Cadaverine can further be converted into its aminopropyl-form (aminopropylcadaverine), which is a close analogue of spermidine and may also be produced in mammalian cells via SRM [32].

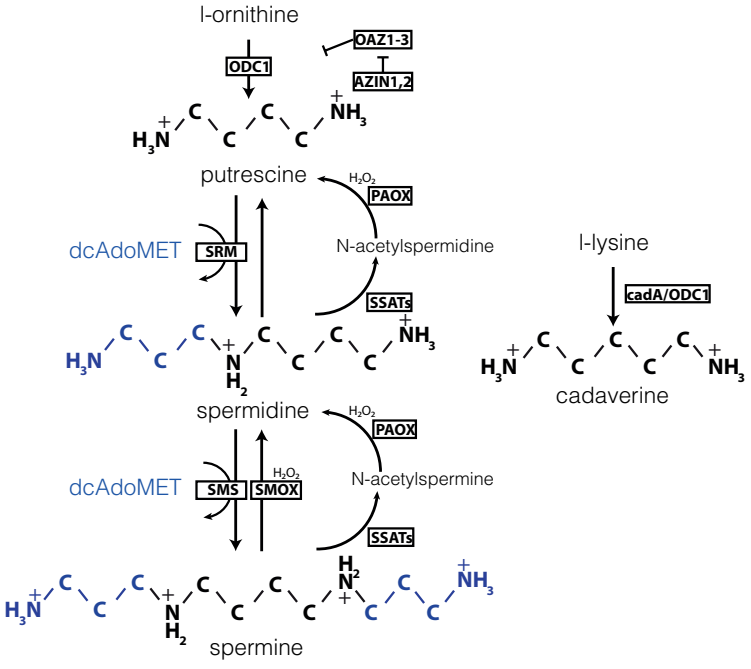



Figure 2.2. **Polyamine metabolism.** First, ornithine is converted into putrescine by ODC1. ODC1 is under tight control by OAZ1-3, which in turn is regulated by the antizyme inhibitor AZIN1-2. Together with dcAdoMET, putrescine can be converted into spermidine. Spermidine in turn can be converted into spermine, again with dcAdoMET as a co-factor. Both spermine and spermidine can be acetylated by SSAT. The acetylated product, but also spermine and spermidine themselves, can then be oxidised by PAOX. Both the acetylation and oxidation reactions produce reactive oxygen species (ROS). Cadaverine is synthesised though decarboxylation of lysine by bacterial cadA. ODC1: ornithine decarboxylase 1; dcAdoMET: s-adenosylmethioninamine; SSATs: spermidine/spermine N-1 acetyl transferases; PAOX: polyamine oxidase; cadA: inducable lysine decarboxylase.

## Regulation of Polyamine Levels

### *Regulation through Intracellular Polyamine Metabolism*

Polyamine levels are tightly controlled within mammalian cells and several processes contribute to their regulation. Intracellular polyamine levels can be controlled through regulation of enzymes involved in polyamine metabolism. ODC1 protein levels are regulated by Antizyme (OAZ1-3), which forms a heterodimer with ODC1 and presents it to the 26S proteasome for ubiquitin-independent degradation [33]. OAZs themselves are regulated by the Antizyme inhibitor (AZIN1 and 2), which, amongst others, are homologous to ODC1 but lack its catalytic activity [34]. Under conditions of low polyamine levels, OAZs are repressed by AZINs, and ODC1 is synthesised. Synthesis of spermine and spermidine is further regulated by S-adenosylmethionine decarboxylase (AMD1), the enzyme that catalyzes the formation of dcAdoMet from SAM. Polyamines

can actively regulate AMD1 expression and activity. AMD1 is transcribed as an inactive pro-enzyme, that undergoes auto-processing to turn into its active form [35]. Putrescine promotes autocatalytic cleavage of the AMD1 pro-enzyme, which leads to higher levels of active AMD1 in the cells, enabling the formation of spermine and spermidine [36]. In addition, putrescine can promote the catalytic activity of the enzyme by binding to the enzyme, which results in a electrostatic change that allows for improved conformation of the catalytic site [36]. Levels of spermine and spermidine are inversely correlated to levels of AMD1. Spermidine predominantly regulates transcription of the *AMD1* gene [37, 38], while spermine seems to have a greater effect on the translation of the enzyme [38]. Furthermore, both spermine and spermidine influence the half-life of AMD1 [37].

The level of AMD1 is also regulated by the Mammalian target of rapamycin complex 1 (mTORC1), a protein complex generally promoting protein synthesis and growth [39]. The pro-enzyme form of AMD1 was shown to be phosphorylated by mTORC1. This prolonged the pro-enzyme's half-life. Thus, phosphorylation of the pro-enzyme contributes to higher levels of the active enzyme [40]. This signifies that in a state of active growth and protein synthesis, mediated through activation of mTORC1, polyamine metabolism is upregulated.

### *Regulation through Uptake and Transport*

Polyamine levels are further regulated through uptake and export. While in prokaryotes uptake and export mechanisms have been described, little is known about these mechanisms in mammalian cells, despite continuous efforts to elucidate them. So far, no polyamine importer has been identified in mammalian cells. Instead, a caveolin-dependent endocytic uptake mechanism was discovered [41-43]. Polyamine export out of mammalian cells is mediated, at least in part, through SLC3A2, a member of the solute carrier family 3, that is involved in transport of L-type amino acids [44]. SLC3A2 is a diamine transporter that exports putrescine and imports arginine. However, interaction of this protein with SSAT suggests that it may also export acetylated polyamines [44].

## Polyamines, Gene-Transcription and Proliferation

### *Polyamines Are the Substrates for the Post-Translational Modification Hypusine*

A key role of polyamines is their ability to activate the eukaryotic translation initiation factor 5A (EIF5A) [45]. The name "eukaryotic translation initiation factor 5A" was given to the protein because initially it was thought that it acted as a translation initiation factor for all proteins. However, later it was discovered that EIF5A actually functions as a translation elongation factor [46, 47]. Despite these findings, the protein is still

known by its original name. Polyamines influence the activity of EIF5A via the post-translational modification of a specific lysine residue. Spermidine can be converted into hypusine (N--(4-amino-2-hydroxybutyryl-lysine)) by deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH) in the cytosol [48]. Because of this modification, activated EIF5A is able to bind to ribosomes, enabling translation [46, 47, 49]. Since putrescine and spermine can both be converted into spermidine, these polyamines contribute to this process in a more indirect way, and the balance between them is dependent on specific cellular demands [50]. So far, EIF5A is the only protein found to have this hypusine post-translational modification. Indeed, the enzymes responsible for hypusination were shown to be EIF5A-specific [51-53]. Under physiological conditions, almost all the EIF5A protein present seems to be present in its active, hypusinated form (around 80%) [54].

### *Regulation of Eukaryotic Translation Initiation Factor 5A (EIF5A) Hypusination through Acetylation*

EIF5A-hypusination was found to be regulated by acetylation of the hypusine residue by SSAT1 [55]. However, both spermine and spermidine are much better substrates for SSAT1, indicating that very little hypusine-acetylation will take place when sufficient levels of these polyamines are present [55]. Isolated acetylated hypusine-EIF5A from bovine testis, as well as recombinant acetylated hypusine-EIF5A, was shown to be inactive [55]. Thus, since hypusine-acetylation inactivates EIF5A, it seems that when there are insufficient levels of polyamines, EIF5A is inhibited and protein translation is decreased. Whether this mechanism also plays a role under physiological conditions remains to be seen, since intracellular polyamine levels are normally tightly controlled. However, it may prove an interesting therapeutic target under conditions where excessive cell proliferation occurs.

### *EIF5A Hypusination Regulates Polyamine Synthesis*

A recent paper revealed how polyamine levels are regulated within the cells through hypusination of EIF5A [56]. The mechanism was shown to occur via AZIN1, the negative regulator of antizyme (OAZ1-3). Normally, translation starts when a 43S pre-initiation complex, consisting of a 40S small ribosomal subunit in combination with various initiation factors, encounters the start codon AUG. However, under certain circumstances, alternative start codons may be recognised, allowing for the transcription of alternative open reading frames [56, 57]. In the presence of high polyamine levels (2 mM spermidine), ribosomes were shown to start queueing before the start of the main open reading frame (mORF), which lead to an increased start of translation at any weak start codon upstream of the mORF [56]. Start of translation at a weak start codon results in failure to properly translate and transcribe the AZIN1

protein, and thus repressed AZIN1 expression. As a result, OAZs are no longer inhibited by AZIN1. This leads to decreased synthesis of polyamines due to repression of ODC1 by OAZs. Thus, in this case polyamines cause stalling of ribosomes, instead of aiding translation elongation through EIF5A [56]. The authors state that high levels of polyamines may interfere with EIF5A hypusination, thus contributing to the stalling of ribosomes. It seems to be the case that optimal hypusination of EIF5A only takes place under low intracellular polyamine conditions, and thus only low concentrations of polyamines are needed to maintain maximal protein synthesis [58, 59]. If this is the case, then increased intracellular concentrations may actually be detrimental for the cell.

## Polyamines and Metabolic Functions

### *Polyamines Induce Mitochondrial Protein and Gene Transcription*

The role of polyamines in transcription can specifically influence mitochondrial function. An overview of how this regulation of polyamines on metabolic function occurs is depicted in Figure 2.3. In macrophages, hypusination of EIF5A was found to induce transcription of mitochondrial genes (Figure 2.3). When macrophages are activated, they can either adapt a pro- or an anti-inflammatory phenotype, depending on cues from the environment. These phenotypic adaptations require, and are even driven by, metabolic changes within the cells [60]. Thus, alterations in metabolic function influence the phenotypic outcome of differentiation from monocyte to macrophage. Puleston *et al.* showed that hypusination of EIF5A plays a crucial role in the metabolic switch of macrophages [61]. In the absence of hypusination of EIF5A, oxygen consumption, and thus mitochondrial function, was greatly reduced [61]. Because of this, macrophages could not adapt an anti-inflammatory phenotype, instead resorting to aerobic glycolysis, which resulted in differentiation of macrophages towards a more pro-inflammatory phenotype [61]. Another study showed that deletion of ODC1, and thus depletion of intracellular polyamine levels, led to a marked increase of pro-inflammatory macrophages [62]. As a result, increased stomach and colonic inflammation was observed. Here, the mechanism was attributed to the altered methylation status of the chromatin, which led to gene transcription that favored pro-inflammatory macrophage differentiation [62].

In kidney cells, the importance of EIF5A-hypusination on mitochondrial protein expression was also observed [63]. Melis *et al.* [63] showed that inhibition of hypusination by intraperitoneal injection of rats with the deoxyhypusine synthase inhibitor N1-guanyl-1,7-diaminoheptane (GC7) led to decreased expression of mitochondrial complexes. This correlated with lower mitochondrial respiration [63]. The decrease in mitochondrial respiration lead to lower reactive oxygen species (ROS)

production during hypoxia. Thus, decreased hypusination could protect kidney cells from oxidative damage through downregulation of mitochondrial protein expression. Based on their observations, higher polyamine levels should thus lead to higher EIF5A hypusination levels, and also higher mitochondrial respiration in kidney cells.

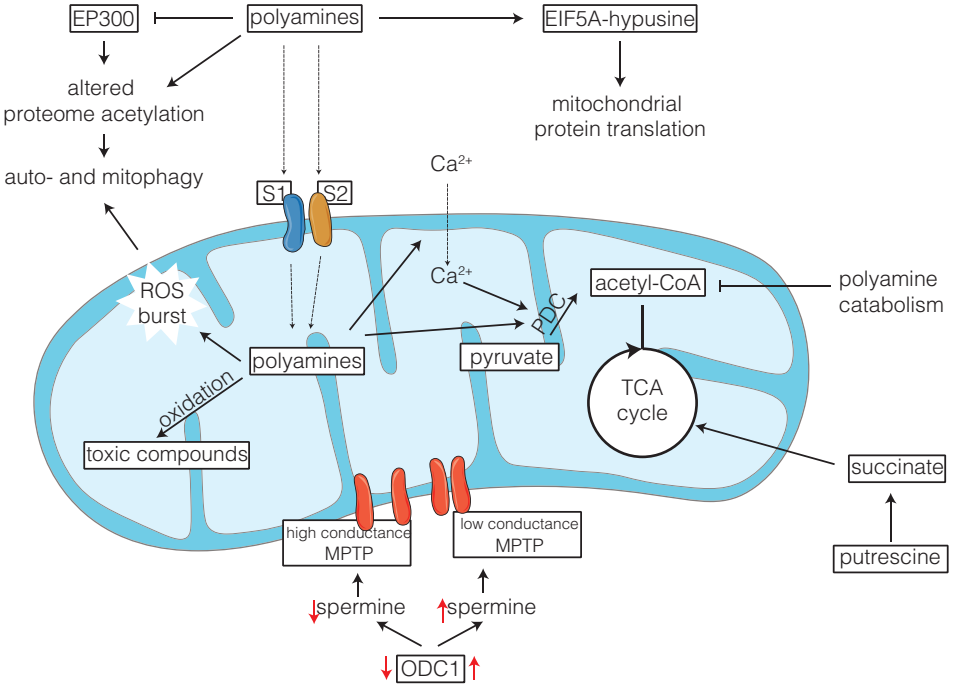



Figure 2.3. **The role of polyamines in metabolism.** Polyamines modify EIF5A by hypusination, which leads to upregulated of mitochondrial protein translation. Polyamines can induce both auto- and mitophagy, through various mechanisms. Firstly, through inhibition of the lysine acetyltransferase EP300, which leads to altered proteome acetylation and autophagy. Secondly, through induction of a ROS burst, which leads to activation of Ataxia-telangiectasia mutated (ATM) protein and more downstream induces mitophagy. Putrescine can, through conversion to succinate, serve as a direct energy source for small intestinal enterocytes. However, polyamine catabolism can deplete acetyl-CoA levels, because it is used as a substrate by SSATs. Polyamines are transported into mitochondria, where then influence respiration directly, through interaction with the pyruvate dehydrogenase complex (PDC), or indirectly by increasing $Ca^{2+}$-concentrations, thereby increasing PDC activity. At the same time, polyamine catabolism within the mitochondrial matrix leads to local production of toxic compounds, which may negatively affect respiratory functions. But, polyamines can also protect mitochondrial membrane integrity through regulation of the mitochondrial permeability transition pore. In red, regulation of spermine levels is indicated. When ODC1 is upregulated, more spermine is produces which induces the formation of the low conductance state of MPTP. This helps to maintain proper membrane integrity. When ODC1 activity is decreased, intracellular spermine concentrations are decreased, and the high conductance state of the MPTP cannot be prevented, resulting in mitochondrial swelling. EP300: E1A-binding protein p300; ROS: reactive oxygen species; MPTP: mitochondrial permeability transition pore; EIF5A: eukaryotic translation initiation factor 5A; TCA: tricarboxylic acid cycle; SSATs: spermidine/spermine N-1 acetyl transferases; S1 and S2: mitochondrial polyamine transporters 1 and 2.

### *Polyamines Can Induce Mitophagy*

Induction of mitophagy has been considered a mechanism to protect from cell death and can even be a mechanism to maintain and optimize metabolic function [64]. Indeed, in a study that examined mitochondria in heart muscle in hypertension and ageing, spermidine was shown to induce mitophagy, thus removing damaged mitochondria, resulting in improved metabolic function and cell survival (Figure 2.3) [65]. This effect was also observed in neuronal cells where ageing was induced [66]. Both mitophagy and autophagy can be induced through decreased mTORC1 activation. However, whether spermidine induces inhibition of mTORC1 is unclear, with some studies reporting inhibition of mTORC1, while others not observing this, even when using the same concentration of spermidine [67, 68]. A consistent finding is that spermidine alters the proteome acetylation status [67-69], at least in part through inhibition of E1A-binding protein p300 (EP300), a lysine acetyltransferase [68]. As a result, increased autophagy was observed.

In human fibroblasts, another mechanism of spermidine-induced mitophagy was observed. Exposure to 50 μM of spermidine was shown to cause mitochondrial depolarization and a ROS burst, which contributed to activation of the protein kinase Ataxia-telangiectasia mutated (ATM) protein [64]. Apart from its role in DNA damage response, ATM also has a function in redox sensing. Activation of ATM contributed to PTEN-induced putative kinase 1 (PINK1) stabilization on the outer membranes of damaged or non-functional mitochondria, which causes translocation of the E3 ubiquitin ligase Parkin (PRKN) to these mitochondria [64]. Ultimately, this leads to the removal of these non-functional mitochondria via mitophagy [64], resulting in improved cell survival.

### *Polyamines Serve as Energy Scource for Enterocytes*

The essential role of polyamines for metabolic function is not restricted to their role in protein transcription or mitochondrial integrity. On a more general metabolic level, putrescine was shown to serve as a direct energy source for intestinal cells. Using labeled putrescine, rat enterocytes were shown to take up putrescine from the lumen [70]. Once taken up, putrescine was in part converted to succinate, and this can be used as a fuel for oxidative phosphorylation (Figure 2.3) [70]. Although the conversion of putrescine to succinate is not high in normal situations, conversion can greatly increase under certain conditions. A fasting period was found to significantly enhance the conversion of putrescine to succinate in these rats, which remained elevated even 12 h after refeeding [70].

### *Polyamines Influence Metabolism by Depleting Acetyl-CoA Levels*

A more indirect way in which polyamines impact metabolism is linked to their dependency on acetyl-CoA as a cofactor for SSAT1 in polyamine acetylation. Polyamine breakdown and export, is for a large part, dependent on the acetylation of the 'higher' polyamines by the enzyme SSAT1, in order remove them faster and more efficiently, where acetyl-CoA is the acetyl-donor [25]. Interestingly, acetyl-CoA levels were shown to be depleted upon overexpression of SSAT1, both *in vitro* and *in vivo* [71-73]. Increased acetylation of polyamines led to induction of ODC1 expression, thus changing overall flux through the polyamine metabolic pathways [73]. Overexpression of SSAT1 in mice led to reduced acetyl-CoA levels in adipose tissue due to the increased acetylation of polyamines, which also correlated with a lean phenotype of the mice [73]. The lean phenotype coincided with increased palmitate and glucose oxidation in the liver [73]. It is likely that increased oxidation is needed to maintain an adequate acetyl-CoA supply. Thus, increased intracellular polyamine levels may deplete cellular acetyl-CoA levels, which may be detrimental if no increase of substrate oxidation can be achieved.

### *Polyamines Are Transported into Mitochondria and Influence Oxidation*

Polyamines also influence metabolic outputs by directly altering mitochondrial functioning. Using isolated mitochondria, it was shown that the mitochondrial matrix can contain high levels of polyamines (around 500 μM for spermine and spermidine, and around 30 μM for putrescine) [74], despite the lack of polyamine biosynthetic pathways within mitochondria. Polyamines within mitochondria, therefore, suggest the presence of a mitochondrial polyamine carrier exists. Indeed, two ATP-dependent mitochondrial carriers have been found for polyamines, termed S1 and S2 (Figure 2.3) [75]. Spermine and spermidine can both bind to and be transported into mitochondria by each of these carriers, while putrescine can only bind to one. Again using isolated mitochondria, polyamine uptake was found to be dependent on membrane potential, as was demonstrated by Grancara *et al.* [76] for spermine, and for all four polyamines by Toninello el al. [77]. Grancara *et al.* [76] found that an increase in the membrane potential from 150 to 180 mV led to a four-fold increase in spermine uptake. Moreover, addition of FCCP, which collapses the membrane potential, caused efflux of spermine [76, 78]. Interestingly, spermine constantly cycled over the mitochondrial membrane. In a normal state, with active ATP production, spermine was transported back and forth over the membrane together with ADP and phosphate, which is mediated by adenine nucleotide translocase (ANT) [76]. However, in a state where ATP synthesis was blocked, either through absence of ADP or by inhibition of ATP synthase with oligomycin, spermine efflux is blocked [76]. Spermine was also shown to have a restorative effect on oxidative functions of isolated aged mitochondria [66, 79] and it

stimulated ATPase activity in bovine heart sub-mitochondrial particles, although it was not mechanistically investigated how this was achieved [80]. Interestingly, spermidine and putrescine exhibited a slightly inhibitory effect on ATPase activity in these sub-mitochondrial particles [80].

Although it is unclear what the precise role of polyamines is in mitochondrial physiology, spermine was shown to regulate calcium transport into mitochondria [81]. The calcium-sensitive pyruvate dehydrogenase complex is important for controlling pyruvate influx and subsequent conversion of pyruvate to acetyl-CoA in the mitochondria. Pezzato *et al.* [78] showed that spermine increased mitochondrial metabolic rate, as seen by a dose-dependent increase in $CO_2$ production from pyruvate in isolated rat liver mitochondria upon exposure to spermine, with a maximum increase observed with 0.5–1 mM of spermine. The authors [78] showed that spermine could influence the pyruvate dehydrogenase complex through increased calcium levels within mitochondria. In addition, spermine had a direct effect on the $E_1$-subunit of the complex (Figure 2.3). Spermine concentrations of up to 0.5 mM led to the highest increase in $CO_2$ production, resulting from dephosphorylation of the $E_1$-subunit. Higher concentrations of spermine led to a gradual re-phosphorylation, which corresponded with a slight decrease in $CO_2$ production. To exclude possible confounding effects of $Ca^{2+}$ signaling, these experiments were carried out in the presence of ethylene glycol tetraacetic acid (EGTA), which chelates calcium from the environment. Thus, the observed increase in metabolic rate at low spermine levels and decrease at high spermine levels, was shown to be a direct effect of spermine itself, independent of intra-mitochondrial calcium concentration [78].

### *Polyamine Catabolism Leads to Toxic By-Product Formation*

In order for a cell to properly regulate the intracellular polyamine levels, polyamines can be oxidised by the amine oxidases polyamine oxidase (PAOX) and spermine/spermidine oxidase (SMOX). These enzymes produce the toxic aldehydes 3-acetoamidopropanal and 3-aminopropanal respectively as by-products during the catabolism of polyamines, as well as $H_2O_2$ [82]. The aldehydes formed by these two oxidases can be converted into acrolein, which is highly toxic. These compounds together may lead to oxidative damage to protein and DNA, which could contribute to cell death [83]. Multiple amine oxidases have also been identified in mitochondria, and oxidation of the polyamines spermine, spermidine and putrescine, together with their acetylated products, was shown to occur within mitochondria (Figure 2.3) [84]. Local oxidation of polyamines results in production of $H_2O_2$ and aldehydes, which may have adverse effects on mitochondrial functioning.

### *Polyamines Regulate Formation of the Mitochondrial Permeability Transition Pore*

Polyamines play a role in opening of the so called mitochondrial permeability transition pore (MPTP), a non-specific pore that allows bidirectional traffic of metabolites and inorganic compounds over the mitochondrial membrane with a size of up to 1.5 kDa (Figure 2.3) [85]. It is suggested that the pore has two conformations: a low-conductance state, which is present in normal cell functioning, and a high-conductance mode, which is thought to be one of the main causes of cell death [85]. Although it is still not completely known which proteins make up the MPTP, a polyanion consisting of ten to hundreds of linked phosphate groups (polyphosphate, or PolyP) seems to be essential for its assembly. Spermine was found to selectively inhibit the high-conductance state of the pore, while maintaining its low-conductance form [85]. The proposed mechanism was that spermine could bind to polyphosphate, thereby inhibiting the induction of MPTP opening [85]. Another study found that spermine could also have direct anti-oxidant capacities at a concentration of 100 μM, which may contribute to its role in preventing the complete opening of the MPTP [86]. Interestingly, the induction of the MPTP by oxidative stress seemed to be tissue specific, since rat brain-derived mitochondria were resistant to oxidative stress, whereas rat liver-derived mitochondria succumbed to the stress [87].

The impact of polyamines on MPTP was shown to be relevant in models of ischeamia/reperfusion injury. Upon ischemia/reperfusion injury of heart muscles, ODC1 was found to be upregulated, thereby elevating the intracellular polyamine content [88]. This was shown to have a positive effect on survival and recovery [88]. In addition, exogenous administration of spermine to rats prior to ischemia/reperfusion was also found to protect heart tissue from injury [89]. Induction of AKT and MAPK1/3 (Erk2/1) pathways, which are known to have cardio-protective effects in ischemia/reperfusion, could upregulate ODC1. It was proposed that the resulting higher polyamine levels prevented the (complete) opening of the MPTP pore, thereby preventing injury to heart tissue (Figure 2.3, regulation indicated in red arrows) [88]. In addition, calcium overload was shown to be prevented by exogenous administration of spermine [89].

This mechanism may also play a role in intestinal mitochondrial membrane integrity. In the small intestine, polyamine metabolism plays a role in maintaining mitochondrial membrane integrity along the crypt–villus axis. In crypts, the activity of ODC1 was found to be lower compared to villus cells [90]. Since the villus cells do not proliferate, and thus have a lesser need for polyamines, this may seem counterintuitive. However, villus cells have higher respiration rates compared to cells at the bottom of the crypt [90, 91]. Perhaps this is because villus cells have an increased energy demand due to active transport of nutrients from the gut lumen. Madsen *et al.* found that blocking

ODC1 activity in rats using -difluoromethylornithine (DMFO) decreased mitochondrial respiration of intestinal cells, and supplementation of spermine was able to reverse this phenotype [90]. Inhibition of ODC1 was shown to specifically disrupt mitochondrial morphology, without damaging the ultrastructure of the cells. Although the mechanism is not clear, ODC1 thus seems to be crucial also in intestinal cells to maintain mitochondrial respiration and membrane integrity [90], the latter possibly by MPTP opening.

## Polyamines as Regulators of Intestinal Physiology

### *Polyamines Regulate Intestinal Barrier Integrity*

The human intestine has a rapid turnover rate, and is thus effectively in a constantly proliferating state [50]. This high level of proliferation coincides with a continuous polyamine requirement to facilitate protein translation. In mice and rats, the proliferative zone of small intestinal and colonic crypts, where the differentiating cells are located, were shown to have especially high polyamine concentrations [92]. The more differentiated cells at the top of the villi had lower intracellular polyamine concentrations [92]. On the other hand, no differences in polyamine levels between villi and crypt cells in isolated human colon cells were found [29]. Although it is unclear whether polyamine levels differ between crypt and villus cells, it is known that they regulate intestinal physiology in various ways, including maintenance of the intestinal barrier function. The intestinal epithelial barrier is maintained by tight junction proteins that form and seal the intestinal barrier. Decreased polyamine levels were shown to result in impaired intestinal barrier integrity [93, 94]. Several polyamine-mediated mechanisms regulating the gut barrier have been discovered. On an mRNA transcription level, polyamines were shown to be important regulators of the transcription factor MYC. MYC controls the expression of E-cadherin, also known as CDH1, a cell-cell adhesion protein [93]. Polyamines also influence E-cadherin expression through a different mechanism. E-cadherin is sensitive to $Ca^{2+}$-levels. In small intestinal epithelial IEC-6 cells, polyamine depletion caused a decrease in cytosolic $Ca^{2+}$ concentrations [95]. Restoration of intracellular $Ca^{2+}$ levels restored E-cadherin expression to normal levels [95]. Also in IEC-6 cells, the tight junction proteins 1 and 2 (TJP1,2), Occludin (OCLN), Claudin 2 and 3 (CLDN2,3) and E-cadherin were shown to be downregulated in the absence of polyamines [94]. For OCLN, polyamines were found to stabilize the mRNA transcripts. In the absence of polyamines, the half-life of OCLN mRNA was ~75 min, while the presence of polyamines increased this to 120 min [94].

### *Polyamines Stimulate Gut Development and Longevity*

Polyamines also play a role in gut development in early life. Both human and animal

breast milk contain polyamines. Interestingly, the concentration of polyamines were significantly higher in human breast milk of mothers feeding pre-term babies than babies born at term [96]. Perhaps this is a way to stimulate gut development of these young babies. Supplementation of polyamines to milk-formulas of piglets was shown to increase expression of maturation markers in the small intestine [97, 98]. In addition, spermine supplementation improved the crypt-to-villus ratio at weaning when administered during the suckling phase in piglets [99]. The crypt-to-villus ratio is often used as a way to assess intestinal morphology and function [100], with 3:1 being considered an optimal ratio for the small intestine. It is interesting to note is that in animal husbandry, antibiotics were previously used in low doses as an in-feed growth promotor during the post-weaning period. This practice was shown to increase polyamine levels, especially that of putrescine, spermidine and cadaverine [101, 102]. By stimulating polyamine levels, the antibiotic treatment may have contributed to maturation of the gut of young piglets. This may have contributed to pig survival, since the intestine of newly weaned piglets is often not fully developed [103]. Although the mechanisms are not well investigated, polyamine supplementation through the diet may well have enabled optimal protein translation in the immature gut.

Additionally, in the mature gut, there is evidence that luminal polyamines continue to play an important role. In humans, it has been shown that polyamine concentrations decline in the fecal content with age [13]. Administration of 4 mM of spermidine in the medium of yeast, or 0.3 and 3 mM in the drinking water of mice, was shown to increase longevity [69]. The increased spermidine content in the intestine was related to hypoacetylation of histone 3 in enterocytes, which during ageing may become hyperacetylated. Increased spermidine-induced acetylation of histone 3 led to upregulation of autophagy-related gene expression, which contributed to increased longevity [69]. The mechanisms of autophagy induction by spermidine described above contribute to increased longevity, as a result of the autophagy-mediated removal of dysfunctional organelles, cells or proteins, preventing cell damage and ultimately cell death [65, 69, 104]. Interestingly, naked mole-rats, rodents that have a 10 times longer life expectancy than mice of the same size, were shown to maintain polyamine levels even during ageing [105].

### *Do luminal Polyamine Levels Influence Intracellular Concentrations in Enterocytes?*

Within the gut, polyamines play a variety of roles; be it in cell proliferation, metabolic regulation or physiological functions of the intestine. Gut polyamines can originate from endogenous production within enterocytes, from the diet, or through bacterial fermentation of protein. In the small intestine, dietary polyamines are the main source, and uptake from the lumen is a rapid process [106]. In the colon, bacterial fermentation

of protein is the main source of polyamines, and the more protein that enters the colon through increased dietary consumption, the more polyamines produced by bacteria [107].

An important question that arises is whether the increased polyamine levels produced by the microbiota in the colon actually influence colonic tissue polyamine levels or gut physiology. From cell culture experiments, we know that polyamines present in the medium do end up in the cells. For example, exposure of human HCT-116 colon cancer cells to putrescine, spermidine and spermine increases the intracellular concentration of the polyamines in a dose-dependent manner [108]. Similarly, when human HT-29 colon cancer cells were exposed to the acetylated polyamine $N^1$,$N^{12}$-diacetylspermine, which is hypothesised to play a role in colon carcinogenesis, this compound could be detected intracellularly 24 h after dosing [109]. In the small intestine of rats, putrescine was also shown to be taken up from the lumen, and could even be used as an energy source [70]. Administration of polyamines through drinking water of piglets was also shown to improve gut maturation of the small intestine [97, 99]. In suckling rats, long-term feeding of a polyamine deficient diet resulted in intestinal hypoplasia, both in the small and the large intestine, although no differences in mucosal polyamine concentrations were observed [110]. These studies indicate that there may at least be an interaction between luminal polyamine concentrations and enterocytes. However, no mechanisms are known as to how luminal polyamines influence the intestinal tissue. Whether *in vivo* increases in luminal polyamine concentrations also increase the colonic tissue content thus remains to be seen. When comparing the levels of polyamines in mid-colonic tissues of germ-free and former germ-free mice, Matsumoto *et al.* [111] observed no increase in the levels of intracellular polyamines. At the same time, the concentrations of putrescine in colon feces was increased seventeen times, and spermidine concentrations were two times higher, in ex-germ-free mice [111]. Thus, although colonic feces concentrations were markedly increased, no reflection of this increase was observed in colonic tissue. A soy protein-based diet was shown to increase luminal polyamine levels in the colon of pigs, coinciding with increased red blood cell polyamine content, but this did not result in increased colonic proliferation or ODC1 expression [21]. Although colonic uptake of luminal putrescine has been observed, this was under conditions of polyamine synthesis inhibition through treatment with DFMO [108]. Thus, it remains unclear whether increased luminal polyamine concentrations result in increased colonic tissue levels under physiological conditions.

## Conclusions

Increased protein intake, shown to be effective as a means to improve weight-loss or sports performance, leads to increased protein fermentation within the colon. As

a consequence, polyamine levels in the lumen of the colon increase. Even though it is known that polyamines play a role in a plethora of cellular functions, much less is known about how luminal polyamines can affect colonic physiology. Perhaps the tight regulation of polyamine levels within the colonocytes is so effective that an increase in luminal polyamine levels will not affect tissue homeostasis. However, since there have been few investigations of the effect of increased luminal polyamines under physiological conditions, no definitive conclusions can be drawn, although it is clear that the outcome will be tissue and concentration-dependent. Studies that specifically explore the interactions between luminal polyamine levels in the colon, EIF5A hypusination, gut proliferation and metabolic function could perhaps further our understanding of how luminal polyamines can influence or interact with normal colonic physiology when high-protein diets are consumed.



**Author Contributions: A.F.B**.: Conceptualizing, Writing—original draft, Writing—review and editing; **J.K.:** Funding acquisition, Writing—review and editing; **W.J.J.G.:** Funding acquisition, Writing—review and editing; **V.C.J.d.B.:** Conceptualization, Funding acquisition, Supervision, Writing—review and editing

## References


1. Berryman, C.E., et al., *Protein intake trends and conformity with the Dietary Reference Intakes in the United States: analysis of the National Health and Nutrition Examination Survey, 2001–2014.* The American Journal of Clinical Nutrition, 2018. **108**(2): p. 405-413.
2. Tomé, D., *Digestibility Issues of Vegetable versus Animal Proteins: Protein and Amino Acid Requirements—Functional Aspects.* Food and Nutrition Bulletin, 2013. **34**(2): p. 272-274.
3. van der Wielen, N., P.J. Moughan, and M. Mensink, *Amino Acid Absorption in the Large Intestine of Humans and Porcine Models.* The Journal of Nutrition, 2017. **147**(8): p. 1493-1498.
4. Ma, N., et al., *Contributions of the Interaction Between Dietary Protein and Gut Microbiota to Intestinal Health.* Current Protein & Peptide Science, 2017. **18**(8): p. 795-808.
5. Gilbert, M.S., et al., *Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2018. **315**(2): p. G159-g170.
6. Leidy, H.J., et al., *The role of protein in weight loss and maintenance.* The American Journal of Clinical Nutrition, 2015. **101**(6): p. 1320S-1329S.
7. Jäger, R., et al., *International Society of Sports Nutrition Position Stand: protein and exercise.* Journal of the International Society of Sports Nutrition, 2017. **14**: p. 20-20.
8. Wen, X., et al., *Fecal scores and microbial metabolites in weaned piglets fed different protein sources and levels.* Animal Nutrition, 2017. **4**(1): p. 31-36.
9. Pieper, R., et al., *Interaction between dietary protein content and the source of carbohydrates along the gastrointestinal tract of weaned piglets.* Archives of Animal Nutrition, 2014. **68**(4): p. 263-280.
10. Pegg, A.E., *Functions of Polyamines in Mammals.* The Journal of Biological Chemistry, 2016. **291**(29): p. 14904-14912.
11. Seiler, N. and F. Raul, *Polyamines and the Intestinal Tract.* Critical Reviews in Clinical Laboratory Sciences, 2007. **44**(4): p. 365-411.
12. Pegg, A.E., *Mammalian Polyamine Metabolism and Function.* IUBMB life, 2009. **61**(9): p. 880-894.
13. Matsumoto, M. and Y. Benno, *The Relationship between Microbiota and Polyamine Concentration in the Human Intestine: A Pilot Study.* Microbiology and Immunology, 2007. **51**(1): p. 25-35.
14. Forget, P., et al., *Fecal polyamine concentration in children with and without nutrient malabsorption.* Journal of Pediatric Gastroenterology and Nutrition, 1997. **24**(3): p. 285-288.
15. Di Martino, M.L., et al., *Polyamines: Emerging players in bacteria–host interactions.* International Journal of Medical Microbiology, 2013. **303**(8): p. 484-491.
16. Macfarlane, G.T., J.H. Cummings, and C. Allison, *Protein degradation by human intestinal bacteria.* Journal of General Microbiology, 1986. **132**(6): p. 1647-1656.
17. Nakamura, A., T. Ooga, and M. Matsumoto, *Intestinal luminal putrescine is produced by collective biosynthetic pathways of the commensal microbiome.* Gut Microbes, 2019. **10**(2): p. 159-171.
18. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(31): p. 11070-11075.
19. Xiao, L., et al., *A reference gene catalogue of the pig gut microbiome.* Nature Microbiology, 2016. **1**(12): p. 16161.
20. Wang, W. and C.M. Higuchi, *Dietary Soy Protein Is Associated with Reduced Intestinal Mucosal Polyamine Concentration in Male Wistar Rats.* The Journal of

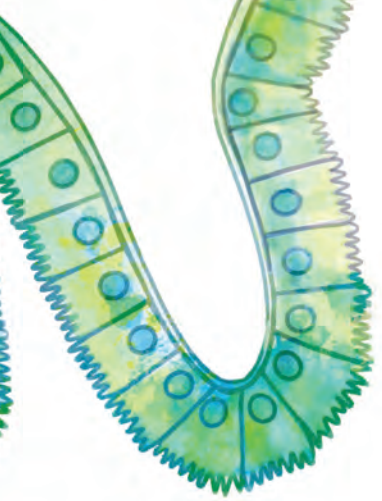
Nutrition, 2000. **130**(7): p. 1815-1820.
21. Benamouzig, R., et al., *Effects of soy protein diet on digestive lumenal polyamines and colonic cell proliferation in pigs.* Reproduction Nutrition Development, 1999. **39**(2): p. 213-221.
22. Holmes, A.J., et al., *Diet-Microbiome Interactions in Health Are Controlled by Intestinal Nitrogen Source Constraints.* Cell Metabolism, 2017. **25**(1): p. 140-151.
23. Walker, A.W., et al., *pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Microbial Communities from the Human Colon.* Applied and Environmental Microbiology, 2005. **71**(7): p. 3692-3700.
24. Wang, Y., et al., *Properties of purified recombinant human polyamine oxidase, PAOh1/SMO.* Biochemical and Biophysical Research Communications, 2003. **304**(4): p. 605-611.
25. Pegg, A.E., *Spermidine/spermine-N1-acetyltransferase: a key metabolic regulator.* American Journal of Physiology-Endocrinology and Metabolism, 2008. **294**(6): p. E995-E1010.
26. Casero, R.A., Jr. and A.E. Pegg, *Spermidine/spermine N1-acetyltransferase--the turning point in polyamine metabolism.* The FASEB journal, 1993. **7**(8): p. 653-661.
27. Tomar, P.C., N. Lakra, and S.N. Mishra, *Cadaverine: A lysine catabolite involved in plant growth and development.* Plant Signaling & Behavior, 2013. **8**(10): p. e25850.
28. Miller-Fleming, L., et al., *Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell.* Journal of Molecular Biology, 2015. **427**(21): p. 3389-3406.
29. Elitsur, Y., M. Gesell, and G.D. Luk, *ODC activity and polyamine levels in isolated human colonocytes.* Life Sciences, 1993. **53**(11): p. 945-952.
30. Hölttä, E. and P. Pohjanpelto, *Polyamine starvation causes accumulation of cadaverine and its derivatives in a polyamine-dependent strain of Chinese-hamster ovary cells.* Biochemical Journal, 1983. **210**(3): p. 945-948.
31. Pegg, A.E. and S. McGill, *Decarboxylation of ornithine and lysine in rat tissues.* Biochimica et Biophysica Acta (BBA) - Enzymology, 1979. **568**(2): p. 416-427.
32. Alhonen-Hongisto, L. and J. Jänne, *Polyamine depletion induces enhanced synthesis and accumulation of cadaverine in cultured Ehrlich ascites carcinoma cells.* Biochemical and Biophysical Research Communications, 1980. **93**(4): p. 1005-1013.
33. Murakami, Y., et al., *Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination.* Nature, 1992. **360**: p. 597.
34. Murakami, Y., et al., *Cloning of Antizyme Inhibitor, a Highly Homologous Protein to Ornithine Decarboxylase.* Journal of Biological Chemistry, 1996. **271**(7): p. 3340-3342.
35. Xiong, H. and A.E. Pegg, *Mechanistic Studies of the Processing of Human S-Adenosylmethionine Decarboxylase Proenzyme: isolation of an ester intermediate.* Journal of Biological Chemistry, 1999. **274**(49): p. 35059-35066.
36. Bale, S., et al., *Structural Basis for Putrescine Activation of Human S-Adenosylmethionine Decarboxylase.* Biochemistry, 2008. **47**(50): p. 13404-13417.
37. Shirahata, A. and A.E. Pegg, *Increased content of mRNA for a precursor of S-adenosylmethionine decarboxylase in rat prostate after treatment with 2-difluoromethylornithine.* Journal of Biological Chemistry, 1986. **261**(29): p. 13833-13837.
38. Shantz, L.M., et al., *Regulation of S-adenosylmethionine decarboxylase activity by alterations in the intracellular polyamine content.* Biochemical Journal, 1992. **288**(Pt 2): p. 511-518.
39. Condon, K.J. and D.M. Sabatini, *Nutrient regulation of mTORC1 at a glance.*

Journal of Cell Science, 2019. **132**(21): p. jcs222570.
40. Zabala-Letona, A., et al., *mTORC1-dependent AMD1 regulation sustains polyamine metabolism in prostate cancer.* Nature, 2017. **547**(7661): p. 109-113.
41. Basu Roy, U.K., et al., *Activated K-RAS Increases Polyamine Uptake in Human Colon Cancer Cells Through Modulation of Caveolar Endocytosis.* Molecular Carcinogenesis, 2008. **47**(7): p. 538-553.
42. Cheng, F., et al., *Nitric oxide-dependent processing of heparan sulfate in recycling S-nitrosylated glypican-1 takes place in caveolin-1-containing endosomes.* Journal of Biological Chemistry, 2002. **277**(46): p. 44431-44439.
43. Belting, M., et al., *Glypican-1 is a vehicle for polyamine uptake in mammalian cells: a pivital role for nitrosothiol-derived nitric oxide.* Journal of Biological Chemistry, 2003. **278**(47): p. 47181-47189.
44. Uemura, T., et al., *Identification and characterization of a diamine exporter in colon epithelial cells.* Journal of Biological Chemistry, 2008. **283**(39): p. 26428-26435.
45. Chattopadhyay, M.K., C.W. Tabor, and H. Tabor, *Spermidine but not spermine is essential for hypusine biosynthesis and growth in Saccharomyces cerevisiae: Spermine is converted to spermidine in vivo by the FMS1-amine oxidase.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(24): p. 13869-13874.
46. Zanelli, C.F. and S.R. Valentini, *Is there a role for eIF5A in translation?* Amino Acids, 2007. **33**(2): p. 351-358.
47. Schuller, A.P., et al., *eIF5A Functions Globally in Translation Elongation and Termination.* Molecular Cell, 2017. **66**(2): p. 194-205.e5.
48. Park, M.H., et al., *Functional significance of eIF5A and its hypusine modification in eukaryotes.* Amino Acids, 2010. **38**(2): p. 491-500.
49. Zanelli, C.F., et al., *eIF5A binds to translational machinery components and affects translation in yeast.* Biochemical and Biophysical Research Communications, 2006. **348**(4): p. 1358-1366.
50. Timmons, J., et al., *Polyamines and Gut Mucosal Homeostasis.* Journal of Gastrointestinal & Digestive System, 2012. **2**(Suppl 7): p. 001.
51. Sievert, H., et al., *Protein-protein-interaction network organization of the hypusine modification system.* Molecular & Cellular Proteomics : MCP, 2012. **11**(11): p. 1289-1305.
52. Kang, K.R., et al., *Specificity of the deoxyhypusine hydroxylase-eukaryotic translation initiation factor (eIF5A) interaction: identification of amino acid residues of the enzyme required for binding of its substrate, deoxyhypusine-containing eIF5A.* Journal of Biological Chemistry, 2007. **282**(11): p. 8300-8308.
53. Joe, Y.A. and M.H. Park, *Structural features of the eIF-5A precursor required for posttranslational synthesis of deoxyhypusine.* Journal of Biological Chemistry, 1994. **269**(41): p. 25916-25921.
54. Nishimura, K., et al., *Independent roles of eIF5A and polyamines in cell proliferation.* The Biochemical Journal, 2005. **385**(Pt 3): p. 779-785.
55. Lee, S.B., et al., *Inactivation of eukaryotic initiation factor 5A (eIF5A) by specific acetylation of its hypusine residue by spermidine/spermine acetyltransferase 1 (SSAT1).* The Biochemical Journal, 2010. **433**(1): p. 205-213.
56. Ivanov, I.P., et al., *Polyamine Control of Translation Elongation Regulates Start Site Selection on Antizyme Inhibitor mRNA via Ribosome Queuing.* Molecular Cell, 2018. **70**(2): p. 254-264.e6.
57. Ivanov, I.P., G. Loughran, and J.F. Atkins, *uORFs with unusual translational start codons autoregulate expression of eukaryotic ornithine decarboxylase homologs.* Proceedings of the National Academy of Sciences, 2008. **105**(29): p. 10079-10084.
58. Venkataramanan, S. and S.N. Floor, *The Traffic Jam: Polyamine Prevalence Pauses Protein Production.* Molecular Cell, 2018. **70**(2): p. 191-192.

59. Shin, B.-S., et al., *Amino acid substrates impose polyamine, eIF5A, or hypusine requirement for peptide synthesis.* Nucleic Acids Research, 2017. **45**(14): p. 8392-8402.
60. Buck, M.D., et al., *Metabolic Instruction of Immunity.* Cell, 2017. **169**(4): p. 570-586.
61. Puleston, D.J., et al., *Polyamines and eIF5A Hypusination Modulate Mitochondrial Respiration and Macrophage Activation.* Cell Metabolism, 2019. **30**(2): p. 352-363.e8.
62. Hardbower, D.M., et al., *Ornithine decarboxylase regulates M1 macrophage activation and mucosal inflammation via histone modifications.* Proceedings of the National Academy of Sciences of the United States of America, 2017. **114**(5): p. E751-E760.
63. Melis, N., et al., *Targeting eIF5A Hypusination Prevents Anoxic Cell Death through Mitochondrial Silencing and Improves Kidney Transplant Outcome.* Journal of the American Society of Nephrology : JASN, 2017. **28**(3): p. 811-822.
64. Qi, Y., et al., *ATM mediates spermidine-induced mitophagy via PINK1 and Parkin regulation in human fibroblasts.* Scientific Reports, 2016. **6**: p. 24700.
65. Eisenberg, T., et al., *Cardioprotection and lifespan extension by the natural polyamine spermidine.* Nature Medicine, 2016. **22**(12): p. 1428-1438.
66. Jing, Y.-H., et al., *Spermidine ameliorates the neuronal aging by improving the mitochondrial function in vitro.* Experimental Gerontology, 2018. **108**: p. 77-86.
67. Morselli, E., et al., *Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome.* The Journal of Cell Biology, 2011. **192**(4): p. 615-629.
68. Pietrocola, F., et al., *Spermidine induces autophagy by inhibiting the acetyltransferase EP300.* Cell Death and Differentiation, 2015. **22**(3): p. 509-516.
69. Eisenberg, T., et al., *Induction of autophagy by spermidine promotes longevity.* Nature Cell Biology, 2009. **11**(11): p. 1305-1314.
70. Bardócz, S., et al., *Putrescine as a source of instant energy in the small intestine of the rat.* Gut, 1998. **42**(1): p. 24-28.
71. Kramer, D.L., et al., *Polyamine Acetylation Modulates Polyamine Metabolic Flux, a Prelude to Broader Metabolic Consequences.* Journal of Biological Chemistry, 2008. **283**(7): p. 4241-4251.
72. Kee, K., et al., *Activated polyamine catabolism depletes acetyl-CoA pools and suppresses prostate tumor growth in TRAMP mice.* Journal of Biological Chemistry, 2004. **279**(38): p. 40076-40083.
73. Jell, J., et al., *Genetically Altered Expression of Spermidine/Spermine N1-Acetyltransferase Affects Fat Metabolism in Mice via Acetyl-CoA.* Journal of Biological Chemistry, 2007. **282**(11): p. 8404-8413.
74. Byczkowski, J.Z., L. Zychlinski, and C.W. Porter, *Inhibition of the bioenergetic functions of isolated rat liver mitochondria by polyamines.* Biochemical Pharmacology, 1982. **31**(24): p. 4045-4053.
75. Dalla Via, L., V. Di Noto, and A. Toninello, *Binding of Spermidine and Putrescine to Energized Liver Mitochondria.* Archives of Biochemistry and Biophysics, 1999. **365**(2): p. 231-238.
76. Grancara, S., et al., *Spermine cycling in mitochondria is mediated by adenine nucleotide translocase activity: mechanism and pathophysiological implications.* Amino Acids, 2016. **48**(10): p. 2327-2337.
77. Toninello, A., et al., *Electrophoretic polyamine transport in rat liver mitochondria.* Amino Acids, 1992. **2**(1-2): p. 69-76.
78. Pezzato, E., et al., *Ca2+ -independent effects of spermine on pyruvate dehydrogenase complex activity in energized rat liver mitochondria incubated in the absence of exogenous Ca2+ and Mg2+.* Amino Acids, 2009. **36**(3): p. 449-456.

79. Phillips, J.E. and R.R.J. Chaffee, *Restorative effects of spermine on oxidative phosphorylation and respiration in heat-aged mitochondria.* Biochemical and Biophysical Research Communications, 1982. **108**(1): p. 174-181.
80. Solaini, G. and B. Tadolini, *Spermine binding to submitochondrial particles and activation of adenosine triphosphatase.* Biochemical Journal 1984. **218**(2): p. 495-499.
81. Nicchitta, C.V. and J.R. Williamson, *Spermine. A regulator of mitochondrial calcium cycling.* Journal of Biological Chemistry, 1984. **259**(21): p. 12978-12983.
82. Pegg, A.E., *Toxicity of Polyamines and Their Metabolic Products.* Chemical Research in Toxicology, 2013. **26**(12): p. 1782-1800.
83. Stevanato, R., et al., *Preliminary kinetic characterization of a copper amine oxidase from rat liver mitochondria matrix.* Amino Acids, 2011. **40**(2): p. 713-720.
84. Bonaiuto, E., et al., *A novel enzyme with spermine oxidase properties in bovine liver mitochondria: Identification and kinetic characterization.* Free Radical Biology and Medicine, 2015. **81**: p. 88-99.
85. Elustondo, P.A., et al., *Spermine selectively inhibits high-conductance, but not low-conductance calcium-induced permeability transition pore.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2015. **1847**(2): p. 231-240.
86. Sava, I.G., et al., *Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria.* Free Radical Biology and Medicine, 2006. **41**(8): p. 1272-1281.
87. Grancara, S., et al., *Mitochondrial oxidative stress induced by Ca2+ and monoamines: different behaviour of liver and brain mitochondria in undergoing permeability transition.* Amino Acids, 2012. **42**(2): p. 751-759.
88. Zhang, H., et al., *Akt and Erk1/2 activate the ornithine decarboxylase/polyamine system in cardioprotective ischemic preconditioning in rats: the role of mitochondrial permeability transition pores.* Molecular and Cellular Biochemistry, 2014. **390**(1): p. 133-142.
89. Wei, C., et al., *Exogenous spermine inhibits hypoxia/ischemia-induced myocardial apoptosis via regulation of mitochondrial permeability transition pore and associated pathways.* Experimental Biology and Medicine, 2016. **241**(14): p. 1505-1515.
90. Madsen, K.L., et al., *Role of ornithine decarboxylase in enterocyte mitochondrial function and integrity.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1996. **270**(5): p. G789-G797.
91. Yang, H., et al., *Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis.* Scientific Reports, 2016. **6**: p. 31917.
92. Fujiwara, K., Y. Masuyama, and T. Kitagawa, *Immunocytochemical localization of polyamines in the gastrointestinal tracts of rats and mice.* Histochemistry and Cell Biology, 1996. **106**(5): p. 465-471.
93. Liu, L., et al., *Polyamines regulate E-cadherin transcription through c-Myc modulating intestinal epithelial barrier function.* American Journal of Physiology-Cell Physiology, 2009. **296**(4): p. C801-C810.
94. Guo, X., et al., *Polyamines are necessary for synthesis and stability of occludin protein in intestinal epithelial cells.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2005. **288**(6): p. G1159-G1169.
95. Guo, X., et al., *Regulation of adherens junctions and epithelial paracellular permeability: a novel function for polyamines.* American Journal of Physiology-Cell Physiology, 2003. **285**(5): p. C1174-C1187.
96. Plaza-Zamora, J., et al., *Polyamines in human breast milk for preterm and term infants.* British Journal of Nutrition, 2013. **110**(3): p. 524-528.
97. Sabater-Molina, M., et al., *Effects of dietary polyamines at physiologic doses in early-weaned piglets.* Nutrition, 2009. **25**(9): p. 940-946.
98. Fang, T., et al., *Spermine: New insights into the intestinal development and*

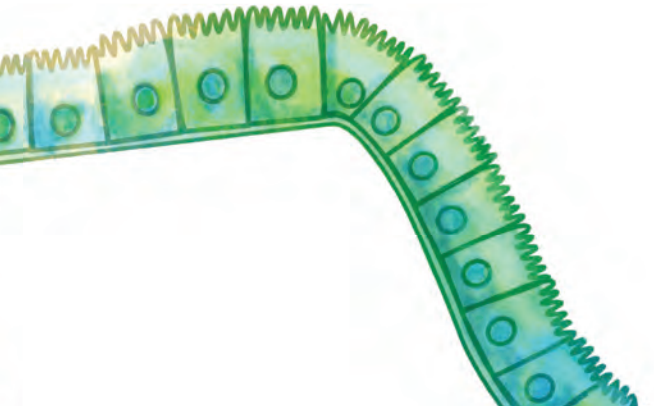
*serum antioxidant status of suckling piglets.* RSC Advances, 2016. **6**(37): p. 31323-31335.
99. van Wettere, W.H., et al., *Effect of oral polyamine supplementation pre-weaning on piglet growth and intestinal characteristics.* Animal, 2016. **10**(10): p. 1655-1659.
100. Pluske, J.R., D.J. Hampson, and I.H. Williams, *Factors influencing the structure and function of the small intestine in the weaned pig: a review.* Livestock Production Science, 1997. ***51***(1): p. 215-236.
101. Mu, C., et al., *Alteration of metabolomic markers of amino-acid metabolism in piglets with in-feed antibiotics.* Amino Acids, 2017. **49**(4): p. 771-781.
102. Gao, K., et al., *Time-course responses of ileal and fecal microbiota and metabolite profiles to antibiotics in cannulated pigs.* Applied Microbiology and Biotechnology, 2018. **102**(5): p. 2289-2299.
103. Moeser, A.J., C.S. Pohl, and M. Rajput, *Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs.* Animal Nutrition, 2017. ***3***(4): p. 313-321.
104. LaRocca, T.J., et al., *The autophagy enhancer spermidine reverses arterial aging.* Mechanisms of Ageing and Development, 2013. **134**(7-8): p. 314-320.
105. Viltard, M., et al., *The metabolomic signature of extreme longevity: naked mole rats versus mice.* Aging, 2019. **11**(14): p. 4783-4800.
106. Uda, K., et al., *Rapid absorption of luminal polyamines in a rat small intestine ex vivo model.* Journal of Gastroenterology and Hepatology, 2003. **18**(5): p. 554-559.
107. Matsumoto, M., et al., *Impact of Intestinal Microbiota on Intestinal Luminal Metabolome.* Scientific Reports, 2012. **2**: p. 233.
108. Uemura, T., et al., *Polyamine transport is mediated by both endocytic and solute carrier transport mechanisms in the gastrointestinal tract.* American Journal of Physiology. Gastrointestinal and Liver Physiology, 2010. **299**(2): p. G517-G522.
109. Johnson, C.H., et al., *Metabolism links bacterial biofilms and colon carcinogenesis.* Cell Metabolism, 2015. **21**(6): p. 891-897.
110. Löser, C., et al., *Dietary polyamines are essential luminal growth factors for small intestinal and colonic mucosal growth and development.* Gut, 1999. **44**(1): p. 12-16.
111. Matsumoto, M., et al., *Colonic Absorption of Low-Molecular-Weight Metabolites Influenced by the Intestinal Microbiome: A Pilot Study.* PloS one, 2017. **12**(1): p. e0169207-e0169207.



# Chapter 3

## Butyrate Alters Pyruvate Flux and Induces Lipid Accumulation in Cultured Colonocytes

Anna F. Bekebrede
Thirza van Deuren
Walter J.J. Gerrits
Jaap Keijer
Vincent C.J. de Boer



***International Journal of Molecular Sciences 2022;***
doi:10.3390/ijms222010937

## Abstract

Butyrate is considered the primary energy source of colonocytes and has received wide attention due to its unique health benefits. Insight into the mechanistic effects of butyrate on cellular and metabolic function relies mainly on research in *in vitro*-cultured cells. However, cells in culture differ from those *in vivo* in terms of metabolic phenotype and nutrient availability. For translation, it is therefore important to understand the impact of different nutrients on the effects of butyrate. We investigated the metabolic consequences of butyrate exposure under various culturing conditions, with a focus on the interaction between butyrate and glucose. To investigate whether the effects of butyrate were different between cells with high and low mitochondrial capacity, we cultured HT29 cells under either low- (0.5 mM) or high- (25 mM) glucose conditions. Low-glucose culturing increased the mitochondrial capacity of HT29 cells compared to high-glucose (25 mM) cultured HT29 cells. Long-term exposure to butyrate did not alter mitochondrial bioenergetics, but it decreased glycolytic function, regardless of glucose availability. In addition, both high- and low-glucose-grown HT29 cells showed increased lipid droplet accumulation following long-term butyrate exposure. Acute exposure of cultured cells (HT29 and Caco-2) to butyrate increased their oxygen consumption rate (OCR). A simultaneous decrease in extracellular acidification rate (ECAR) was observed. Furthermore, in the absence of glucose, OCR did not increase in response to butyrate. These results lead us to believe that butyrate itself was not responsible for the observed increase in OCR, but, instead, butyrate stimulated pyruvate flux into mitochondria. Indeed, blocking of the mitochondrial pyruvate carrier prevented a butyrate-induced increase in oxygen consumption. Taken together, our results indicate that butyrate itself is not oxidized in cultured cells but instead alters pyruvate flux and induces lipid accumulation.

**Keywords**: butyrate; glucose oxidation; metabolite interactions

## Introduction

Butyrate is a short-chain fatty acid (SCFA) that, in humans, is mostly produced through fibre fermentation by microbiota in the colon. Butyrate is mainly known as the primary source of energy for healthy colon cells [1], and has also been found to have beneficial health effects. In the colon, it is found to protect against colorectal cancer [2], and evidence from pre-clinical models shows that butyrate can be used as a treatment for intestinal bowel diseases (IBD) [3] and even diarrheal diseases [4]. Apart from local effects in the colon, butyrate was also found to affect whole-body metabolism, such as the prevention of high-fat-diet-induced obesity [5] and fatty liver disease caused by obesity and a high-fat diet [5].

Butyrate exerts its beneficial effects through various mechanisms, of which the most interesting one is by serving as a direct mitochondrial substrate. Apart from thermogenic effects in adipocytes [6] and effects on colonocyte-neighbouring immune cells [7], the effects of butyrate have prominently been studied in colonocytes. As an energy source in colonocytes, butyrate provides cellular energy in the form of adenosine triphosphate (ATP) [8]. Germ-free mice lack butyrate-producing bacteria, and their colonocytes were found to be energy deprived [9]. Re-administration of butyrate restored colonic energy levels, indicating that butyrate is indeed a crucial energy source [9]. Because butyrate catabolism in mitochondria utilizes oxygen for oxidation in colonocytes, it lowers oxygen levels, which prevents vascular oxygen from leaking into the anoxic colonic lumen [10]. In some diseases, such as IBD, butyrate oxidation is decreased, especially during phases of active disease [11-13]. The mechanistic explanation for the decrease in butyrate oxidation is likely the ongoing inflammation, which alters the ability of colonocytes to take up and metabolize butyrate [14], highlighting that butyrate metabolism is central to many of its physiological functions.

Studies that aim to identify mechanisms for how butyrate affects metabolic and cellular functions often rely on *in vitro*-cultured colonic cell lines. However, the metabolic phenotype of cultured cells often differs from that of cells *in vivo* [15, 16]. Cells in the physiologic context of the human body are exposed to different and changing nutrient and oxygen levels, whereas in vitro cultured cells are typically grown in a culture medium that is rich in nutrients and oxygen. Interestingly, the *in vitro* effects of butyrate were found to be influenced by the culture medium composition, showing the importance of understanding how different nutritional factors impact the outcome of butyrate exposure [17, 18]. In addition, cultured colonic cells display the Warburg effect, which means that they preferentially oxidize glucose as an energy substrate in normoxic conditions, instead of butyrate, which is typically highly available *in situ* in the colon and preferred by *in vivo* colonocytes [16]. Nutritional conditions and consequential nutrient interactions thus differ from the *in vivo* situation, which may impact the effects

of butyrate exposure and therefore pose limitations to the translatability of the *in vitro* findings to *in vivo* human physiology.

Cultured cells can be steered towards oxidative metabolism, making them more comparable to the oxidative phenotype of *in vivo* colonocytes. Attempts at more physiological culturing of colonic cells have been performed extensively, by changing the medium nutrient composition [19-21], as well as by co-culturing with other cell types or by growing cells as spheroids or organoids. Supplying low glucose levels or replacing glucose with galactose stimulates mitochondrial metabolism because of the lower ATP yields in the glycolytic pathway [19-22]. The obtained increased mitochondrial metabolism could increase mitochondrial butyrate oxidation, but other metabolic routes of butyrate utilization have also been described. For example, butyrate was shown to be incorporated into lipids in cultured colonic cells [23]. Butyrate can also be used as a substrate for post-translational protein acylation modification, either when converted to acetyl-CoA as a source for protein acetylation or more directly via butyryl-CoA as a source for protein butyrylation [24, 25].



An important remaining question is how different metabolic phenotypes, i.e., glycolytic or oxidative, and nutritional environments influence the effects of butyrate exposure. In particular, we are interested in the interaction between butyrate and glucose, since cultured cells are often cultured in media containing high levels of glucose. Interestingly, glucose was found to dictate whether butyrate was able to induce apoptosis in cultured colonocytes [26]. In addition, butyrate exposure led to increased glucose uptake in Caco-2 cells [27], but decreased glucose uptake in HT29, HCT116 and LoVo cells [28, 29]. In contrast, butyrate was also found to impact the oxidation of glucose, although the direction of this effect differed between studies [30-34]. To better understand the fate of butyrate under different metabolic and nutritional conditions, we studied butyrate utilization in colonic cells in detail. We used a panel of colonic cell lines as well as colon-derived primary cells to demonstrate that butyrate is likely not oxidized by mitochondria but instead rewires pyruvate flux and fuels lipid droplets, even in conditions of high mitochondrial oxidative metabolism.

## Methods

### *Cell Culture*

The human colorectal (adeno)carcinoma cell lines HT29 (HTB-38), Caco-2 (HTB-37) and HCT116 (CCL-247) were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 25 mM glucose-cultured HT29, Caco-2 and HCT116 cells were maintained in 25 mM glucose Dulbecco's Modified Eagle's Medium (DMEM) (42430-025, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA, 227289Gibco),

supplemented with 10% v/v fetal bovine serum (FBS), 25 mM HEPES (15630-056, Gibco), 1 mM sodium pyruvate (11360-039, Thermo Fisher ScientificGibco), 1% v/v glutamax 100x (35050-038, Thermo Fisher ScientificGibco, 35050-038) and 1% v/v antibiotic–antimycotic (15240-062, Thermo Fisher ScientificGibco, 15240-062). The 0.5 mM glucose-cultured HT29 cells were cultured in DMEM (11966-025, Gi Thermo Fisher Scientificbco) supplemented with 0.5 mM glucose, 10% v/v FBS, 25 mM HEPES, 1 mM sodium pyruvate, 1% v/v glutamax 100x and 1% v/v antibiotic–antimycotic. The 0.5 mM glucose cells were passaged at least 10 times before being used for experiments. All cell cultures were grown in T75 flasks and kept in a humidified incubator at 37 °C in 95% air and 5% CO2. Cells were passaged or used for experiments when a confluency of 80–90% was reached.

### *Intestinal Colon Cell Isolation*

Colon samples were obtained from slaughterhouse material of approximately 10-week-old piglets. A 20 cm section of the mid-sigmoid colon was obtained immediately after slaughter, and intestines were placed in aerated Krebs Henseleit Buffer containing 5 mM glucose (hereafter referred to as modified-KHB, K3753, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2.5 g/L Bovine serum albumin (BSA, A7906, Sigma-Aldrich, St. Louis, MO, USA). After this, the intestines were flushed with modified-KHB. Then, they were inverted, and a sac was created using dialysis clamps by filling them with modified-KHB. The sacs were incubated for 20 min in $Ca^{2+}$-free KHB buffer containing 20 mM EDTA and 10 mM DTT in a shaking 37 °C water bath. Following this washing step, intestines were reverted and filled with an isolation buffer containing $Ca^{2+}$-free KHB buffer, 2.5 g/L BSA and 400 U/mL hyaluronidase type IV (3884, Sigma-Aldrich, St. Louis, MO, USA). After a fifteen-minute incubation, the intestines were gently massaged and cells were collected, washed and counted using a Cellometer K4 (Nexcelom Bioscience, Lawrence, MA, USA), and viability was simultaneously assessed by staining with ViaStain (CS2-0106, Nexcelom Bioscience, Lawrence, MA, USA). Cells were taken up in KHB medium containing 1 mM HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) and 2.5 mM glucose, pH 7.4.

### *Metabolic Flux Analysis with Seahorse XFe96 Analyzer*

To investigate the long-term metabolic consequences of butyrate, both the 25 mM and 0.5 mM glucose-cultured HT29 cells were exposed to 1 mM butyrate for 72h prior to metabolic analysis. Cells were first exposed for 48h in a T75 flask, after which they were detached and taken up in fresh medium containing 1 mM butyrate. Then, cells were counted and seeded in an XF96 cell plate at $3\times10^4$ cells/well (25 mM glucose-cultured cells) or $3.5\times10^4$ (0.5 mM glucose-cultured cells). After an additional 24h of exposure to 1 mM butyrate, the medium was switched to an XF DMEM assay medium

supplemented with either 25 or 0.5 mM XF glucose (for the 25 and 0.5 mM glucose-cultured HT29 cells, respectively), 2 mM XF glutamine and 1 mM XF pyruvate. In addition, 1 mM sodium butyrate was added to the exposed cells. XF96 cell plates were kept in a non-CO2 incubator set to 37 °C for one hour prior to metabolic flux analysis.

To assess the acute metabolic consequences of butyrate, 25 mM glucose-cultured HT29, Caco-2 and HCT116 cells were plated in an XF96 cell plate at $3\times10^4$ cells/well and left to attach overnight. One hour prior to the metabolic analysis, the culture medium was replaced with bicarbonate-free KHB supplemented with 2.5 mM glucose and 1 mM HEPES, set at pH 7.4 at 37 °C. XF96 cell plates were kept in a non-CO2 incubator set to 37 °C for one hour prior to metabolic flux analysis.



The primary isolated colonocytes were taken up in a KHB medium containing 1 mM HEPES and 2.5 mM glucose set at pH 7.4, and plated at a concentration of 9 × 104 cells/well in XF96 cell plates that were coated with Cell-Tak (354240, Corning, New York, NY, USA), according to the manufacturer's protocol. XF96 cell plates were kept in a non-CO2 incubator set to 37 °C for one hour prior to metabolic flux analysis.

A Seahorse Extracellular XFe96 analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA, USA) was used to assess the metabolic consequences of butyrate pre-treatment or acute exposure. To assess the metabolic consequences of 72h exposure to butyrate, extracellular flux analyses (XF assays) were performed using serial injections of 1.5 µM Oligomycin (O4875), 1.5 µM carbonyl cya-nide-p-trifluoromethoxyphenylhydrazone (FCCP; C2920), a combination of 1.25 µM Ro-tenone (R8875) and 2.5 µM Antimycin A (A8674), all purchased from Sigma-Aldrich. The XF assay protocol typically consisted of 12 measurement cycles of 3 min, with 2 min of mixing between measurements. To measure the acute effects of butyrate, XF assays were performed using serial injections of 0, 0.5, 5 or 10 mM sodium butyrate, 50 mM 2-deoxyglucose (2DG) and a combination of 1.25 µM Rotenone and 2.5 µM Antimycin A. To block the mitochondrial pyruvate carrier, 8 µM UK5099 (PZ0160, Sigma-Aldrich, St. Louis, MO, USA).

### *Imaging Procedure*

Following the XF analysis, cells were fixed using a 4% neutrally buffered formalin solution (NBF, 252549, Sigma-Aldrich, St. Louis, MO, USA). Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA), and images were taken of the middle of each well using the Cytation 1 (Nexcellom Biosciences, Lawrence, MA, USA). Images were processed in ImageJ (Win64, version 1.52, NI, USA). First, the background was subtracted. Then, the nucleus-covered area was determined by setting an automatic threshold, and the number of nucleus-covered

pixels was determined. The number of pixels covered with nuclei was then used to normalize the XF assays.

### *Oil-Red-O Staining and Image Processing*

To assess lipid accumulation following 72h butyrate exposure, 0.5 mM and 25 mM glucose-cultured HT29 cells were exposed to 1 mM butyrate in a T75 flask for 48 h. Then, they were plated in tissue-culture-treated 12-well plates for an additional exposure of 24h to 1 mM butyrate, after which they were stained with Oil-Red-O (ORO, O0625, Sigma-Aldrich, St. Louis, MO, USA). A working solution of 0.3% v/v ORO in 60% isopropanol was used to stain lipid droplets. First, cells were fixed using 4% NBF, followed by ORO and DAPI staining to stain lipid droplets and cell nuclei, respectively.

Images were obtained using the Leica DM8 inverted microscope, using a 40× magnification. Gain, image intensity and exposure time were kept equal for all images obtained. ImageJ was used to quantify areas covered by lipid droplets. First, the image was converted to an 8-bit image and inverted. Then, the background was subtracted using a rolling ball algorithm. The threshold was then manually set and kept the same for all images, and the selected area was measured. In addition, the DAPI images were used to count the number of nuclei, and the area covered was corrected for the number of cells in the picture.

### *WST-1 Cell Viability Assay*

To assess cell viability following 48h butyrate exposure, 25 mM glucose-cultured HT29 cells were seeded at 30,000 cells/well. Following overnight attachment, cells were exposed to only medium or 1 mM butyrate for 48h. Then, WST-1 reagent (5015944001, Roche, Basel, Switzerland) was added according to the manufacturer's protocol, and absorbance was read using the Synergy™ HT Multi-Detection Microplate Reader (BioTek In-struments, Inc., Winooski, VT, USA) at 450 nm. Cell viability is reported as a percentage of control.

### *RNA Extraction and Semi-Quantitative Real-Time Polymerase Chain Reaction (qPCR)*

Cells were washed with cold Hanks' Balanced Salt Solution (HBSS) and directly scraped using RLT buffer supplemented with 1% -mercaptoethanol. RNA was then isolated using the RNeasy mini kit (74106, Qiagen, Hilden, Germany). The quality and quantity of the purified RNA was determined using a NanoDrop spectrophotometer (ND-1000). cDNA was synthesized with the iSCRIPT cDNA synthesis kit (170-8891, BioRad, Hercules, CA, USA) in the Eppendorf-Master cycler (5' 25 °C, 30 42 °C, 5

85 °C, 10 °C ∞). Gene expression was measured using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYPBR green master mix (1725006CUST, BioRad, Hercules, CA, USA). The cycling program was set as follows: 3 95 °C, 40 cycles of 15" 95 °C and 45" 60 °C, 1 95 °C and 1 65 °C, followed by melt curve analysis by increasing temperature every 10"with increments of 0.5 °C. Primers were designed using NCBI Primer-BLAST. Normalized expression was calculated according to the Cq method, by making use of multiple reference genes (*RSP15* and *B2M*), using the CFX Maestro software (BioRad, Hercules, CA, USA). An overview of the primers used can be found in Table 3.1.

Table 3.1. **Details of primers.**

| Symbol | RefSeq | Forward Primer * | Reverse Primer * | bp |
|---|---|---|---|---|
| *RSP15* | NM_003194.5 | AGAAGCCGGAAGTGGTGAAGAC | AGAGGGATGAAGCGGGAGGAG | 220 |
| *B2M* | NM_004048.4 | TGCCGTGTGAACCATGTG | GCGGCATCTTCAAACCTC | 92 |
| *ACLY* | NM_001096.3 | GGACTTCGGCAGAGGTAGAG | TGATCAGCTGGTCTGGCTTG | 227 |
| *ACACA* | NM_198834.3 | GGGCTAGGTCTTTTTGGAAGTG | GGCCAAGGGAGATGGTTCAT | 104 |
| *G6PD* | NM_001360016.2 | AAGCGCAGACAGCGTCAT | TGAAGGTGTTTTCGGGCAGA | 215 |
| *PGD* | NM_002631.4 | TGTGACTGGGTGGGAGATGA | TCCCTGATCTTTGGCAGCAG | 257 |

* from 5 to 3, bp = fragment length.

### *Statistical Analysis and Data Representation*

Data are presented as mean ± SD, unless stated otherwise. Statistical analyses and data visualizations were performed using GraphPad Prism v.9 (GraphPad Software, CA, USA). Statistical testing was performed using Student's t-test and two-way or repeated-measures ANOVA, followed by Bonferroni's post-hoc analysis when appropriate and as stated in the figure legends. A p-value of $<0.05$ was considered statistically significant.

## Results

### *Long-Term Exposure to Butyrate Decreases Glycolytic Function of Both High- and Low-Glucose-Cultured HT29 Cells*

To investigate whether the long-term effects of butyrate on mitochondrial function were affected by glucose, we cultured HT29 cells using standard high-glucose medium (25 mM) or custom low-glucose medium (0.5 mM glucose). HT29 cells cultured in 0.5 mM glucose had higher basal oxygen consumption rate (OCR; increase of 9.9 ± 2.7 pmol/min/1×$10^5$ area, $p$ = 0.0067), higher maximal oxidative capacity (increase of 32.3 ± 6.4 pmol/min/1×$10^5$ area, $p$ = 0.001), higher spare respiratory capacity (increase of 38.4 ± 14% of basal OCR, $p$ = 0.0026) and lower basal extracellular acidification rate (ECAR; (reduction of 2.1 mpH/min/1×$10^5$ area, $p$ = 0.0012) as compared to cells cultured in 25 mM glucose, which indicated that our low-glucose-cultured HT29 cells relied more on mitochondrial metabolism than high-glucose cultured HT29 cells (Figure 3.1). In addition, 0.5 mM glucose-cultured cells had lower lipid levels than 25 mM glucose-cultured HT29 cells (a reduction of ±10.8 ORO-stained pixels/cell; Figure 3.2). Since butyrate is a short-chain fatty acid that can be converted to acetyl-CoA in the mitochondria, we expected that long-term butyrate exposure would affect mitochondrial parameters. Surprisingly, butyrate did not alter mitochondrial parameters but changed glycolytic parameters significantly. Butyrate lowered basal ECAR by 36% in 25 mM glucose-cultured HT29 cells and by 38% in 0.5 mM glucose HT29 cells ($p$ = 0.0023; Figure 3.1B) and maximal ECAR by approximately 20% in both culture conditions ($p$ = 0.0025). Although 25 mM and 0.5 mM glucose-cultured cells had significantly different mitochondrial functions, the effect of butyrate on metabolic flux was not different between these conditions. We hypothesized that instead of increased mitochondrial flux, *de novo* lipogenesis could be affected by butyrate exposure. We did not observe altered gene expression of two *de novo* lipogenesis-related genes (Supplementary Figure S3.1), indicating that lipogenesis upon butyrate exposure is likely not regulated on the gene expression level but instead could be regulated on the enzymatic or post-translational level. Remarkably, exposure to butyrate increased lipid accumulation to the same extent in 25 mM and 0.5 mM glucose-cultured HT29 cells (in both cases, a significant increase of approximately 150%, $p < 0.0001$; Figure 3.2). In the literature, it is often suggested that the pentose phosphate pathway (PPP) is increased following butyrate exposure to regenerate NADPH for lipogenesis [23, 28]. However, we did not observe altered gene expression of key PPP genes after 72h butyrate exposure, in either the 25 or the 0.5 mM glucose-cultured HT29 cells (Supplementary Figure S3.1). Thus, long-term exposure to butyrate reduced the glycolytic function of HT29 cells without affecting mitochondrial flux parameters. The effect of butyrate was independent of culture conditions, since there was no difference

between 25 and 0.5 mM glucose-cultured HT29 cells. To ensure that our findings were not affected by altered cell viability or cell death following butyrate exposure, we investigated cell viability and cell counts in response to 1 mM butyrate exposure. Cell viability was not changed in high-glucose-cultured HT29 cells after 48h exposure to 1 mM butyrate (Supplementary Figure S3.2A), and the cell count in high- and low-glucose-cultured HT29 cells was also not significantly affected by 48h exposure to 1 mM butyrate (Supplementary Figure S3.2B).

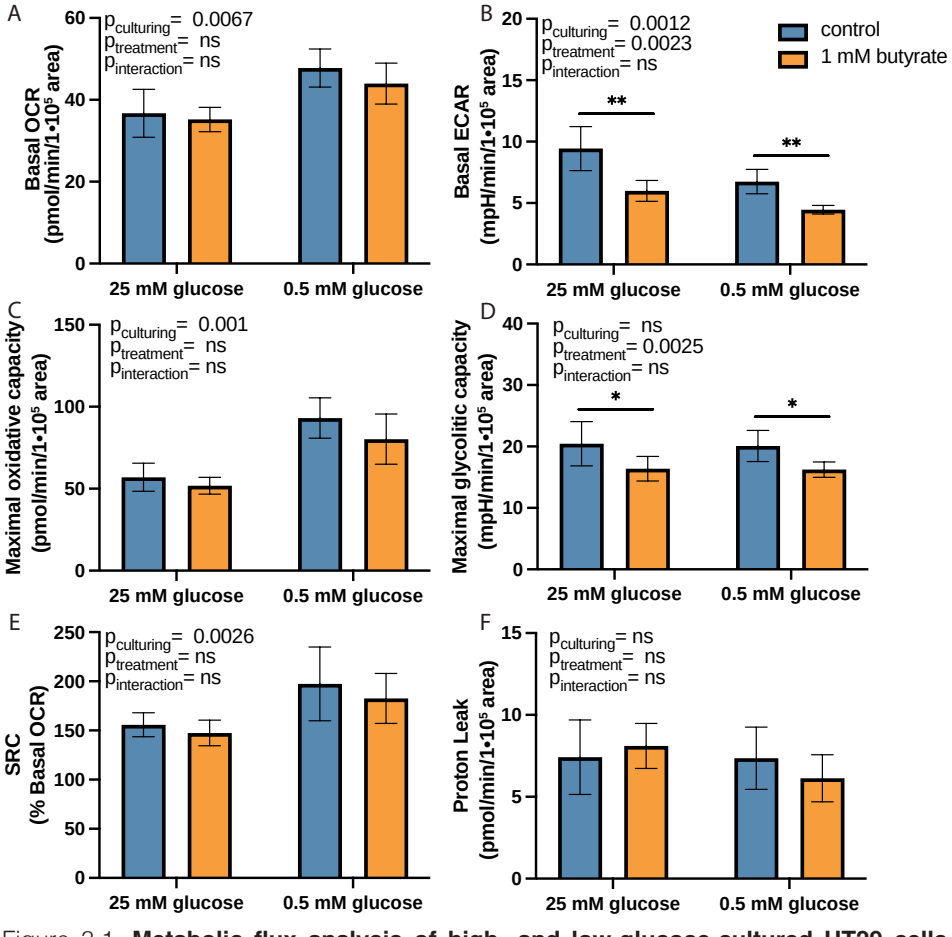



Figure 3.1. **Metabolic flux analysis of high- and low-glucose-cultured HT29 cells following 72h exposure to 1 mM butyrate.** (**A**) Basal OCR. (**B**) Basal ECAR. (**C**) Maximal oxidative capacity. (**D**) Maximal glycolytic capacity. (**E**) SRC (%) and (**F**) Proton leak. (N = 3. Data are presented as mean ± SD; significance was determined using two-way ANOVA with factors culturing (25 mM or 0.5 mM glucose level in culture medium) and treatment (only medium or butyrate exposure), followed by Bonferroni post-hoc analysis. * = $p < 0.05$, ** = $p < 0.01$).

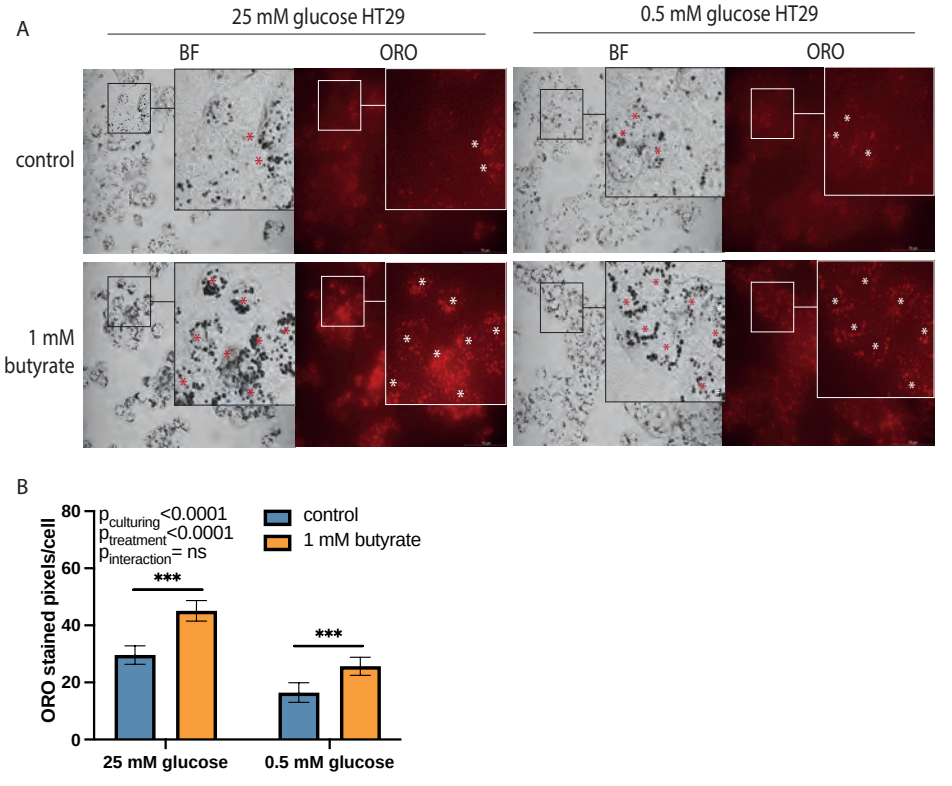



Figure 3.2. **Lipid accumulation in high- and low-glucose-cultured HT29 cells following 72h exposure to 1 mM butyrate.** (**A**) Representative images showing brightfield (BF), nuclear (DAPI) and Oil-Red-O (ORO) staining in 25 mM and 0.5 mM glucose-cultured HT29 cells following 72h exposure to 1 mM butyrate. Red and white stars (*) indicate lipid droplets stained using Oil-Red-O, while hashes (#) indicate DAPI-stained nuclei. (**B**) Representative bar graph showing quantification of ORO-stained image area in pixels corrected for cell number. (Images and the bar graph are from a representative experiment of a total of N = 2. Data are presented as mean ± SD; significance was determined using two-way ANOVA followed by Bonferroni's post-hoc analysis. $p < 0.001$ = ***).

## *Butyrate Acutely Lowers Glycolytic Function in Multiple Colon-Derived Cell Lines*

Since we observed a decrease in glycolytic function only after longer-term butyrate exposure, we went on to investigate the acute effects of butyrate on metabolic flux. To investigate this, we exposed high-glucose-cultured HT29, Caco-2 and HCT116 cells to an acute injection of butyrate during the Seahorse Extracellular Flux analysis. The 25 mM glucose-cultured HT29 cells and Caco-2 cells responded to acute butyrate exposure with increased OCR in a dose-dependent manner (Figure 3.3A,B,E,F). Given that 2DG (2-deoxyglucose) blocks glycolysis, which necessitates the sole use of mitochondria for ATP generation, we expected that 2DG would further increase the

butyrate-induced OCR. Strikingly, OCR was attenuated by subsequent 2DG injection (Figure 3.3A,B,E,F). The decrease in OCR following 2DG injection suggests that butyrate is not the only substrate responsible for the increase in OCR, and that at least part of the observed increase in oxidation rate is fueled by glucose. This notion is strengthened by the observation that acute exposure to butyrate strongly reduced ECAR in a dose-dependent manner in the 25 mM glucose-cultured HT29 and Caco-2 cell lines (Figure 3.3C,D,G,H). Interestingly, the HCT116 cells did not respond to the acute butyrate exposure by increasing their OCR, but there was also an inhibitory effect on ECAR, significant at the highest butyrate concentration of 10 mM (Figure 3.3I–L).



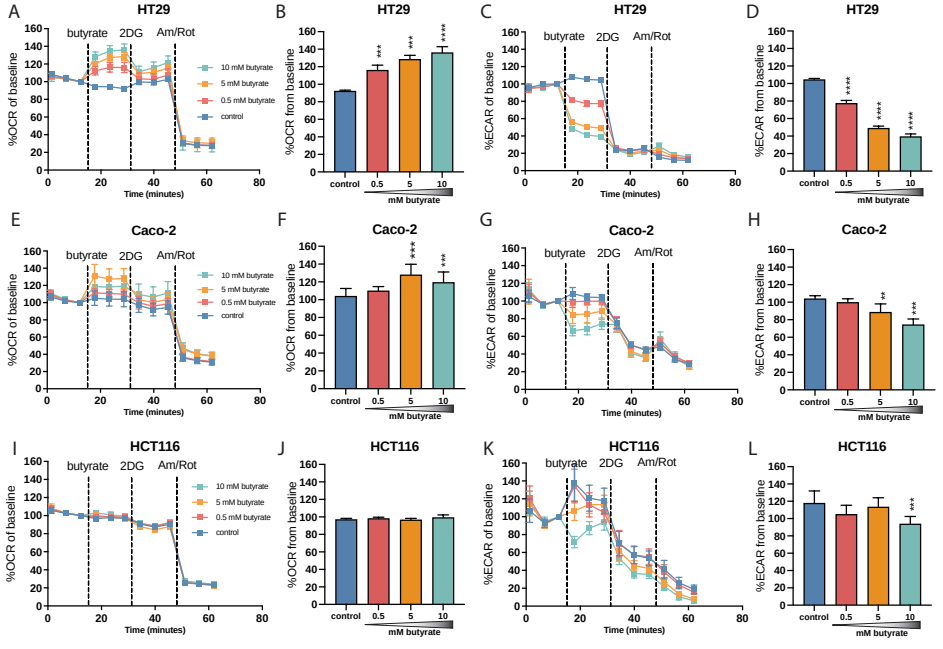



Figure 3.3. **Metabolic flux analysis of multiple colon-derived cell lines following acute butyrate exposure.** (**A-D**) 25 mM cultured HT29 cells (representative experiment of N = 4 independent experiments consisting of n = 5–6 wells). (**E-H**) Caco-2 cells (representative experiment of N = 3 independent experiments consisting of n = 9–15 wells). (**I-L**) HCT116 cells (representative experiment of N = 3 independent experiments consisting of n = 12 wells; significance was determined for one representative experiment using repeated-measures ANOVA followed by Bonferroni's post-hoc analysis. $p = 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****).

To verify whether the effect of butyrate on OCR and ECAR was not limited to merely cultured cell lines, we isolated primary pig colonocytes and analyzed mitochondrial and glycolytic parameters in response to an acute butyrate injection in these primary cells. Similar to HCT116 cells and different from Caco-2 and HT29 cells, primary pig colonocytes did not show any changes in OCR upon acute exposure to 5 mM of butyrate (Figure 3.4A,B). However, as with all cultured cell lines tested, upon injection of butyrate, pig colonocytes showed reduced glycolytic flux by approximately 50% of

the total inhibition that was achieved by 2DG (Figure 3.4C,D). This indicates that the acute effects of butyrate on glycolysis are not restricted to cultured cell

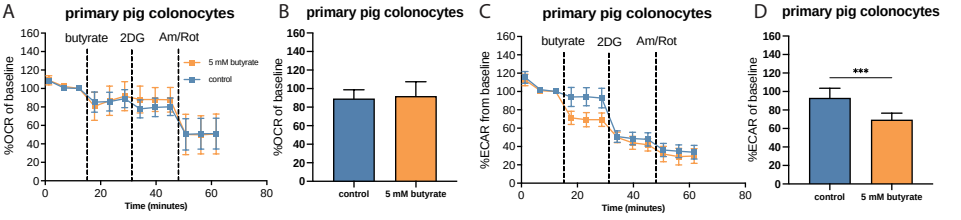



Figure 3.4. **Metabolic flux analysis of primary pig colonocytes following acute butyrate exposure in the presence of 2.5 mM glucose.** (**A,B**) Time-course and bar graph showing the acute effect of control or butyrate injection and subsequent injection of 2DG and Antimycin A/Rotenone on OCR. (**C,D**) Time-course and bar graph showing the acute effect of control or butyrate injection and subsequent injection of 2DG and Antimycin A/Rotenone on ECAR in primary pig colonocytes. (n = 3. Data are shown for colonocyte isolation of one representative pig and are presented as mean ± SD for n = 12 wells; significance was determined using the replicates of one individual using repeated-measures ANOVA followed by Bonferroni's post-hoc analysis. $p < 0.001$ = ***).

### *Butyrate Alters Pyruvate Flux in Cultured Colon Cell Lines*

Our results suggest that at least part of the increased oxidation rate is due to the oxidation of substrates other than butyrate. Glucose-derived pyruvate seems to be the most likely substrate for two reasons. Firstly, acute butyrate exposure increased OCR, which was at least partly reversed by exposure to 2DG, as 2DG inhibits glycolysis and thus pyruvate production. This decreased pyruvate supply for mitochondrial oxidation is the most likely cause for the decrease in OCR observed upon 2DG injection. Secondly, acute butyrate exposure led to a simultaneous increase in OCR and a decrease in ECAR. Altered pyruvate flux seems to be the most likely cause, since pyruvate can either enter the mitochondria, which contributes to OCR, or be converted into lactate, which contributes to ECAR.

To test our hypothesis that glucose-derived pyruvate flux is altered by acute exposure to butyrate, we exposed 25 mM cultured HT29 cells to butyrate in the presence or absence of 2.5 mM glucose. We observed that the increase in OCR upon butyrate exposure did indeed not occur when there was no glucose present in the medium (Figure 3.5A,B). In addition, the decrease in ECAR upon acute butyrate exposure was less apparent when no glucose was available (Figure 3.5C,D). These results show that glucose is required to achieve the acute increase in OCR and decrease in ECAR upon butyrate exposure. To confirm that butyrate specifically alters pyruvate flux toward mitochondrial oxidation and away from lactate production, we inhibited the mitochondrial pyruvate carrier (MPC) with UK5099. Exposure to UK5099 induced a slight but significant ($p < 0.0001$) decrease in OCR and increase in ECAR. This confirms that UK5099 indeed inhibits MPC, since pyruvate can no longer directly enter

the mitochondria and is therefore converted into lactate. When MPC was blocked with UK5099, the increased OCR (Figure 3.5E,F) and decreased ECAR (Figure 3.5G,H) following acute butyrate exposure were no longer observed. We, therefore, believe that the increase in OCR observed in the cultured cell lines HT29 and Caco-2, as well as the decrease in ECAR that could be seen in HCT116 and primary pig colonocytes, was not due to direct oxidation of butyrate but instead resulted from a rerouting of the pyruvate away from lactate production and towards mitochondrial oxidation.

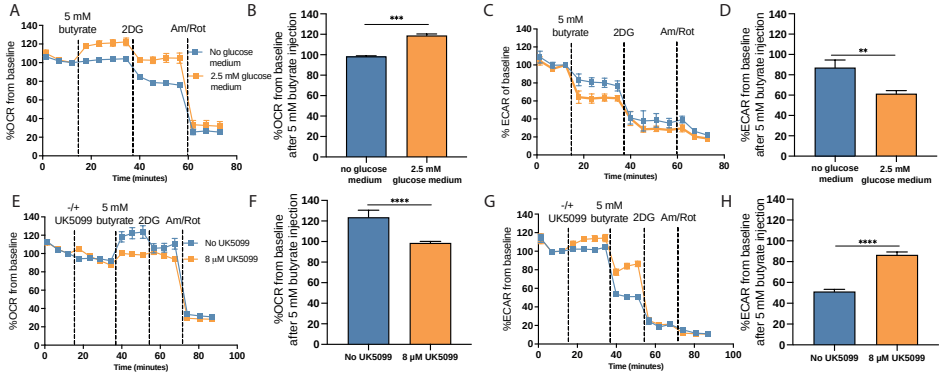



Figure 3.5. **The altered response to acute butyrate exposure as a consequence of glycolysis inhibition in HT29 cells.** (**A,B**) OCR response following butyrate exposure in the absence or presence of 2.5 mM glucose in the medium (representative experiment of N = 2 independent experiments consisting of n = 6 wells). (**C,D**). ECAR response following butyrate exposure in the absence or presence of 2.5 mM glucose in the medium (representative experiment of N = 2 independent experiments consisting of n = 6 wells). (**E,F**) OCR response upon inhibition of pyruvate oxidation using 8 µM UK5099 in the presence of 2.5 mM glucose (representative experiment of N = 3 independent experiments consisting of n = 13–15 wells). (**G,H**) ECAR response upon inhibition of pyruvate oxidation using 8 µM UK5099 (representative experiment of N = 3 independent experiments consisting of n = 13–15 wells; significance was determined for one representative experiment using repeated-measures ANOVA followed by Bonferroni's post-hoc analysis. $p = 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****).

## Discussion

Although butyrate is considered the main energy source for *in vivo* colonocytes [1], it is unclear whether *ex vivo* and cultured colonocytes are able to oxidize butyrate. Whether or not oxidation can take place in a certain cell is an important issue with regard to the usability of these models when investigating butyrate's translatable mechanisms of action. In this study, we have used both cultured and primary cells, as well as high- and low-glucose culturing conditions, to show that butyrate does not seem to be primarily oxidized in cultured cells but instead fuels lipid droplets and alters pyruvate flux away from lactate production and towards mitochondrial oxidation. These findings imply that the nutritional environment is an important determinant of the effects of butyrate in cultured cells. Our research shows that a better understanding of the *in vivo* nutrient composition, and how different nutritional environments may affect the impact of

butyrate, is needed to further study the effects of butyrate on colonocyte metabolism.

We hypothesize that the effects that we observed on pyruvate flux and lipid accumulation are intertwined. Even though butyrate oxidation is sometimes even observed in cultured cells [35], we observed that butyrate primarily alters the flux of pyruvate towards mitochondrial oxidation. We conclude this based on four main findings: (1) increased OCR upon butyrate exposure elicits a simultaneous decrease in ECAR, indicating that less lactate is being produced; (2) the increased OCR following butyrate exposure is largely blunted by the addition of 2DG, indicating that part of the OCR is derived from glucose; (3) the absence of glucose in the medium prevented the butyrate-induced increase in OCR; (4) UK5099, which blocks pyruvate entry into mitochondria, likewise largely prevents the butyrate-induced increase in OCR. At the same time, we saw a clear increase in lipid droplets, even in the more oxidative low-glucose-cultured HT29 cells, indicating that this was not solely due to the diminished mitochondrial capacity of the cells. Likely, butyrate is directly incorporated into lipids, because it does not have to be broken down completely into acetyl-CoA but can be directly elongated from butyryl-CoA [36]. As others have suggested before us, we hypothesize that mitochondrial pyruvate oxidation is increased to generate the NADPH and acetyl-CoA that are needed to convert butyryl-CoA into lipids. There are multiple pathways through which NADPH can be generated. One is through the PPP, which has been observed to be upregulated upon butyrate exposure by some [23, 28-30], but not by us and others [29]. NADPH can also be generated through the cytosolic conversion of malate to pyruvate or citrate to oxoglutarate, which regenerates cytosolic NADPH. For this, pyruvate would need to be (partially) oxidized in the TCA cycle first, which would explain the need for the increased pyruvate oxidation observed in our experiments. Mechanistically, butyrate possibly alters pyruvate flux by post-translationally inhibiting pyruvate dehydrogenase kinase (PDK), which alleviates the inhibition of PDK on pyruvate dehydrogenase (PDH) [37]. Butyrate was also shown to inactivate SIRT3, resulting in increased acetylation and activation of PDH [38]. In this way, more pyruvate is able to be converted into acetyl-CoA and enter the TCA cycle. Butyrate was also shown to increase the expression of pyruvate kinase M2 (PKM2), which stimulates the conversion of phosphoenolpyruvate (PEP) to pyruvate [39]. Another possibility is that butyrate directly interacts with the PDH complex through butyrylation or crotonylation, but we have not yet found any clear evidence for this, neither in our own experiments nor in the literature. Although it remains unclear by which means, our data clearly indicate the upregulation of pyruvate oxidation, which is possibly driven by an increased need for NADPH.

Butyrate is already known to be incorporated into lipids [40]. Butyrate possibly contributes to *de novo* lipogenesis through a shared pool with ketone bodies that is separate from that of acetate and propionate [41]. Interestingly, the degree to which

butyrate contributes to *de novo* lipogenesis seems to be largely dependent on substrate availability. The presence of glucose in the medium was found to greatly increase the amount of butyrate that is converted into lipids [40, 41]. Corresponding with our findings, butyrate was found to increase lipogenesis in cultured colonocytes [23]. However, on a whole-body level, butyrate administration mainly decreased lipid accumulation in the liver [42, 43]. The decreased lipid storage in the liver upon butyrate exposure could be due to increased lipolysis in hepatocytes themselves and other cells such as adipocytes [44-47], but possibly also because of increased lipid storage in other organs, such as the muscle [45] or even intestine. Nonetheless, it is unclear whether butyrate can induce lipid storage *in vivo* in the colon, and how the nutritional environment affects the outcome of butyrate exposure in the colon. In our experiments, there was no difference in lipogenesis between high- and low-glucose-cultured cells, but the low-glucose-cultured cells still had sufficient pyruvate and glutamine available to them, which could partly replace the role of glucose break-down as a source for NADPH generation. Our experiments thus show that it is important to further investigate the impact of metabolic phenotype as well as nutritional environment to better understand the effects of butyrate.



In our experiments, it remains undetermined whether the lipids are directly derived from butyrate, or perhaps originate from other substrates. We, therefore, propose to perform isotope-tracing studies for future experiments to deepen our understanding of the fate of butyrate under different nutritional environments and metabolic phenotypes. Nevertheless, our experiments have already generated novel insights into the role of butyrate in regulating colonocyte metabolism. An interesting proposition for why cultured cells store butyrate as lipid droplets, instead of oxidizing this substrate, is that these cells are maintained in a nutrient-rich environment, and butyrate is an excess nutrient that can be stored for later use.

**Acknowledgements:** We would like to thank Karthikeyan Gunasekaran for the technical support.

**Author Contributions:** **A.F.B**: Conceptualization, Data curation, Writing—original draft, Writing—review and editing; **T.v.D.:** Conceptualization, Data curation, Writing—review and editing; **W.J.J.G:** Funding acquisition, Writing—review and editing; **J.K.:** Funding acquisition, Writing—review and editing; **V.C.J.d.B.**: Conceptualization, Funding acquisition, Supervision, Writing—review and editing.

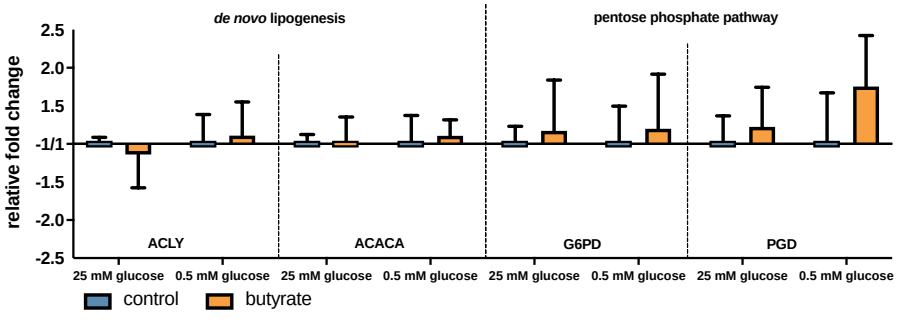
## References


1. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-429.
2. Sengupta, S., J.G. Muir, and P.R. Gibson, *Does butyrate protect from colorectal cancer?* J Gastroenterol Hepatol, 2006. **21**(1 Pt 2): p. 209-18.
3. Chen, J. and L. Vitetta, *Butyrate in Inflammatory Bowel Disease Therapy.* Gastroenterology, 2020. **158**(5): p. 1511.
4. Krokowicz, L., et al., *Sodium butyrate and short chain fatty acids in prevention of travellers' diarrhoea: A randomized prospective study.* Travel Medicine and Infectious Disease, 2014. **12**(2): p. 183-188.
5. van der Beek, Christina M., et al., *Distal, not proximal, colonic acetate infusions promote fat oxidation and improve metabolic markers in overweight/obese men.* Clinical Science, 2016. **130**(22): p. 2073-2082.
6. Wang, D., et al., *LSD1 mediates microbial metabolite butyrate-induced thermogenesis in brown and white adipose tissue.* Metabolism, 2020. **102**: p. 154011.
7. Bachem, A., et al., *Microbiota-Derived Short-Chain Fatty Acids Promote the Memory Potential of Antigen-Activated CD8+ T Cells.* Immunity, 2019. **51**(2): p. 285-297. e5.
8. Zhang, L., et al., *Butyrate in Energy Metabolism: There Is Still More to Learn.* Trends in Endocrinology & Metabolism, 2021. **32**(3): p. 159-169.
9. Donohoe, D.R., et al., *The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon.* Cell Metabolism, 2011. **13**(5): p. 517-526.
10. Kelly, C.J., et al., *Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function.* Cell Host Microbe, 2015. **17**(5): p. 662-671.
11. Den Hond, E., et al., *In vivo butyrate metabolism and colonic permeability in extensive ulcerative colitis.* Gastroenterology, 1998. **115**(3): p. 584-590.
12. Ahmad, M.S., et al., *Butyrate and glucose metabolism by colonocytes in experimental colitis in mice.* Gut, 2000. **46**(4): p. 493.
13. Chapman, M.A., et al., *Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis.* Gut, 1994. **35**(1): p. 73-76.
14. Ferrer-Picón, E., et al., *Intestinal Inflammation Modulates the Epithelial Response to Butyrate in Patients With Inflammatory Bowel Disease.* Inflammatory Bowel Diseases, 2019. **26**(1): p. 43-55.
15. Heiden, M.G.V., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation.* Science, 2009. **324**(5930): p. 1029-1033.
16. Warburg, O., *On the Origin of Cancer Cells.* Science, 1956. **123**(3191): p. 309.
17. Fleming, S.E. and R. Gill, *Aging Stimulates Fatty Acid Oxidation in Rat Colonocytes But Does Not Influence the Response to Dietary Fiber.* The Journals of Gerontology: Series A, 1997. **52A**(6): p. B318-B330.
18. Hofmanová, J., et al., *Human fetal colon cells and colon cancer cells respond differently to butyrate and PUFAs.* Molecular Nutrition & Food Research, 2009. **53**(S1): p. S102-S113.
19. Zweibaum, A., et al., *Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose.* Journal of Cell Physiology, 1985. **122**(1): p. 21-29.
20. Rossignol, R., et al., *Energy Substrate Modulates Mitochondrial Structure and Oxidative Capacity in Cancer Cells.* Cancer Research, 2004. **64**(3): p. 985.
21. Kase, E.T., et al., *Remodeling of Oxidative Energy Metabolism by Galactose Improves Glucose Handling and Metabolic Switching in Human Skeletal Muscle Cells.* PLOS ONE, 2013. **8**(4): p. e59972.
22. JanssenDuijghuijsen, L.M., et al., *Mitochondrial ATP Depletion Disrupts Caco-2 Monolayer Integrity and Internalizes Claudin 7.* Frontiers in Physiology, 2017. **8**: p. 794.

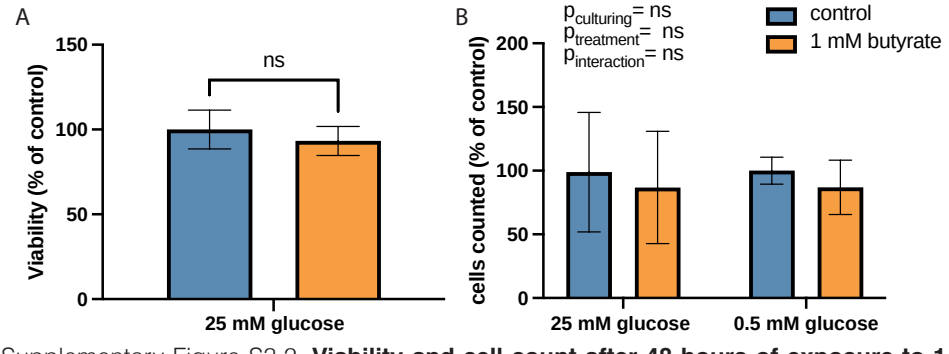
23. Leschelle, X., et al., *Butyrate metabolism upstream and downstream acetyl-CoA synthesis and growth control of human colon carcinoma cells.* European Journal of Biochemistry, 2000. **267**(21): p. 6435-6442.
24. Donohoe, Dallas R., et al., *The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation.* Molecular Cell, 2012. **48**(4): p. 612-626.
25. Xu, J.-Y., et al., *Protein Acetylation and Butyrylation Regulate the Phenotype and Metabolic Shifts of the Endospore-forming Clostridium acetobutylicum.* Molecular & Cellular Proteomics : MCP, 2018. **17**(6): p. 1156-1169.
26. Singh, B., A.P. Halestrap, and C. Paraskeva, *Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources.* Carcinogenesis, 1997. **18**(6): p. 1265-1270.
27. Marcil, V., et al., *Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2002. **283**(2): p. G340-G346.
28. Boren, J., et al., *The Stable Isotope-based Dynamic Metabolic Profile of Butyrate-induced HT29 Cell Differentiation.* Journal of Biological Chemistry, 2003. **278**(31): p. 28395-28402.
29. Geng, H.-W., et al., *Butyrate Suppresses Glucose Metabolism of Colorectal Cancer Cells via GPR109a-AKT Signaling Pathway and Enhances Chemotherapy.* Frontiers in Molecular Biosciences, 2021. **8**: p. 634874-634874.
30. Tan, H.T., et al., *Quantitative and Temporal Proteome Analysis of Butyrate-treated Colorectal Cancer Cells.* Molecular & Cellular Proteomics, 2008. **7**(6): p. 1174-1185.
31. Ghorbaniaghdam, A., O. Henry, and M. Jolicoeur, *A kinetic-metabolic model based on cell energetic state: study of CHO cell behavior under Na-butyrate stimulation.* Bioprocess and Biosystems Engineering, 2013. **36**(4): p. 469-487.
32. Matthews, G.M., G.S. Howarth, and R.N. Butler, *Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism.* Chemotherapy, 2012. **58**(2): p. 102-109.
33. Butler, R.N., et al., *Pyruvate sparing by butyrate and propionate in proliferating colonic epithelium.* Comparative Biochemistry and Physiology Part B, 1990. **97**(2): p. 333-337.
34. Tabuchi, Y., et al., *Identification of genes responsive to sodium butyrate in colonic epithelial cells.* Biochemical and Biophysical Research Communications, 2002. **293**(4): p. 1287-1294.
35. Han, A., et al., *Cellular Metabolism and Dose Reveal Carnitine-Dependent and -Independent Mechanisms of Butyrate Oxidation in Colorectal Cancer Cells.* Journal of Cellular Physiology, 2016. **231**(8): p. 1804-1813.
36. Long, R.W. and J.W. Porter, *Incorporation of the Butyryl Unit of Butyryl Coenzyme A into Palmitic Acid by the Pigeon Liver System.* Journal of Biological Chemistry, 1959. **234**(6): p. 1406-1410.
37. Blouin, J.-M., et al., *Butyrate elicits a metabolic switch in human colon cancer cells by targeting the pyruvate dehydrogenase complex.* International Journal of Cancer, 2011. **128**(11): p. 2591-2601.
38. Xu, S., et al., *Butyrate induces apoptosis by activating PDC and inhibiting complex I through SIRT3 inactivation.* Signal Transduction and Targeted Therapy, 2017. **2**(1): p. 16035.
39. Fung, K.Y.C., et al., *Proteomic Analysis of Butyrate Effects and Loss of Butyrate Sensitivity in HT29 Colorectal Cancer Cells.* Journal of Proteome Research, 2009. **8**(3): p. 1220-1227.
40. Roediger, W.E.W., O. Kapaniris, and S. Millard, *Lipogenesis from n-butyrate in colonocytes.* Molecular and Cellular Biochemistry, 1992. **116**(2): p. 113-118.
41. Zambell, K.L., M.D. Fitch, and S.E. Fleming, *Acetate and Butyrate Are the Major Substrates for De Novo Lipogenesis in Rat Colonic Epithelial Cells.* The Journal of Nutrition, 2003. **133**(11): p. 3509-3515.

42. den Besten, G., et al., *Short-Chain Fatty Acids Protect Against High-Fat Diet–Induced Obesity via a PPARγ-Dependent Switch From Lipogenesis to Fat Oxidation.* Diabetes, 2015. **64**(7): p. 2398-2408.
43. Du, Y., et al., *Butyrate protects against high-fat diet-induced atherosclerosis via up-regulating ABCA1 expression in apolipoprotein E-deficiency mice.* British Journal of Pharmacology, 2020. **177**(8): p. 1754-1772.
44. Yang, T., et al., *Amelioration of non-alcoholic fatty liver disease by sodium butyrate is linked to the modulation of intestinal tight junctions in db/db mice.* Food & Function, 2020. **11**(12): p. 10675-10689.
45. Zhang, H., et al., *Transcriptomic Responses Induced in Muscle and Adipose Tissues of Growing Pigs by Intravenous Infusion of Sodium Butyrate.* Biology, 2021. **10**(6): p. 559.
46. Rumberger, J.M., J.R.S. Arch, and A. Green, *Butyrate and other short-chain fatty acids increase the rate of lipolysis in 3T3-L1 adipocytes.* PeerJ, 2014. **2**: p. e611.
47. Zhao, L., et al., *Low and high concentrations of butyrate regulate fat accumulation in chicken adipocytes via different mechanisms.* Adipocyte, 2020. **9**(1): p. 120-131.

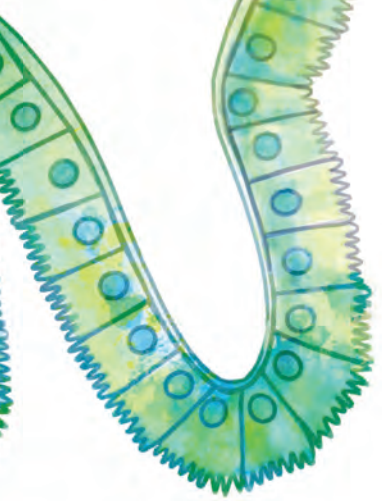
## Supplementary material




Supplementary Figure S3.1. **Relative gene expression of *de novo* lipogenesis and pentose phosphate pathway genes in long-term butyrate exposed high and low glucose HT29 cells.** Relative gene expression of ATP citrate lyase (ACLY), acetyl-Coenzyme A carboxylase alpha (ACACA) glucose-6-phosphate dehydrogenase (G6PD) and 6-Phosphogluconate Dehydrogenase (PGD) in 25 mM and 0.5 mM glucose cultured HT29 cells exposed to only medium or 1 mM butyrate for 72 hours. (Data are derived from N=3-4 independent experiments and are presented as mean ± SD; significance was determined using two-way ANOVA followed by Bonferroni's post-hoc analysis).



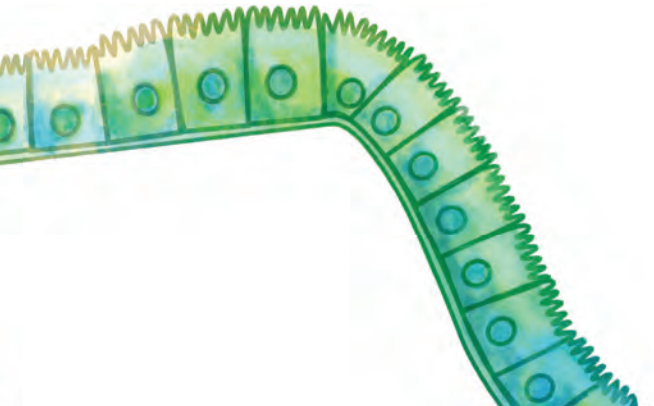

Supplementary Figure S3.2. **Viability and cell count after 48 hours of exposure to 1 mM butyrate.** (**A**) Viability of high glucose (25 mM) cultured HT29 cells after 48 hours of exposure to 1 mM butyrate (N=2) (**B**) Cell count of high (25 mM) and low glucose (0.5 mM) cultured HT29 cells after 48 hours of exposure to 1 mM butyrate (N=3; data are presented as mean ± SD; significance was determined using Student's t-test (**A**) or two-way ANOVA followed by Bonferroni's post-hoc analysis (**B**)).



# Chapter 4

## Mitochondrial and Glycolytic Extracellular Flux Analysis Optimization for Isolated Pig Intestinal Epithelial Cells

Anna F. Bekebrede
Jaap Keijer
Walter J.J. Gerrits
Vincent C.J. de Boer



***Scientific Reports 2021;***
doi:10.1038/s41598-021-99460-0

## Abstract

Intestinal epithelial cells (IECs) are crucial to maintain intestinal function and the barrier against the outside world. To support their function they rely on energy production, and failure to produce enough energy can lead to IEC malfunction and thus decrease intestinal barrier function. However, IEC metabolic function is not often used as an outcome parameter in intervention studies, perhaps because of the lack of available methods. We therefore developed a method to isolate viable IECs, suitable to faithfully measure their metabolic function by determining extracellular glycolytic and mitochondrial flux. First, various methods were assessed to obtain viable IECs. We then adapted a previously in-house generated image-analysis algorithm to quantify the amount of seeded IECs. Correcting basal respiration data of a group of piglets using this algorithm reduced the variation, showing that this algorithm allows for more accurate analysis of metabolic function. We found that delay in metabolic analysis after IEC isolation decreases their metabolic function and should therefore be prevented. The presence of antibiotics during isolation and metabolic assessment also decreased the metabolic function of IECs. Finally, we found that primary pig IECs did not respond to Oligomycin, a drug that inhibits complex V of the electron transport chain, which may be because of the presence of drug exporters. A method was established to faithfully measure extracellular glycolytic and mitochondrial flux of pig primary IECs. This tool is suitable to gain a better understanding of how interventions affect IEC metabolic function.

**Key words**: intestinal epithelial cells; metabolism; isolation; normalization; antibiotics

## Introduction

The intestine forms a physical barrier against the outside milieu. Intestinal barrier function can be diminished by multiple factors, of which the most common ones are stress [1, 2], infectious agents [3, 4] and drug use [5-7]. If the barrier fails, this can lead to intestinal problems such as diarrhea. Diarrheal diseases are a serious health concern, especially in third world countries [8] and in animal husbandry [9, 10]. Diarrhea in early life can have long lasting effects; the malnutrition it causes has been shown to increase the susceptibility for developing metabolic disorders in later life [11]. Intestinal epithelial cells have a central role in maintaining the intestinal barrier function, for which they rely on energy supply by mitochondria and glycolysis.

Intestinal epithelial cell (IEC) energy metabolism, and especially mitochondrial energy production, is essential for maintaining the intestinal barrier function both *in vitro* and *in vivo* [6, 12, 13]. Tight junction proteins that are responsible for tethering the IECs together rely on sufficient energy generation, since inhibition of mitochondrial ATP production internalized the tight junction protein claudin 7 because of an energy crises, resulting in a loss of barrier [12]. Also other intestinal functions, such as nutrient digestion, uptake and metabolism, rely on energy generation by the mitochondria. After a meal, when nutrient processing is highly upregulated, small intestinal oxygen consumption doubles to satisfy energy needs [14]. In the colon, IECs are faced with additional metabolic challenges, since IECs are then in constant contact with the microbiome. For example, bacteria target and inhibit mitochondrial function of colonocytes to increase their virulence [3, 4], which decreases intestinal barrier function and promotes bacterial translocation [15]. Thus, IEC metabolic function is important for maintenance of intestinal barrier function, as well as the response to external and internal stressors.

Assessment of intestinal metabolic function in both animal and human *in vivo* experiments is not often considered as an outcome parameter of interventions that target intestinal function. Part of this hiatus is caused by difficulties in measuring intestinal epithelial energy metabolism, especially mitochondrial respiration and the enterocyte glycolytic flux. Measurement of IEC metabolism is extra challenging because of the high turnover rate of the intestinal epithelium of every 5-7 days [16], which can indicate that the fully differentiated enterocytes only have a short life-span, and isolation of these cells likely rapidly leads to apoptosis [17]. We set out to design a procedure to isolate intestinal epithelial cells and characterize their metabolism. We chose to optimize the isolation procedure for the colon, because microbial fermentation end-products present in this intestinal compartment can both positively and negatively impact mitochondrial function of IECs. For example, butyrate, a short-chain fatty acid produced through fibre fermentation, is known to be an important energy source for colon cells and thus supports mitochondrial function [18, 19]. On the other hand, hydrogen sulfide, which is

produced through protein fermentation, decreases mitochondrial function [20]. We use the pig both as a model and a relevant target species. Pigs are an important agricultural species [21], and are also the best human translatable animal model to study large organs systems, such as the intestinal tract [22]. Apart from the high similarity between pigs and humans in GI tract anatomy, a great advantage for using pigs as a model lies in the similar clinical manifestations and their susceptibility to many enteric pathogens and intestinal diseases afflicting humans [23, 24]. Other commonly used animal models like mice are less similar to humans than pigs with regard to their intestinal metabolism, microbial pathways and their response to nutritional interventions [24-26]. A more practical consideration for using pigs, is the availability of adequate amounts of healthy intestinal material.

Here, we designed an IEC isolation technique to harvest viable primary IECs from the pig colon, that is suitable for measurement of mitochondrial respiration and glycolytic flux. We optimized the flux analysis for chemical induced signal stability and normalization procedures for cell number correction. Our results showed that the optimized cell isolation technique was suitable for measurement of metabolic function of pig IECs. This technique will be a useful tool to evaluate the effect of interventions on intestinal function.



## Methods

### *Animals*

Intestines were either harvested from pigs at a slaughterhouse or from control pigs from dedicated animal experiments sacrificed at our Animal Facility, that were all approved by the Animal Care and Use Committee of Wageningen University. However no animals were sacrificed specifically for the purpose of this study. The majority of material was derived from slaughterhouses. We were unable to get all details for pig breeds, age and sex, because we choose for a rapid, relatively easy organ collection procedure instead of a detailed dissection. This offers ease in performing multiple experiments on scheduled cell isolation days, and shows the flexibility of the isolation procedure. Overall, the pigs were from both sexes, multiple breeds, weighed between 20-100 kg and were aged between 12-32 weeks.

### *IEC isolation*

Following excision from the abdominal cavity, an approximately 20 cm long segment of the colon was taken for IEC isolation and placed in aerated Krebs Henseleit Buffer containing 5 mM glucose (#K3753, Sigma Aldrich; hereafter referred to as modified-KHB), containing 2.5 g/L Bovine serum albumin (BSA, #A7906, Sigma-Aldrich).

Samples were transferred to the lab and isolation commenced within 2 hours after killing the animal.

Multiple steps were taken to optimize the isolation procedure. Initially, two methods described in literature were used as a basis for the design of the procedure. First, a method described by Roedinger et al. [27] was assessed. We refer to this method as the vigorous method. The colon segments were first flushed with room temperature (RT) modified-KHB, inverted and then a sac was created using dialysis clamps (#Z371092, Sigma Aldrich) and the sac was filled with modified-KHB. The sacs were then placed in $Ca^{2+}$-free KHB with 5 mM Ethylenediaminetetra-aceticacetic acid (EDTA) and 2.5 g/L BSA. After a 30-minute incubation in a shaking 37 °C water bath, the buffer was removed and replaced by fresh $Ca^{2+}$-free KHB containing 2.5 g/L BSA. The intestines were stirred vigorously by hand for two minutes to dissociate the IECs. IECs were then passed over a 70 µM cellulose filter top to remove large tissue pieces and debris. After washing cells twice using modified-KHB contained 2.5 g/L BSA, cells were taken up in pH 7.4 buffered XF DMEM medium (#103575-100, Agilent Technologies) supplemented with 10 mM glucose (#103577-100, Agilent Technologies), 2 mM glutamine (#103579-100, Agilent Technologies) and 1 mM pyruvate (#103578-100, Agilent Technologies) and counted using a Cellometer K4 (Nexcelom Bioscience) and viability was simultaneously assessed by staining with ViaStain (#CS2-0106, Nexcelom Bioscience). The second method tested, to which we refer as the gentle method, was a modification of the one described by Darcy-Vrillon [19]. In this method, the intestine was first flushed with modified-KHB, and then immediately a sac was created using dialysis clamps. The sac was filled with $Ca^{2+}$-free KHB containing 10 mM EDTA, 5 mM Dithiothreitol (DTT), and 2.5 g/L BSA. After a 20-minute incubation in a shaking 37 °C water bath, the sac was emptied and refilled with the same buffer, followed by another fifteen-minute incubation. Afterwards, the intestines were gently massaged, and cells were collected, washed and counted, as described for the first procedure.

The third method we tested (which was also the optimized method we used for downstream analysis of metabolic function) was a combination of steps from the above two methods combined with a hyaluronidase enzymatic dissociation step and optional washing steps. We refer to this method as the ‘enzyme’ method or ‘enzyme + extra wash’ method (see supplementary materials for a stepwise lab protocol of the final optimized method). First, intestines were flushed with modified-KHB. Then, they were inverted, and a sac was created using dialysis clamps by filling them with modified-KHB. Inversion of the intestines at this stage facilitated exchange between buffer and mucosa, since the amount of buffer can be much higher than if the intestines are not inverted. The sacs were first incubated for 20 minutes in $Ca^{2+}$-free KHB buffer containing 20 mM EDTA and 10 mM DTT in a shaking 37 °C water bath. Following this

washing step, intestines were re-verted and filled with an isolation buffer containing $Ca^{2+}$-free KHB buffer, 2.5 g/L BSA and 400 U/mL hyaluronidase type IV (#3884, Sigma-Aldrich), an enzyme that catalyzes the breakdown of hyaluronic acid which is present in the extracellular matrix of IECs. The re-version of the sacs at this stage is convenient, since cells will be collected in a smaller volume. In addition, the amount of buffer and enzyme needed can thus be reduced, which is cost-effective. After a fifteen-minute incubation, the intestines were gently massaged and cells were collected, washed and counted as previously described. This protocol was finally adjusted by adding a 20-minute washing step before the enzymatic digestion to facilitate increased removal of mucus from the intestines.

For the experiments where we analyzed the effects of antibiotics on metabolic function of IECs, all washing and isolation buffers used in the isolation procedure were supplemented with 1% v/v penicillin-streptomycin (#15140122, Fisher Scientific).

### *Metabolic flux analysis with Seahorse XFe96 analyzer*



Isolated IECs were plated in a XF96 cell plates that were coated with Cell-Tak (#354240, Corning, New York, USA) according to manufacturer's protocol, no longer than one week prior to the assay. Cells were plated at concentrations ranging from 25,000-150,000 cells/well in 50 µL pH 7.4 balanced XF DMEM assay medium supplemented with 10 mM XF glucose, 2 mM XF glutamine and 1 mM XF pyruvate. For the normalization optimization and Oligomycin response experiments, cells were plated at 100,000 cells/well, left to settle for 5 min prior to spin-down (200 x *g* for 2' with zero break). After spin-down, cell plates were imaged as described below, while kept at 37 °C. Following imaging, an additional volume of 130 µL assay medium was added and cell plates were incubated for another 20 minutes in a non-$CO_2$ 37 °C incubator. For the optimization of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; #C2920, Sigma-Aldrich), cells were isolated using the 'gentle' method and final concentrations of 0.5-1.18 µM were injected into the wells. For optimization of Oligomycin (a mix of A, B and C Oligomycin, #O4875, Sigma-Aldrich) concentration, a final concentration of either 1.5 or 15 µM was used. Extracellular flux analyses (XF assays) was performed using the Seahorse XFe96 (Seahorse Bioscience, Agilent Technologies, Santa Clara, USA). Most often, XF assays were performed using serial injections of 1.5 µM Oligomycin, 1 µM FCCP, a combination of 1.25 µM Rotenone and 2.5 µM Antimycin A and finally 50 mM 2-deoxyglucose (2-DG). The XF assay protocol typically consisted of 12 measurement cycles of 3 minutes, with 2 minutes of mixing in between measurements. For the measurements with antibiotics, assay medium also contained 1 % v/v penicillin-streptomycin. Cell plates were kept in a non-$CO_2$ 37 °C incubator for 1.5h prior to the start of the assay for the delayed measurement experiments.

### *Imaging procedure*

Brightfield images of the inner probe area of each well in the XF96 cell plates were obtained prior to the XF assay run using a 37 °C equilibrated Cytation 1 Cell Imaging Multi-Mode Reader (BioTek, Winooski, Vermont, USA) using a 4x objective. A LED intensity of 5 and integration time of 80 milliseconds was kept constant for all cell plates, image focus height was adjusted as needed to get the optimal image quality, as was determined by visual inspection. For optimization of the normalization procedure cell nuclei were either stained using 8 µM Hoechst 33342 (Hoechst, #B2261, Sigma-Aldrich), or fixed using 4% paraformaldehyde (#252549, Sigma-Aldrich) and then stained with 4,6-diamidino-2-phenylindo (DAPI, #D9564, Sigma-Aldrich), followed by image acquisition using a 4x objective with a 365 nm LED in combination with an EX337/EM447 filter cube.

### *Brightfield image analysis in R*

Brightfield images obtained prior to the XF assay run were processed and quantified using an in-house generated R-script that uses the EBImage package available for Bioconductor [28]. Image processing was performed in a similar manner as previously published [29], with an adjustment of the image quantification. Briefly, a Gaussian blur low-pass filter was applied to generate a background image, followed by subtraction of the background image from the original. The background corrected image was then inverted to generate a "white-objects-on-black-background image". This image was subsequently cropped by 5% to remove potential noise from the XF assay plate molded stops, that are present on the plates to prevent the sensors from disrupting the cell monolayer. Images were then analyzed to calculate pixel intensity values for all the pixels in the image. All the pixels with an intensity >1 was counted as representing the presence of a cell, and we refer to these as "cell-pixels". For conversion of cell-pixels back to cells, an external calibration curve was generated. To do this, a second order polynomial fit analysis was performed on the combined data of three individual pig standard curves. The best-fit curve that matched the data was then used as an external calibration curve to convert cell-pixel values of every plate back to cell numbers. These cell numbers were subsequently used for normalization of the Seahorse XF assays. The R-script is available from GitHub (https://github.com/vcjdeboer/seahorse-data-analysis-PIXI).

### *Statistical analysis and data visualization*

Data are presented as mean ± s.d., unless stated otherwise. The standard score, or z-score, was calculated using equation (1): *z-score = (well – mean(all wells per subject))/s.d.(all wells per subject).* Statistical analyses and data visualizations were

performed using GraphPad Prism v.9 (GraphPad Software, CA, USA). Statistical testing was performed using student's t-test or one-way ANOVA when appropriate and as stated in the Figure legends. A p-value of <0.05 was considered statistically significant.

## Results

### *Pig primary IEC isolation optimization for analyzing metabolic activity*

We set out a strategy to set-up our methodology using a number of shearing and isolation methods described in literature (Fig. 4.1A). For reference, we compared the methods described by Roedinger and Truelove [27] and Darcy-Vrillon et al. [19]. The former used vigorous shaking while the latter used gentle massaging of the intestinal segments, and we will thus refer to them as "vigorous" and "gentle", respectively. Both methods were performed in physiological buffer without enzymatic treatment. To be able to compare methods, we first scored the presence of crypts in the isolates as a sign of isolation robustness. More crypts means that less single cells are isolated, which makes the method less suitable. The vigorous as well as the gentle method both gave crypts (Fig. 4.1B), although slightly less in the gentle method, and the overall yield and viability was not different between both techniques (Fig. 4.1C-D). Although viability was not different, basal OCR was significantly lower for the vigorous method (Supplementary Fig. S4.1A). Therefore, we decided to further optimized the gentle method, by inverting the intestine during the washing steps to increase the exchange between buffer and intestinal surface area. In addition, we added an enzymatic dissociation step in order to improve single-cell yield ('enzyme' method). After including these steps, we observed less crypts compared to the method without inversion and enzymatic dissociation (Fig. 4.1B). Next, to be able to isolate IECs from piglets that received different feeds that could possibly affect the thickness of the mucus layer, we added an extra washing step to the isolation protocol to increase mucus removal prior to enzymatic cell dissociation ('enzyme+wash' method; final protocols see supplemental method for a stepwise description). Introduction of the extra washing step did not result in lower cell yield (Fig. 4.1E), and increased cell viability (Fig. 4.1F, p=0.0018), demonstrating that extra washing can be included without negatively impacting cell viability and yield, but allowing for a flexible isolation protocol based on the feeding status of the animals. In the subsequent experiments, the 'enzyme" and "enzyme + extra wash" methods were used, unless otherwise stated.

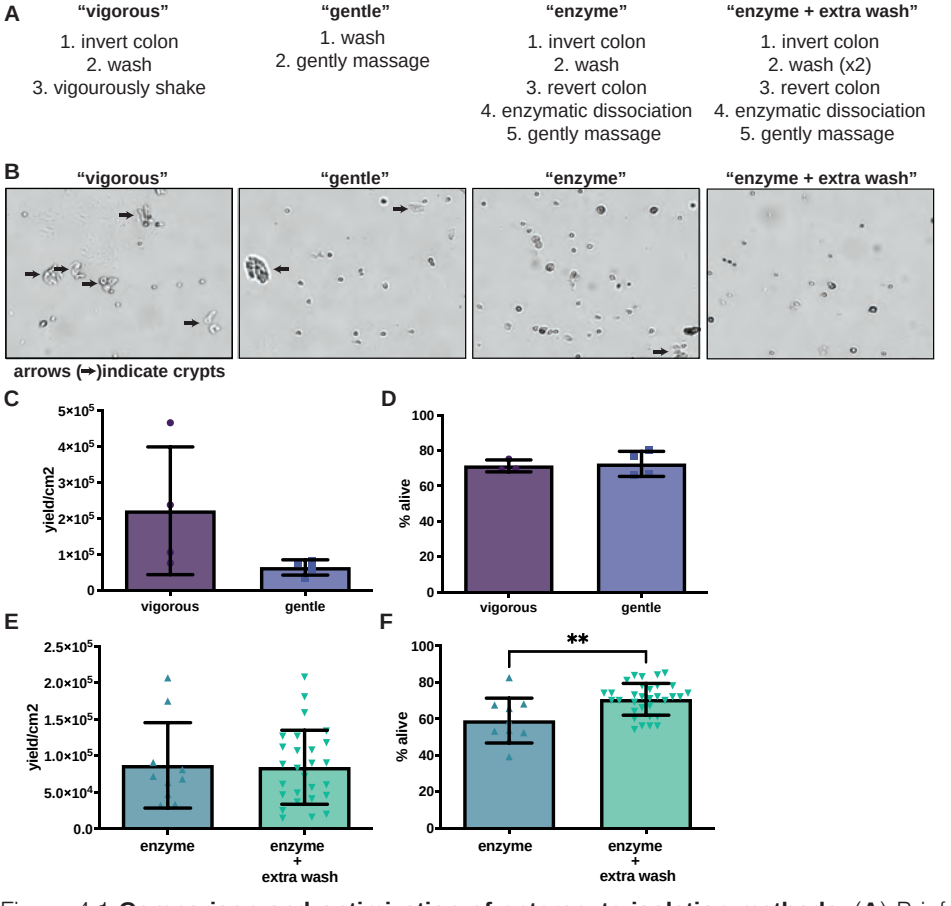



Figure 4.1 **Comparison and optimization of enterocyte isolation methods**. (**A**) Brief overview of the experimental procedures of for the different isolation methods used during the optimization procedure. (**B**) Representative brightfield images of the isolated cell populations for the different isolation methods; crypts are indicated with arrows. (**C,D**) Analysis of yield and viability of the vigorous and gentle isolation methods (n=4 pigs per isolation method). (**E,F**) Analysis of yield and viability of enzymatic (enzyme) and enzymatic with additional washing step (enzyme+ extra wash) dissociation methods (n=8 pigs for enzyme procedure, n=27 pigs for enzyme + extra wash). Student's t-tests were performed to compare yield and viability of the enzyme and enzyme + wash methods, ** indicates a p-value of ≤ 0.01. Each dot in the bar-graphs represents one an IEC isolation from a single pig.

## *Optimization of normalization methodology for pig IEC metabolic measurements*

Next, we analyzed extracellular $O_2$ (OCR) and pH (ECAR) fluxes of primary isolated pig IECs using the Seahorse XFe96 analyzer. This allows for simultaneous measurement of oxygen consumption, as a measure for mitochondrial respiration, and extracellular acidification, as a measure for glycolytic function. An important step in this analysis is

adjustment of the data to the actual cell number in the well, which can fluctuate despite cell counting prior to the addition of the cells to the plate. We therefore optimized the normalization method. First, we tested nuclear staining with Hoechst, but noticed that not all cells were stained, as can be observed from the non-blue stained cells present in the picture (Fig. 4.2A). This was likely due to active efflux drug transporters in the IECs [30]. Next, we fixed the cells in wells and stained them with DAPI. Because the IECs were only loosely attached to the assay plate, and fixation and DAPI staining required several washing steps, cells were often washed away after the procedure (Fig. 4.2B). Next, we used brightfield (BF) images of the wells, obtained prior to the Seahorse XF analysis, similar to a method performed for primary PBMCs [29] (Fig. 4.2C). We observed a correlation coefficient of 0.95-0.99 between the number of cells we seeded from three pigs and the number of cell-pixels counted using our R-algorithm (Fig. 4.2D). A drawback of this correction method is that there can be inter-plate differences in image acquisition, necessitating the addition of a standard curve on each plate [29]. However, we observed that differences in image intensity did not result in different amounts of counted cells, making the use of in-plate calibration curve unnecessary for our experimental set-up and cell type (Fig. 4.2E). We combined the data of the three piglets (Fig. 4.2D) and performed a second order polynomial fit analysis, which we used as a calibration curve. Following transformation of pixel intensities to cell number using the formula obtained from the combined data of the three pigs ($R^2$=0.96), we applied this normalization method to a group of piglets (n=7) that received an experimental diet for two weeks. We found that normalization indeed reduced the within subject variation of basal OCR values, as can be analyzed using the average z-score (or standard score, see equation (1) for calculation; Fig. 4.2F). The standard score represents the variation within the technical replicates (n=4-10 wells/pig) that are included during the Seahorse XF analysis. The arithmetic mean of the standard score decreased from 0.78 to 0.76 after correction for cell number and the range of standard scores was smaller (Fig. 4.2F). The coefficient of variation (CV), which represent the between pig variation, decreased from 31% to 15% (Fig 4.2). These results indicate that normalization using bright field imaging for pig IEC Seahorse analysis lowers both within and between subject variation, which benefits statistical interpretation and the number of replicates that are needed.

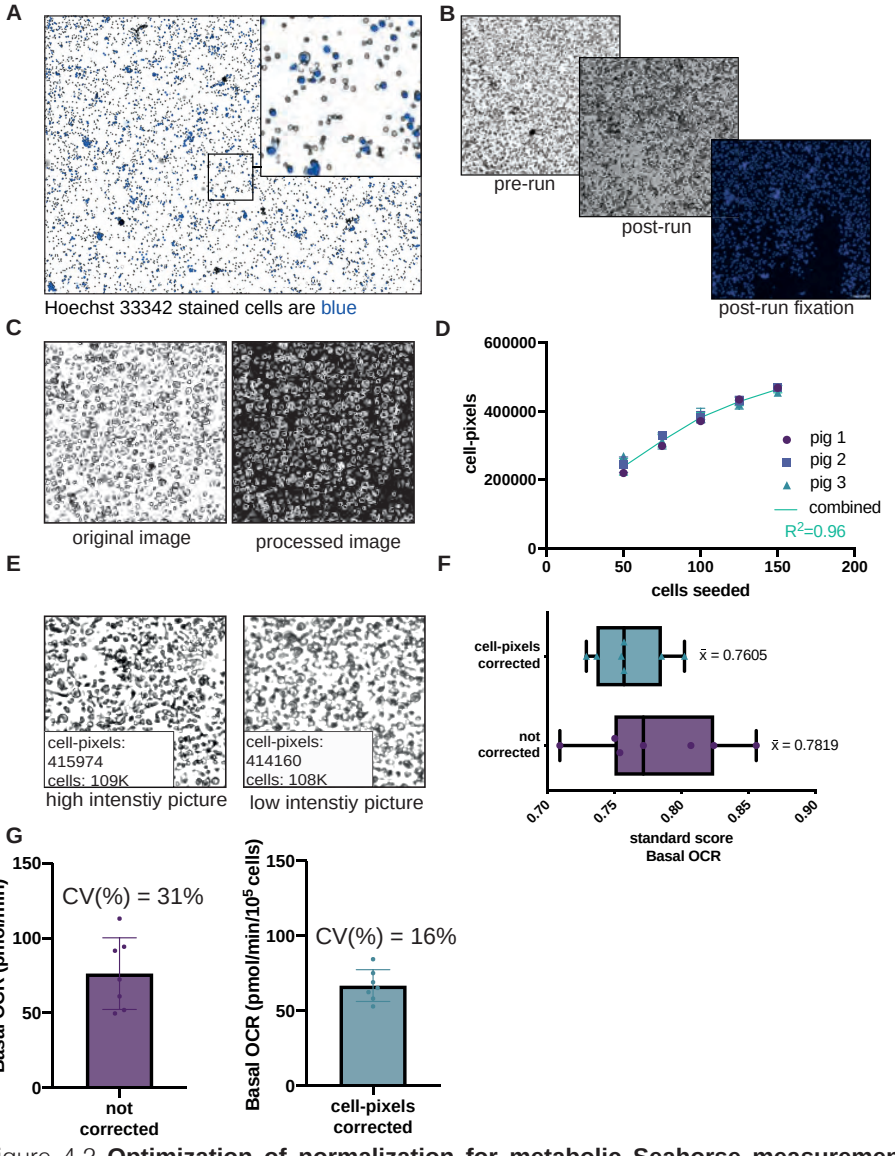



Figure 4.2 **Optimization of normalization for metabolic Seahorse measurements**. (**A**) Hoechst 33342 staining of isolated IECs. (**B**) Images depicting the same well pre-run, post-run and post-fixation and DAPI staining. (**C**) Pre-run brightfield images are processed for image analysis, yielding a white-object-on-black-background image. (**D**) Correlation of cell-pixels to the number of cells expected to be seeded for three pigs and their combined correlation coefficient (50,000-150,000 cells/well/n=4 wells per cell concentration). The combined calibration curve for the three pigs was used to convert cell-pixel values back into cell numbers. (**E**) Images with varying image intensities show similar cell-pixel values, with corresponding also similar cell number values. (**F**) Within-pig variation, as represented by standard score, of basal OCR measurement in a group of piglets corrected using our cell-pixel image analysis (n=7 pigs). (**G**) Coefficient of variation (%), as a measure of between-pig variation, of basal OCR measurement in the same group of piglets (n=7). Representative images are shown.

### *Optimization of metabolic analysis using isolated pig primary IECs*

After establishing the proper strategy to correct for cell number, we determined the number of cells needed to obtain a sufficient signal during a Seahorse run. We observed a strong correlation between the number of seeded cells and basal OCR (Fig. 4.3A) as well as basal ECAR when cells were seeded within a range of 50,000-150,000 cells per well (Fig. 4.3B), showing that this range was suitable for analysis. Spare respiratory capacity is an important metabolic parameter that often can distinguish metabolic states between cell populations. Therefore, we studied the optimal FCCP concentration needed for uncoupling. Both too low and too high concentrations of this drug will result in sub-optimal uncoupling. We noticed that at a low cell seeding density (25,000 cells per well) spare respiratory capacity (SRC) was not detectable. At 50,000 cells per well and at 120,000 cells per well a different concentration FCCP was observed to induce the highest SRC. For 50,000 cells per well 1.18 µM FCCP induced the highest increase in respiration, while this was 0.75 µM FCCP at 115,000 cells per well, although there were no significant differences between the different FCCP concentrations (Fig. 4.3C). To achieve experimental uniformity, we decided to continue our experiments using 100,000 cells per well and 1 µM FCCP.



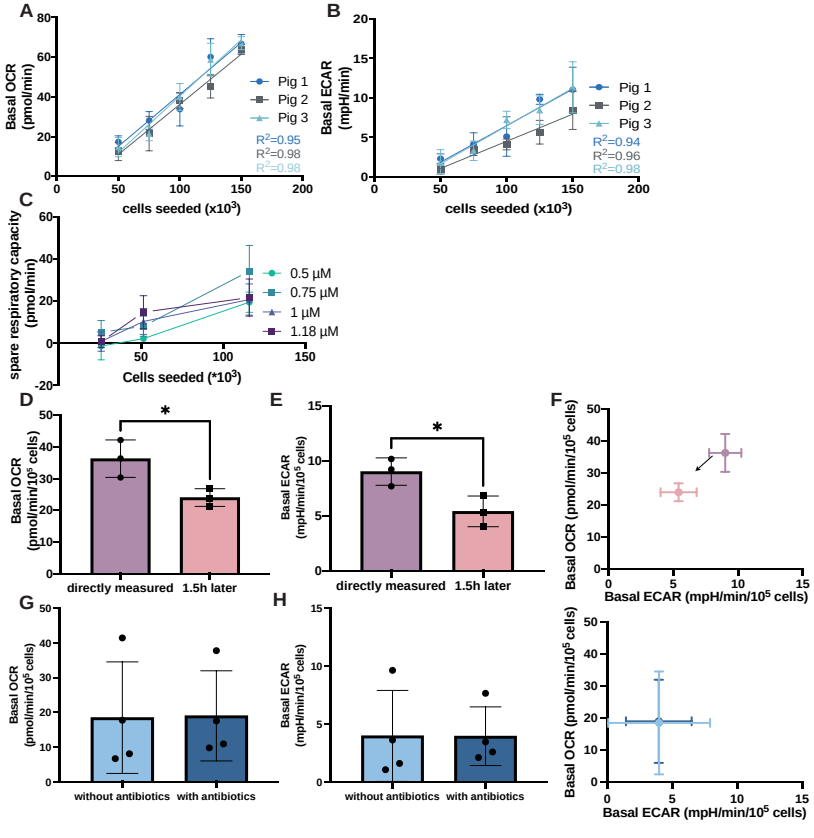


Figure 4.3 **Optimization of Seahorse metabolic flux analysis of isolated pig IECs.** (**A,B**) Comparison of the linear correlations between basal OCR and basal ECAR versus seeded cells, in a range of 50,000-150,000 cells (n=4 wells per concentration). (**C**) Optimization of FCCP concentration for different cell concentrations, ranging from 25,000-115,000 cells/well (n=3-5 wells per concentration, cells were isolated using the 'gentle' method). (**D-F**) Effect of leaving IECs in the plate for 1.5h on their OCR, ECAR and energetic phenotype (n=3 pigs). (**G-I**) Effect of addition of antibiotics to the isolation buffers and assay medium of IECs on their OCR, ECAR and energetic phenotype (n=4 pigs with and n=4 pigs without antibiotics). One-way ANOVA was performed to identify the optimal FCCP concentration, while student's t-tests were performed to compare OCR and ECAR after leaving cells in the plate for 1.5h and the effect of antibiotics, * indicates a p-value of ≤ 0.05.

Given that isolated primary IECs do not remain viable for extended periods of time, we evaluated the effect of delaying metabolic function analysis by leaving cells in the plate after isolation. We observed that leaving the cells in the plate for 1.5h prior to metabolic measurement resulted in a significant decrease of basal OCR as well as ECAR, compared to immediate measurement (Fig. 4.3D-E). In Figure 4.3F, when plotting OCR and ECAR in an energetic phenotype plot, it can be seen that a delay of 1.5h already resulted in movement of the energetic phenotype towards the less energetic quadrant. These results indicated that the metabolic function should be analyzed as soon as possible after isolation and at least at a defined time interval smaller than 1.5h.

Microbial contamination of the isolated cell population can disturb the metabolic measurements of isolated cells. Therefore, antibiotics have been used in enterocyte isolations to lower the risk of contamination. However, antibiotics have also been shown to alter cellular metabolism [31, 32]. We therefore investigated the effect of isolation of cells in the absence or presence of 1% (v/v) penicillin/streptomycin. We did not observe differences in basal OCR and ECAR of intestinal cells isolated in the presence of antibiotics as compared to absence of antibiotics (Fig. 4.3G-I). Even in absence of antibiotics, bacterial contamination is unlikely to contribute substantially to basal OCR and ECAR, because bacteria are much less dense than cells and it is thus likely that during spin-down at low g-force most of the bacteria are still in the supernatant fraction. In addition, the bacteria need to be attached to the bottom of the plate and be present in the small transient measurement chamber during XF analysis to contribute to the OCR and ECAR, which is likely not the case. To show that the OCR we measure is not due to bacterial contamination, we collected the supernatant after the final wash-step and added this to the plate. The gentle spin resulted in some cells being present in the supernatant fraction (Supplementary Figure S4.2A). However, OCR levels of supernatants were lower than the 20 pmol/min detection limit that is typically used as a cut-off for reliable Seahorse OCR measurements (Supplementary Figure S4.2B), whereas the cells had OCR levels ranging from 28-110 pmol/min (Supplementary Figure S4.2B). Thus, the OCR that is measured cannot originate from bacterial contamination, but is instead associated with the intestinal cellular fraction. Therefore, we decided not

to use antibiotics and practiced robust sterile working conditions and proper washing of cell isolates.

During XF analysis, Antimycin A and Rotenone are used to attain maximal inhibition of mitochondrial respiration by respectively blocking complex III and I of the electron transport chain. We have titrated AM/Rot for several cell lines in the past and always obtained maximal inhibition with the doses we use here. During analysis of IEC metabolic flux analysis, we observed an additional decreased in OCR following addition of 2DG (Fig. 4.4A). This additional decrease could be due to the presence of non-mitochondrial oxidases, that may respond physiologically to inhibition of glycolysis by injection of 2DG. Some of the known processes that can contribute to non-mitochondrial respiration are NADPH oxidases and even electron cycling at the plasma membrane [33], which can be dependent on glycolytic metabolism to generate NADPH or NADH substrates, and are therefore inhibited by 2DG.



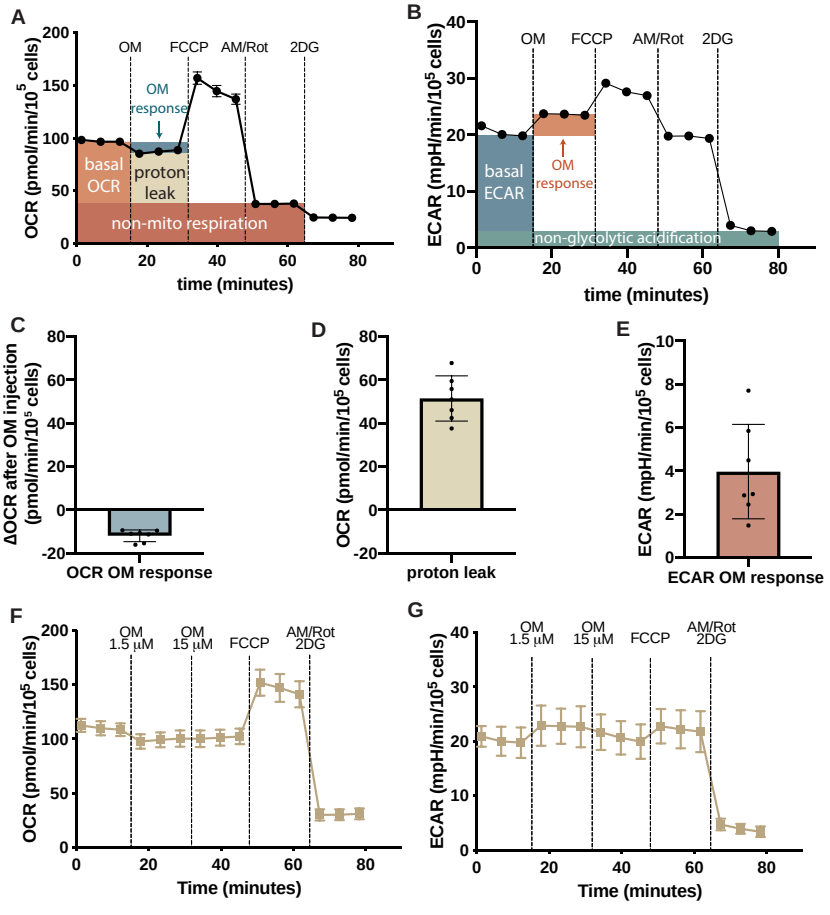



Figure 4.4 **Effect of Oligomycin on oxidative and glycolytic function of isolated IECs.** (**A,B**) The average time-course trace for OCR and ECAR of n=7 pigs, following

subsequent injections of Oligomycin, FCCP, Antimycin A, Rotenone and 2-DG, depicting which time-points are used for the calculations. (**C**) Change in OCR of isolated pig IECs after Oligomycin injection (n=7). (**D**) Calculated proton leak of isolated pig IECs (n=7). (**E**) Effect of Oligomycin on ECAR in pig IECs (n=7). (**F,G**) Time-course traces of OCR and ECAR, showing the effect of addition of a ten-fold higher Oligomycin concentration (average traces of n=6 pigs).

Apart from FCCP, Antimycin A and Rotenone, the drug Oligomycin, a blocker of $F_0F_1$-ATPase (Complex V) is used to assess mitochondrial respiration and glycolytic flux. Typically, Oligomycin lowers OCR and increases ECAR substantially in a response of the cells to compensate for loss of mitochondrial ATP production. However, the average OCR and ECAR time-course traces of primary IECs show only a slight effect of 1.5 µM OM (Fig. 4.4A-B). Indeed, we observed only a limited decrease in oxidative respiration, and a concomitant limited increase in glycolytic function (Fig. 4.4C-E). As a result, the isolated IECs seem to have a relatively high proton leak (Fig. 4.4D). We investigated whether the unresponsiveness to Oligomycin could be due to the enzyme used during the dissociation method, but found that IECs isolated using the non-enzymatic 'gentle' isolation method also did not respond to 1.5 µM Oligomycin (Supplementary Fig. S4.3A). To rule out that unresponsiveness to Oligomcyin could be due to bacterial contamination, we investigated the response to Oligomycin in the presence and absence of antibiotics. If bacterial contamination indeed contributes to the lack of response of Oligomycin, antibiotics treated cells should show an Oligomycin response. However, we did not observe a significant difference between Oligomycin inhibition of cells isolated with and without antibiotics (Supplementary Fig. S4.3B). Another explanation was that the Oligomycin is unable to properly block complex V activity, which has also been observed for other cell types [34-36]. Indeed, increasing the Oligomycin concentration 10-fold did not elicit an additional response in OCR and ECAR, indicating that Oligomycin is likely not able to properly inhibit complex V in isolated primary pig IECs (Fig. 4.4F-G).

## Discussion

Intestinal barrier function is decreased when energy production is impaired, which can occur through inhibition by drugs, redirection of blood flow during strenuous exercise or even upon bacterial infection [3, 6, 12]. Thus, IEC metabolism plays an important role in supporting intestinal barrier function. However, there is a lack of availability of good research protocols to study IEC metabolism. In this paper we successfully developed a method to isolate primary pig IECs and optimized the analysis of their energy metabolism. The method yielded a population of viable, single cells and can be easily adjusted to facilitate e.g. better mucus removal by altering the number of washing steps, without decreasing cell viability. In addition, the technique can be applied to a wide range of pig ages, sexes and breeds, as we have demonstrated by using a mix of ages, sexes and breeds throughout the optimization procedure.

We also adapted an algorithm to normalize the Seahorse XF data for the number of cells in the assay using brightfield images obtained before the run, which reduced the coefficient of variation of basal respiration between the pig IEC isolates. Furthermore, our data showed that delay of measurement after IEC isolation as well as the use of antibiotics negatively impact the metabolic function of the primary IECs. Measurement immediately after isolation is advised. Combined with optimization of cell densities, medium and chemical composition and concentrations, we have obtained a robust procedure to measure metabolic function of primary isolated pig IECs using the Seahorse Extracellular Flux analyzer.

The method described in this paper will improve our understanding on the role of mitochondrial function in intestinal health in pigs as well as humans. Applying this method can be especially interesting for studies with interventions that alter the luminal environment of the intestine, such as dietary interventions. Because such interventions can be performed *in vivo,* this enables all the complex interactions within the intestinal environment to take place. Currently, our knowledge on the impact of dietary interventions on intestinal epithelial cell metabolic function is limited. There are some reports of how dietary interventions, such as high fiber diets [19], can affect intestinal mitochondrial function, but these are mostly limited to oxygen consumption analysis. The use of Seahorse Extracellular Flux analysis facilitates simultaneous measurement of oxygen consumption and pH change, and thus allows for the simultaneous assessment of oxidative and glycolytic metabolism. Our isolation technique mainly results in the isolation of fully differentiated IECs, which have a metabolically active phenotype, with both high mitochondrial as well as glycolytic function [37, 38]. It is therefore important to include both these pathways in metabolic analysis of intestinal epithelial cells. It will be interesting to investigate whether interventions that reduce mitochondrial oxidative functions also reduce glycolytic function, or if glycolytic function can actually be increased to compensate for the loss of mitochondrial ATP production, as is sometimes observed (e.g. [39]).

In this study, we used primary IECs to investigate the metabolic consequence of antibiotics. In literature, antibiotics were found to inhibit mitochondrial function, increase production of reactive oxygen species, disrupt mitochondrial biogenesis and induce mitochondrial-mediated apoptosis in cancer cells [40, 41]. Specifically, antibiotics were found to reduce the expression of the respiratory chain complexes in mouse ileal tissue [42]. Also in cultured cell lines, including those derived from the intestine, antibiotics were found to decrease the function of mainly complex I and III of the electron transport chain, with a concomitant increase in reactive oxygen species (ROS) production [32, 43, 44]. Antibiotics are typically used routinely in cell culture, even when those cells are later used for metabolic assessments. In some IEC isolation procedures, antibiotics are

also added to the isolation medium, which is intended to reduce bacterial contamination [19, 45]. This, however, may not be ideal if subsequent metabolic parameters are analyzed. Since mitochondrial function impacts a wide array of cellular functions [14, 46], including barrier function [12], the use of antibiotics will likely also affect other processes. Therefore, we do not recommend the use of antibiotics during isolation of IECs, even though our results show no direct effect of antibiotics on cellular intestinal metabolism fluxes (Fig. 4.3G-I). In addition, the lack of effect of antibiotics on OCR and ECAR and the low OCR and ECAR measured in the supernatants (Supplementary Fig. S4.2) also indicates that bacterial contamination does not significantly contribute to the OCR and ECAR that we measure. Thus, antibiotic use may not be necessary to accurately measure metabolic function of isolated IECs. Inclusion of additional wash steps in the IEC isolation procedure may help to reduce bacterial contamination and permits the omission of antibiotics during the isolation procedure. We showed that more washing steps can be included, e.g. if the mucus layer is thick, without affecting cell viability and yield (Fig. 4.1E-F).

During analysis of isolated pig IEC metabolism we observed a relatively small change in OCR and ECAR upon oligomycin injection (Fig. 4.4). A possible reason for a lower responsiveness of our cells to Oligomycin may be the presence of active efflux drug transporter in these primary IECs [30]. Oligomycin analogues have been shown to inhibit P-glycoprotein mediated calcein-AM transport, indicating that these oligomycin analogues interact with these efflux transporters [47]. Furthermore, it has also been shown that the mitochondrial ATPase enzyme itself can be less sensitive to Oligomycin [34]. Mutations in complex V have been shown to block Oligomycin binding [35, 48], but without compromising proton translocation [35], indicating that unresponsiveness to Oligomycin is not always paralleled by uncoupled respiration. Also certain pig cells, boar sperm, were found to be insensitive to Oligomycin with regard to decreases in ATP production and oxygen consumption [36]. Interestingly, Oligomycin did result in decreased sperm motility, indicating that Oligomycin probably caused off-target effects in these cells [36]. With regard to IECs specifically, a inhibitory effect of Oligomycin has been reported for the pig-derived cell line IPEC-J2 [49]. However, these are cultured cells that are in long-time culture, and have possibly accumulated mutations which can affect their metabolic responses. In mice, Fan *et al.* [50] also observed a small increase in ECAR in response to Oligomycin and reported that the oxygen used for ATP production was half of that contributing to the proton leak in isolated mouse colonic crypts [50], which is in line with our observed Oligomycin responses.

In conclusion, we have successfully developed a method to isolate viable pig primary IECs and faithfully measure their extracellular glycolytic and mitochondrial flux. As a whole, the method we present may likely be a useful tool to be included for functional

analysis of the effects of various interventions on intestinal health, thus providing new insight into the complex interactions in the intestinal environment; the intestine that is simultaneously the gateway and the gatekeeper towards the rest of the body.

**Author contributions: A.F.B**. designed the research, performed the experiments, analysed the data, performed data interpretation and wrote the original draft; **W.J.J.G.** performed data interpretation and edited the final manuscript; **J.K.** performed data interpretation and edited the final manuscript; **V.C.J.d.B.** conceived and designed the research, analysed the data, performed data interpretation and edited the final manuscript.

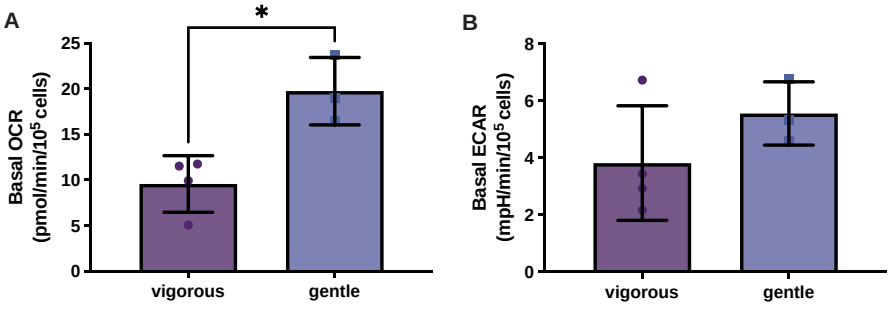
## References

1. Cao, S.T., et al., *Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets.* Journal of Animal Science, 2018. **96**(3): p. 1073-1083.
2. Li, Y., et al., *Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function, and alters neuro-immune mediator and receptor expression.* PLOS ONE, 2017. **12**(2): p. e0171617.
3. Ma, C., et al., *Citrobacter rodentium infection causes both mitochondrial dysfunction and intestinal epithelial barrier disruption in vivo: role of mitochondrial associated protein (Map).* Cellular Microbiology, 2006. **8**(10): p. 1669-1686.
4. Josephson, H., et al., *Pseudomonas aeruginosa N-3-Oxo-Dodecanoyl-Homoserine Lactone Impacts Mitochondrial Networks Morphology, Energetics, and Proteome in Host Cells.* Frontiers in Microbiology, 2020. **11**(1069).
5. Mahmud, T., et al., *Nonsteroidal antiinflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation.* Arthritis & Rheumatism, 1996. **39**(12): p. 1998-2003.
6. Somasundaram, S., et al., *Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat.* Alimentary Pharmacology & Therapeutics, 2000. **14**(5): p. 639-650.
7. Bjarnason, I. and K. Takeuchi, *Intestinal permeability in the pathogenesis of NSAID-induced enteropathy.* Journal of Gastroenterology, 2009. **44**(19): p. 23-29.
8. GBD 2019 Diseases and Injuries Collaborators *Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019.* The Lancet, 2020. **396**(10258): p. 1204-1222.
9. Van Breda, L.K., et al., *Pre- and post-weaning scours in southeastern Australia: A survey of 22 commercial pig herds and characterisation of Escherichia coli isolates.* PLOS ONE, 2017. **12**(3): p. e0172528.
10. Hong, T.T.T., et al., *Survey on the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam.* Tropical Animal Health and Production, 2006. **38**(5): p. 397-405.
11. DeBoer, M.D., et al., *Early childhood diarrhea and cardiometabolic risk factors in adulthood: the Institute of Nutrition of Central America and Panama Nutritional Supplementation Longitudinal Study.* Annals of Epidemiology, 2013. **23**(6): p. 314-320.
12. JanssenDuijghuijsen, L.M., et al., *Mitochondrial ATP Depletion Disrupts Caco-2 Monolayer Integrity and Internalizes Claudin 7.* Frontiers in Physiology, 2017. **8**: p. 794.
13. JanssenDuijghuijsen, L.M., et al., *Endurance Exercise Increases Intestinal Uptake of the Peanut Allergen Ara h 6 after Peanut Consumption in Humans.* Nutrients, 2017. **9**(1).
14. Rolfe, D.F. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals.* Physiological Reviews, 1997. **77**(3): p. 731-758.
15. Matarrese, P., et al., *Clostridium difficile Toxin B Causes Apoptosis in Epithelial Cells by Thrilling Mitochondria: involvement of ATP-sensitive mitochondrial potassium channels.* Journal of Biological Chemistry, 2007. **282**(12): p. 9029-9041.
16. Potten, C.S., C. Booth, and D.M. Pritchard, *The intestinal epithelial stem cell: the mucosal governor.* International Journal of Experimental Pathology, 1997. **78**(4): p. 219-243.
17. Grossmann, J., et al., *New isolation technique to study apoptosis in human intestinal epithelial cells.* The American Journal of Pathology, 1998. **153**(1): p. 53-62.
18. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-429.
19. Darcy-Vrillon, B., et al., *Metabolic Characteristics of Pig Colonocytes after Adaptation to a High Fiber Diet.* The Journal of Nutrition, 1993. **123**(2): p. 234-243.
20. Libiad, M., et al., *Hydrogen sulfide perturbs mitochondrial bioenergetics and*

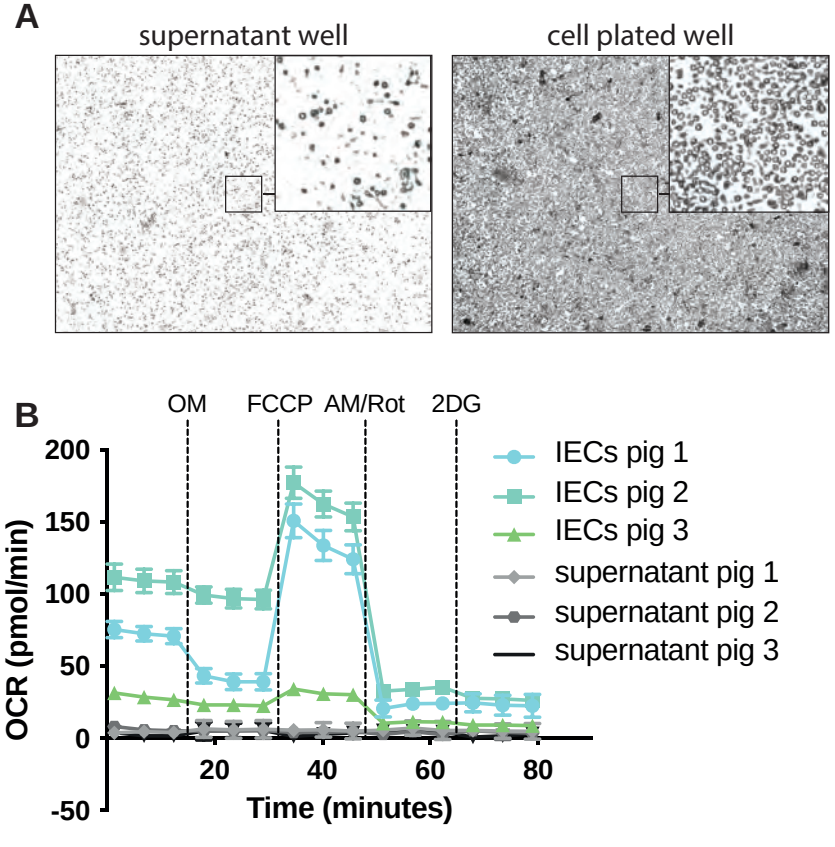
*triggers metabolic reprogramming in colon cells.* Journal of Biological Chemistry, 2019. **294**(32): p. 12077-12090.
21. FAO, *Meat market review: Overview of global meat market developments in 2020.* 2021.
22. Ziegler, A., L. Gonzalez, and A. Blikslager, *Large Animal Models: The Key to Translational Discovery in Digestive Disease Research.* Cellular and Molecular Gastroenterology and Hepatology, 2016. **2**(6): p. 716-724.
23. Zhang, Q., G. Widmer, and S. Tzipori, *A pig model of the human gastrointestinal tract.* Gut Microbes, 2013. **4**(3): p. 193-200.
24. Gonzalez, L.M., A.J. Moeser, and A.T. Blikslager, *Porcine models of digestive disease: the future of large animal translational research.* Translational Research: the Journal of Laboratory and Clinical Medicine, 2015. **166**(1): p. 12-27.
25. Heinritz, S.N., R. Mosenthin, and E. Weiss, *Use of pigs as a potential model for research into dietary modulation of the human gut microbiota.* Nutrition Research Reviews, 2013. **26**(2): p. 191-209.
26. Sciascia, Q., G. Da, and C.C. Metges, *REVIEW: The pig as a model for humans: Effects of nutritional factors on intestinal function and health.* Journal of Animal Science, 2016. **94**(suppl_3): p. 441-452.
27. Roediger, W.E. and S.C. Truelove, *Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies.* Gut, 1979. **20**(6): p. 484-488.
28. Pau, G., et al., *EBImage--an R package for image processing with applications to cellular phenotypes.* Bioinformatics (Oxford, England), 2010. **26**(7): p. 979-981.
29. Janssen, J.J.E., et al., *Novel standardized method for extracellular flux analysis of oxidative and glycolytic metabolism in peripheral blood mononuclear cells.* Scientific Reports, 2021. **11**(1): p. 1662-1662.
30. Takano, M., R. Yumoto, and T. Murakami, *Expression and function of efflux drug transporters in the intestine.* Pharmacology & Therapeutics, 2006. **109**(1-2): p. 137-161.
31. Wüst, R.C.I., R.H. Houtkooper, and J. Auwerx, *Confounding factors from inducible systems for spatiotemporal gene expression regulation.* Journal of Cell Biology, 2020. **219**(7).
32. Ryu, A.H., et al., *Use antibiotics in cell culture with caution: genome-wide identification of antibiotic-induced changes in gene expression and regulation.* Scientific Reports, 2017. **7**(1): p. 7533.
33. Herst, P.M., et al., *Cell surface oxygen consumption by mitochondrial gene knockout cells.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2004. **1656**(2): p. 79-87.
34. Pagliarani, A., S. Nesci, and V. Ventrella, *Modifiers of the oligomycin sensitivity of the mitochondrial F1F0-ATPase.* Mitochondrion, 2013. **13**(4): p. 312-319.
35. Breen, G.A., et al., *Mitochondrial DNA of two independent oligomycin-resistant Chinese hamster ovary cell lines contains a single nucleotide change in the ATPase 6 gene.* Journal of Biological Chemistry, 1986. **261**(25): p. 11680-11685.
36. Ramió-Lluch, L., et al., *Oligomycin A-induced inhibition of mitochondrial ATP-synthase activity suppresses boar sperm motility and in vitro capacitation achievement without modifying overall sperm energy levels.* Reproduction, Fertility and Development, 2014. **26**(6): p. 883-897.
37. Yang, H., et al., *Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis.* Scientific Reports, 2016. **6**: p. 31917.
38. Lindeboom, R.G., et al., *Integrative multi-omics analysis of intestinal organoid differentiation.* Molecular Systems Biology, 2018. **14**(6): p. e8227.
39. Yang, M., et al., *Inhibition of mitochondrial function by metformin increases glucose uptake, glycolysis and GDF-15 release from intestinal cells.* Scientific Reports, 2021. **11**(1): p. 2529.
40. Yadav, V. and P. Talwar, *Repositioning of fluoroquinolones from antibiotic to anti-cancer agents: An underestimated truth.* Biomedicine & Pharmacotherapy, 2019. **111**: p. 934-946.

41. Abad, E., et al., *Common Metabolic Pathways Implicated in Resistance to Chemotherapy Point to a Key Mitochondrial Role in Breast Cancer.* Molecular & Cellular Proteomics, 2019. **18**(2): p. 231-244.
42. Morgun, A., et al., *Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks.* Gut, 2015. **64**(11): p. 1732-1743.
43. Kalghatgi, S., et al., *Bactericidal Antibiotics Induce Mitochondrial Dysfunction and Oxidative Damage in Mammalian Cells.* Science Translational Medicine, 2013. **5**(192): p. 192ra85-192ra85.
44. Esner, M., et al., *Targeting cancer cells through antibiotics-induced mitochondrial dysfunction requires autophagy inhibition.* Cancer Letters, 2017. **384**: p. 60-69.
45. Chougule, P., et al., *Isolation and characterization of human primary enterocytes from small intestine using a novel method.* Scandinavian Journal of Gastroenterology, 2012. **47**(11): p. 1334-1343.
46. Berger, E., et al., *Mitochondrial function controls intestinal epithelial stemness and proliferation.* Nature Communications, 2016. **7**: p. 13171.
47. Salim, A.A., et al., *Oligomycins as inhibitors of K-Ras plasma membrane localisation.* Organic & Biomolecular Chemistry, 2016. **14**(2): p. 711-715.
48. Slott, E.F., Jr., R.O. Shade, and R.A. Lansman, *Sequence analysis of mitochondrial DNA in a mouse cell line resistant to chloramphenicol and oligomycin*. Molecular and Cellular Biology, 1983. **3**(10): p. 1694-1702.
49. Tan, B., et al., *The profiles of mitochondrial respiration and glycolysis using extracellular flux analysis in porcine enterocyte IPEC-J2*. Animal Nutrition, 2015. **1**(3): p. 239-243.
50. Fan, Y.-Y., et al., *A bioassay to measure energy metabolism in mouse colonic crypts, organoids, and sorted stem cells.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2015. **309**(1): p. G1-G9.

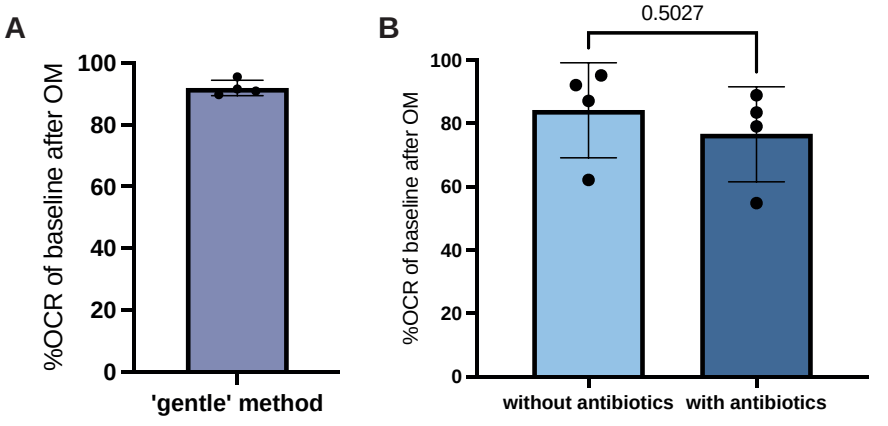
## Supplementary material




Supplementary Figure S4.1 **Comparison of metabolic function following non-enzymatic enterocyte isolation methods.** (**A**) Basal OCR and (**B**) basal ECAR of colonocytes isolated with the vigorous or gentle methods (n=4 for vigorous method and n=3 for gentle method). Student's t-tests were performed to compare metabolic parameters of the vigorous and gentle methods, * indicates a p-value of ≤ 0.05.




Supplementary Figure S4.2 **Comparison of respiration of supernatant and IECs to rule out bacterial contamination.** (**A**) representative image showing a well where only supernatant was plated ('supernatant well') and a well where cell suspension was plated ('cell plated well'). Some cells are present in the supernatant plated well. (**B**) OCR time-course graph of supernatant compared to IEC plated wells.




Supplementary Figure S4.3 **Effect of Oligomycin IECs isolated using non-enzyme method and in the presence of antibiotics.** (**A**) OCR plotted as a % of basal OCR after Oligomycin (OM) injection for cells isolated using the non-enzymatic 'gentle' method. (**B**) OCR plotted as a % of basal OCR after Oligomycin injection for cells isolated in the absence (without) or presence (with) antibiotics. Student's t-tests were performed to compare OCR response in the presence or absence of antibiotics, *p*-value is shown in the graph.

## Supplementary protocol for pig primary intestinal epithelial cells isolation and metabolic analysis

### *Isolation procedure*

1) Prepare in advance:
   Modified Krebs Henseleit buffer (KHB; #K3753, Sigma Aldrich), according to manufacturer's protocol and set to pH 7.4. Can be stored at 4 °C for 2 weeks.
   a) Modified KHB buffer (#K3753, Sigma Aldrich), containing 2.5 g/L bovine serum albumin (BSA: #A7906, Sigma-Aldrich) and set to pH 7.4. Prepare on the day prior to the assay and store at 4 °C until use.
   b) $Ca^{2+}$-free KHB (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 $KH_2PO_4$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) with 20 mM Ethylenediaminetetra-aceticacetic acid (EDTA) and 10 mM Dithiothreitol (DTT) and set to pH 7.4. Preferably make fresh on the day of assay, but no more than one day ahead.
   c) $Ca^{2+}$-free KHB (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 $KH_2PO_4$, 10 mM HEPES) with 10 mM DTT and 400 U/mL hyalurondisase type IV (#3884, Sigma-Aldrich) and set to pH 7.4. Preferably make fresh on the day of assay, but no more than one day ahead.
   d) Pre-coat Seahorse XF 96 well assay plate with CellTak (#354240, Corning, New York, USA) according to manufacturer's protocol. Seal the places and store at 4 °C until use, but no more than one week in advance. On the morning of the assay, plate at room temperature (RT).
   e) Oxygenate the buffers prior to use with pure $O_2$.
2) Kill the animal and remove the intestine.
3) Separate the intestine and determine where the desired segment of intestine is located. For us, this was at 80% of the colon (from proximal).
4) Remove a ± 20 cm tissue piece and place in oxygenated modified KHB buffer containing 2.5 g/L BSA.
5) Once all the intestines are removed, flush the intestines using modified KHB buffer. We recommend to include no more than 8 animals per isolation round. This allows for fast isolation and fast metabolic analysis, in addition to allowing for sufficient replicates on the Seahorse XF assay plate.
6) Invert the intestines, using for example a long crochet hook. Make sure to remove any mesentery or fat tissue left on the segment, to allow for quick reversion in subsequent steps.
7) Clamp off the ends of the segments and fill the intestines with modified KHB buffer. Use a 50 mL syringe and standardize the amount of buffer between samples. With a 12-week-old pig we used 50 mL for a 20 cm long segment of the colon. The

intestine should be somewhat distended to increase surface contact between the buffer and the intestinal mucosa.

8) Place the inverted segment in an Erlenmeyer with screwcap, containing about 150 mL $Ca^{2+}$-free KHB with 20 mM EDTA, 10 mM DTT and 2.5 g/L BSA.
9) Incubate for 20 min at 37 °C in a shaking water bath.
10) Pour away the buffer and refill the Erlenmeyer with another 150 mL of new $Ca^{2+}$-free KHB with 20 mM EDTA, 10 mM DTT and 2.5 g/L BSA.
11) Incubate for 20 min at 37 °C in a shaking water bath
12) Revert the intestines and fill them with $Ca^{2+}$-free KHB with 10 mM DTT and 400 U/mL hyaluronidase type IV (#3884, Sigma-Aldrich). Use about the same amount as previously, making sure the intestine is slightly distended to increase surface contact area between buffer and mucosa.
13) Place the intestinal segments in an Erlenmeyer containing 150 mL modified KHB and incubate for 15 min at 37 °C in a shaking water bath.
14) Gently massage the intestines for about 15 seconds and collect the contents in a conical 50 mL falcon tube.
15) Using a 70 µm cell strainer, filter the cells and place in a new 50 mL falcon tube
16) Spin the cells down for 5 min at 400 *x g*.
17) Discard the supernatant, pour about 10 mL modified KHB buffer containing 2.5 g/L BSA and shake the pellet loose.
18) Spin the cells down for 5 min at 400 *x g*.
19) Discard the supernatant, wash twice with 10 mL pH buffered Seahorse XF assay medium (#103575-100, Agilent Technologies) supplemented with 10 mM pH balanced glucose (#103577-100, Agilent Technologies), 2 mM pH balanced glutamine (#103579-100, Agilent Technologies), and 1 mM pH balanced pyruvate (#103578-100, Agilent Technologies).
20) Count the cells using the Bürker chamber.
21) Simultaneously measure viability with the K4 (Nexcelom biosciences) using ViaStain (#CS2-0106, Nexcelom Bioscience).
22) Prepare cell suspensions, seeding cells at 100,000 cells/well in 80 µL in a CellTak coated Seahorse XF 96 well assay plate.
23) Leave the cells to settle at RT for 10 minutes.
24) Spin down for 1 min at 200 G with zero break.

### *Imaging*

25) Take Brightfield images of the inner probe area of each well in the XF96 cell plates using a 37 °C equilibrated Cytation 1 Cell Imaging Multi-Mode Reader (BioTek, Winooski, Vermont, USA) with a 4x objective. Set the LED intensity to 5 and integration time to 80 milliseconds. Use the 'User trained autofocus' option to obtain

the best image quality.

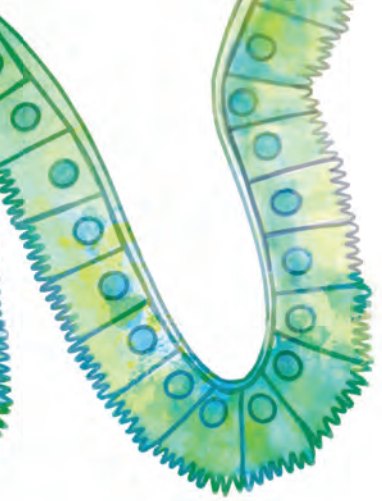
26) After images are obtained, add another 100 µL of supplemented pH buffered Seahorse XF assay medium and place the plate into a non-$CO_2$ 37 °C incubator.

### *Seahorse XF assay*

27) On the day prior to the assay, equilibrate the Seahorse XF analyzer to 37 °C
28) Hydrate a cartridge overnight in a non-$CO_2$ 37 °C incubator, using 200 µL sterilized milli-Q water (MQ) per well.
29) Replate MQ for XF Calibrant no less than 1 hour prior to the assay and incubate in a non-$CO_2$ 37 °C incubator.
30) Prepare 10x injections in supplemented pH buffered Seahorse XF assay medium and pipet into the Seahorse cartridge:

    Injection A (20 µL): 15 µM Oligomycin (#O4875, Sigma-Aldrich)
    Injection B (22 µL): 10 µM carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone (FCCP; #C2920, Sigma-Aldrich
    Injection C (25 µL): 12.5 µM Rotenone (#R8875, Sigma-Aldrich) and 25 µM Antimycin A (#A8674, Sigma-Aldrich)
    Injection D (28 µL): 500 mM 2-deoxyglucose (2-DG; #D8375, Sigma -Aldrich)

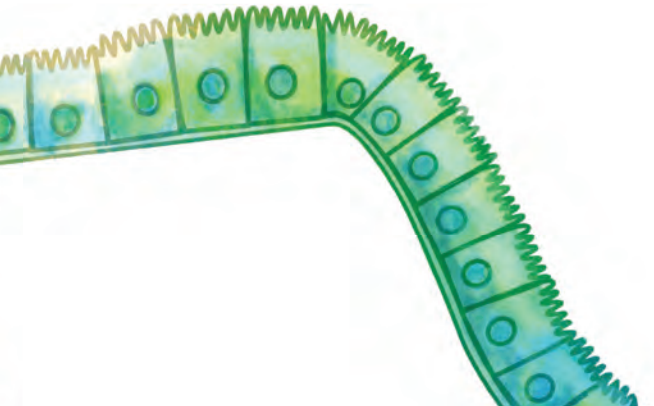
31) Run Seahorse protocol. Include an equilibration step, and measure 3 times for 3 minutes, with 2 minutes of mixing between each measurement. Then, inject port A, and mix and measure another 3 times. Subsequently inject all other ports, always measuring and mixing 3 times prior to the net injection.
32) Optional: After the Seahorse XF assay run, image the plate in a similar manner as previously described, using the “user trained auto-focus” method to obtain the best quality images. This step can be included to check the amount of cell detachment.



# Chapter 5

## Functional Metabolic Capacity of Pig Colonocytes is Differentially Modulated by Fermentable Fibre and Poorly Digestible Protein

Anna F. Bekebrede
Lonneke Noorman
Jaap Keijer
Vincent C.J. de Boer
Walter J.J. Gerrits



***Animal 2022;***
doi:10.1016/j.animal.2022.100625

## Abstract

The intestine is a highly metabolic organ that relies on energy production within the intestinal cells to sustain its functions. In the colon, intestinal cell metabolic function could be affected both positively and negatively by microbiota derived metabolites. Protein fermentation metabolites are known to negatively impact intestinal metabolic function, while fibre fermentation metabolites are generally thought beneficial. We aimed to investigate whether proteins of different digestibility in the absence and presence of fibres impact energy metabolism of colonocytes, with potentially adverse health effects. We fed 32, 9-week-old boars one of four experimental diets for 14 days in a 2x2 factorial arrangement. Whey and collagen were added as a well and a poorly digestible protein source, respectively, and fibre was either included at 5 or 23%. We examined the effects of the diets on flux of fermentation metabolites in colon digesta and assessed the impact of the diets on functional metabolic capacity of isolated colonocytes using the Seahorse XF analyzer. Feeding the poorly digestible protein source collagen indeed increased nitrogen flow into the colon by 135% compared to the well-digestible whey-protein source. Feeding high fermentable fibre increased colonic fluxes of both fibre-derived metabolites acetate, propionate, butyrate and caproate, but also increased flux of protein-derived metabolites ammonia, isobutyrate, isovalerate, valerate and isocaproate. To analyse the impact of the diets and the induced differential metabolic composition of the intestinal lumen on functional metabolic capacity of the intestine, we used extracellular flux analysis on freshly isolated pig colonocytes. Colonocytes isolated from high fermentable fibre fed pigs in the whey-protein diet, but not in the collagen-protein diet had a reduced mitochondrial capacity, as indicated by a 35% reduction of maximal respiration (interaction $p<0.05$) and a 20% reduction of spare respiratory capacity (interaction $p<0.05$). Colonocytes from high fermentable fibre fed pigs had a 37% decreased glycolytic activity as compared to the colonocytes isolated from the low fermentable fibre fed pigs ($p<0.001$). This indicated that different diets, and in particular different protein sources and fibre levels differentially affect colonic epithelial cell metabolism in pigs. Especially high-fermentable fibre lowered both colonocyte mitochondrial and glycolytic metabolism, indicating that high fibre intake in pigs could lower colonocyte energetic status. Because metabolic capacity of colonocytes is tightly linked with their functionality, assessment of intestinal cell metabolic capacity may be a valuable tool for future research.

**Keywords:** diet, fermentation, metabolism, short-chain fatty acids, colon

## Implications

Sufficient energy production in intestinal epithelial cells (IECs) is important to support intestinal functions. In this study, we investigated whether dietary interventions, designed to impact colonic microbial fermentation, could alter metabolic function of colonocytes. To do this, we isolated pig colonocytes and directly measured their metabolic capacity. We found that pig colonocyte metabolic capacity was indeed modulated by two-week-long dietary interventions, and that protein source and fibre level differentially affected metabolic capacity. These results not only give us new insights into how diets can impact intestinal health, but also show that assessment of IEC metabolic function may be a sensitive tool to investigate the effects of diets on the intestine.

## Introduction

The intestine is a major metabolic organ, which facilitates the selective entry of nutrients into the body, while simultaneously providing a barrier against pathogens. The intestine's absorptive and barrier functions are actualised by intestinal epithelial cells (IECs), a type of cell that is continuously generated from stem cells in the crypts, which migrate to the villus top as they mature, in a 5-7 day cycle [1]. IECs form a physical barrier to protect the host, comprising a tight layer with actively maintained cell-cell junctions and specialised cells that secrete mucus to create a protective layer on top of the IECs [2]. To perform its complex role, the intestine is divided into various regions with distinct functions, which together comprise an enormous surface area, resulting in a large, dynamic and active tissue, with high energy demand. The specific energy demand for the intestinal processing of nutrients is illustrated by a 25% increase in resting metabolic rate for humans [3], and 15-20% for growing pigs [4]. This increase reflects a doubling of intestinal energy use, which is required to support digestion, absorption and metabolism of sugars, proteins and lipids. In addition, adequate cellular energy production was shown to be important for barrier integrity, and thus to maintain intestinal health [5-7]. Disruption of intestinal energy production, for example due to the absence of energy substrates or because of drug use, was found to increase intestinal apoptosis and permeability [8-10]. As such, intestinal energy metabolism emerges as an area of interest for the overall health of organisms.

For their metabolism, IECs use dietary energy substrates, of which the availability differs across the intestinal tract. Rapidly digestible carbohydrates and proteins are metabolised and absorbed in the small intestine [11]. Complex carbohydrates like fibres serve as substates for microbiota, mainly in the colon [11, 12]. Microbial fibre fermentation results in, among others, the production of short-chain fatty acids (SCFAs). One of the SCFAs, butyrate, is regarded as a preferred energy substrate of colonocytes [13]. Similar to carbohydrates, undigested proteins are subject to fermentation in the

colon. Whereas colonic proteins can be used for microbial protein synthesis, they can also be used as microbial energy sources, leading to the production of a wide variety of protein fermentation metabolites [14]. Protein fermentation metabolites are thought to contribute to intestinal dysfunction by negatively impacting intestinal epithelial cell function, such as by disrupting colonocyte metabolic function [12, 15]. A good examples of a harmful protein fermentation metabolite is ammonia. Ammonia was shown to decrease the ability of colonocytes to oxidize butyrate, their primary energy source [13, 16]. In this study, we addressed whether functional colonocyte metabolism is challenged by protein fermentation metabolites. Most studies have not measured colonocyte energy metabolism directly, but we are able to do this with a recently optimised protocol to assess energy metabolism of isolated IECs, measuring their oxidative and glycolytic fluxes [17]. This allows us to study the effects of feeds on colonic energy metabolism.

Given the upcoming feed transition to increased use of high-fibre co-products that are also rich in moderately digestible proteins [18], we were interested in better understanding the interactions between protein and fibre in the colon. In general, the addition of fermentable fibre into pig feeds is thought to improve animal health through multiple mechanisms [19, 20]. Firstly, it provides butyrate to colonocytes. Secondly, it could reduce the production of metabolites originating from the fermentation of undigested proteins [21, 22]. Thirdly, fibre fermentation metabolites, in particular butyrate, are known for their beneficial effects, such as by improving barrier function [5], and acting as an anti-inflammatory [23] and anti-tumorigenic agents [24, 25].



In the current study, we investigated whether proteins of different digestibility in the absence and presence of fibres impact colonic energy metabolism, thereby potentially affecting pig health. To investigate this, we fed pigs diets in a 2x2 factorial arrangement, with protein source and fibre level as factors. Protein sources were either highly (whey) or poorly digestible (collagen), and for each protein source fibre was included at either 5% or 23%, where starch was exchanged for soybean- and oat hulls in the high-fibre diets. We examined the effects of the diets on colonic fermentation metabolites and assessed the impact of the diets on functional metabolic capacity of IECs.

## Methods

### *Animals*

A total of 32 boars, 9 weeks of age, 22.8 kg BW (SEM=0.5) (TN70; Large White x Norwegian Landrace, TopigsNorsvin, Vught, The Netherlands) originating from a specific pathogen-free breeding farm were transported to CARUS, the research facility of Wageningen University, the Netherlands. Pigs were group-housed with four pigs per

pen, in 5 $m^2$ partly slatted pens with thick rubber mats for lying comfort. Upon arrival, pigs were weighed and allocated to the pens based on their initial body weight, to minimize variation in BW between pens and treatments. Temperature was kept between 22 and 25°C with a humidity of 65%, and lights and radio were switched on between 7:00 and 19:00h and lights were dimmed to 5% and radio was off from 19:00 till 7:00h. The first two days, pigs were gradually switched to the experimental diets, which they received throughout the next two weeks. Health and welfare were assessed visually twice a day during feeding, and faecal consistency score was assessed (score of 1-5, 1 = liquid diarrhea, 5 = hard faeces).

The feeds were produced in meal form and produced by Research Diet Services (RDS BV, Wijk bij Duurstede, The Netherlands) in a single batch. Collagen protein and the amino acids L-Phenylalanine and L-Leucine were mixed into the feeds at the research facility. Diets contained different protein sources and fibre levels in a 2x2 factorial design (Table 5.1). The experimental diets contained either whey as a highly digestible protein source or collagen as a poorly digestible protein source. For each protein source, there was a low fibre diet that contained only 5% dietary fibre, and a high fibre diet that contained 23% dietary fibre. To create the high fibre diets, maize starch was exchanged for soybean hulls and oat hulls. Synthetic amino acids were added to the collagen diets to meet the minimal requirements for essential amino acids. Pigs were fed twice a day at 8:00h and 16:00h, and received 50% of the daily feed allowance per meal. Feed intake was set at 1.4 times the maintenance energy requirement using the formula from CVB (NE maintenance = 750 x $BW^{0.60}$, [26]), to ensure that all pigs consumed the same amount of feed. To determine nutrient digestibility and the mean intestinal retention time of the diets, $TiO_2$ was included at 2 g/kg as an indigestible marker. Based on previous studies, it was modelled that feeding pigs every 6 hours for a period of at least 36h could provide a constant flow of digesta through the colon [27-29]. Therefore, starting 36 hours before slaughter, pigs were fed every 6 hours with 25% of the daily feed allowance, and constant flux of digesta through the colon was assumed for all diets in this study. We verified that each pig consumed its own portion of feed by visual observation. Water was available ad libitum throughout the entire study period. At the end of the study period, pigs were sedated using intramuscular injection of Zoletil+Xylazine (5:2 ratio, 1 mg/10 kg BW), and euthanised by lethal injection with pentobarbital (24 mg/kg BW).

Table 5.1 **Ingredient and calculated nutrient composition of the four experimental diets fed to pigs for two weeks, expressed on an as fed basis**[1].

| | Whey | | Collagen | |
|---|---|---|---|---|
| Components | Low Fibre | High Fibre | Low Fibre | High Fibre |
| *Ingredient composition (%)* | | | | |
| Whey Protein isolate | 21 | 21 | - | - |
| Bovine Collagen | - | - | 18.1 | 18.1 |
| Soy Protein Concentrate | 3.1 | 0 | 3.1 | 0 |
| L-lysine HCl | 0.05 | - | 0.64 | 0.58 |
| DL-Methionine | - | - | 0.5 | 0.5 |
| L-threonine | - | - | 0.4 | 0.4 |
| L-tryptophan | - | - | 0.2 | 0.2 |
| L-isoleucine | - | - | 0.3 | 0.3 |
| L-Histidine | - | - | 0.3 | 0.3 |
| L-Phenylalanine | - | - | 0.3 | 0.3 |
| L-Leucine | - | - | 0.6 | 0.6 |
| L-Valine | - | - | 0.3 | 0.3 |
| Sucrose | 10 | 10 | 10 | 10 |
| Wheat gluten meal | 0.8 | - | 0.8 | - |
| Maize starch | 53.2 | 29 | 52.9 | 28.9 |
| Soybean Hulls | - | 15.5 | - | 15.5 |
| Oat Hulls | - | 13.2 | - | 13.2 |
| Cellulose | 3 | 3 | 3 | 3 |
| Rape seed oil | 3 | 3 | 3 | 3 |
| Premix (vitamins + minerals)[2] | 1 | 1 | 1 | 1 |
| MgO | 0.2 | 0.1 | 0.2 | 0.1 |
| CaCO3 | 1.5 | 1.2 | 1.5 | 1.2 |
| $Ca(H_2PO_4)_2$ | 1.5 | 1.5 | 1.6 | 1.6 |
| $KCO_3$ | 0.3 | - | 0.5 | - |
| KCl | 0.3 | 0.3 | 0.1 | 0.1 |
| $NaHCO_3$ | 0.5 | 0.5 | 0.5 | 0.5 |

| | | | | |
|---|---|---|---|---|
| $TiO_2$ | 0.2 | 0.2 | 0.2 | 0.2 |
| Total | 100 | 100 | 100 | 100 |
| *Nutrient composition (g/kg)* | | | | |
| Dry Matter | 920 | 921 | 924 | 925 |
| Crude Protein | 220 | 220 | 220 | 220 |
| Digestible Crude Protein | 194 | 179 | 177 | 162 |
| Crude Fat | 36 | 40 | 34 | 38 |
| Crude Ash | 40 | 49 | 42 | 49 |
| Starch | 513 | 303 | 510 | 302 |
| Sugars | 113 | 111 | 112 | 109 |
| Dietary Fibre[3] | 47.1 | 231.5 | 47 | 231.3 |
| NE (MJ/kg) | 11.6 | 9.5 | 11.4 | 9.4 |

Abbreviations: NE = net energy.
[1] Nutrient composition was calculated based on ingredient composition and table values for the composition of the ingredients [30].
[2] Supplied per kilogram of feed: retinyl acetate, 10,000 IU; cholecalciferol, 2,000 IU; DL-a-tocopherol, 40 mg: menadione, 1.5 mg; thiamine 1.0 mg; riboflavin, 4 mg; pyridoxin-HCl, 1.5 mg; cyanocobalamin, 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; Choline chloride, 150 mg; Folic acid, 0.4 mg; Biotin, 0.05 mg; iron(II)sulphate monohydrate, 331 mg; copper(II)sulphate pentahydrate, 80 mg; manganese(II)oxide, 49 mg; zinc sulphate monohydrate, 194 mg, potassium iodate, 1 mg; sodium selenite, 0.56 mg.
[3]Dietary fibre was calculated as follows: organic matter – crude protein – crude fat – starch – sugar.

### *Enterocyte isolations*

Colonocytes were isolated according to [17]. Briefly, following excision from the abdominal cavity, the entire intestine was separated from the mesentery, and the colon was located. At 66% of the length of the colon, a clamp was placed to delineate the distal part of the colon. Then, a piece of 20 cm proximal of the clamp was removed and placed in aerated modified Krebs Henseleit Buffer (KHB) containing 5 mM glucose (#K3753, Sigma Aldrich, hereafter referred to as modified-KHB), supplemented with 2.5 g/L bovine serum albumin (BSA, #A7906, Sigma-Aldrich). To ensure that isolated cells maintained good viability, cell isolations for each slaughter-day were performed simultaneously. Only eight pigs were slaughtered per day to ensure that each pig had at least eight replicates for the metabolic analysis, which is performed on a 96-well plate. When all samples were obtained, intestines were washed thoroughly with modified KHB and inverted. Using dialysis clamps (#Z371092, Sigma Aldrich), a sac was created by filling the inverted intestine with modified-KHB, which was then placed in 118 mM

NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 $KH_2PO_4$, 10 mM HEPES ($Ca^{2+}$-free KHB, pH 7.4), supplemented with 10 mM Dithiothreitol (DTT), 20 mM Ethylenediaminetetraacetic acid (EDTA) and 2.5 g/L fatty-acid free BSA (#3117057001, Sigma-Aldrich) to wash the mucus away. The intestines were incubated in this wash buffer for 20 minutes in an oscillating water bath at 37 °C. Then, the buffer was discarded and the washing step was repeated for another 20 minutes, using the same $Ca^{2+}$-free KHB buffer supplemented with 10 mM DTT, 20 mM EDTA and 2.5 g/L fatty-acid free BSA. The intestinal sacs were then reverted and filled with $Ca^{2+}$-free KHB buffer containing, 10 mM DTT, 400 U/mL hyaluronidase type IV (#3884, Sigma-Aldrich) and 2.5 g/L fatty acid-free BSA. After a fifteen-minute incubation, the intestinal sacs were gently massaged for 15 seconds, after which the content of the sacs was collected in 50 mL tubes. The cells were then passed through a 70 µM cellulose filter top remove debris and large tissue pieces. The collected cells were washed twice using modified-KHB containing 2.5 g/L BSA and once with pH balanced XF DMEM assay medium supplemented with pH balanced 10 mM XF glucose, 2 mM XF glutamine and 1 mM XF pyruvate. Cells were spun down at 400 *x g* for 5 min. Then, cells were counted using the Bürker chamber, and cell viability was assessed by staining cells with ViaStain (#CS2-0106, Nexcelom Bioscience) and imaging them using the Cellometer K4 (Nexcelom Bioscience).

### *Metabolic measurements*



Metabolic function of the isolated colonocytes was measured in real-time using the XFe96 Seahorse Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, USA). Isolated colonocytes were plated into Seahorse 96-well plates at 100,000 cells/well. Because isolated colonocytes are non-adherent cells, the plates were pre-coated with CellTak (#354240, Corning, New York, USA) according to the manufacturer's protocol to ensure that cells adhere to the bottom of the plate throughout the measurement. After calibration and initialization, basal metabolic function of the plated colonocytes was measured. Then, 1 µM FCCP (#C2920, Sigma-Aldrich), 2.5 µM Antimycin A (#A8674, Sigma-Aldrich) and 1.25 µM Rotenone (#R8875, Sigma-Aldrich) and 2-deoxyglucose (2-DG; #D8375, Sigma-Aldrich) were consecutively injected into the wells. The responses of the colonocytes to the different drug injections can then be used to calculate non-mitochondrial respiration, basal oxygen consumption rate (basal OCR), maximal respiration and spare respiratory capacity (SRC) from the oxygen consumption rate. Extracellular acidification rate (ECAR) is measured simultaneously and can be corrected for the contribution of mitochondrial $CO_2$-production using the buffer capacity factor of the medium to approximate lactate flux, which is expressed as the glycolytic proton efflux rate (glycoPER).

### *Normalisation*

Seahorse XF assays were normalised using an adaptation of a previously in-house generated R-script, that uses the EBImage package available for Bioconductor [31, 32]. The normalization method was validated and optimised together with the enterocyte isolation protocol [17]. Brightfield images were obtained before the Seahorse XF assay, using the Cytation 1 (BioTek Instruments, Inc., Winooski, VT, USA), with a 4x objective. The images were processed as follows: a Gaussian blur low-pass filter was applied to generate a background image, followed by subtraction of the background image from the original. The background-corrected image was then inverted to generate a "white-objects-on-black-background image". This image was subsequently cropped by 5% to remove potential noise from the XF assay plate moulded stops, that are present on the plates to prevent the sensors from disrupting the cell monolayer. Images were then analysed to calculate pixel intensity values for all the pixels in the image. All the pixels with an intensity >1 was counted as representing the presence of a cell, and we refer to these as "cell-pixels". For conversion of cell-pixels back to cell numbers, an external calibration curve was generated, using a second-order polynomial fit analysis on data obtained from three individual pig colonocyte standard curves. These cell numbers were subsequently used for normalization of the Seahorse XF assays.

### *Citrate synthase activity*

To investigate mitochondrial density in colonic mucosa we used the Citrate Synthase (CS) activity assay kit according to the manufacturer's instructions (#CS0720, Sigma-Aldrich). In short, intestinal scrapings were first crushed with a pestle in liquid nitrogen. The crushed scrapings were then added to lysis buffer containing 50 mM Tris-HCl pH 7.4 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease and deacetylase inhibitors (2 µM trichostatin A, 10 mM nicotinamide and one tablet per 10 mL of both protease and phosphatase inhibitor cocktail (Roche)). Samples were first placed in a thermoshaker at maximum speed for 5 minutes in a cold room, and were then submitted to a freeze-thaw cycle (-80°C freezing, 4°C thawing), after which they were sonicated on ice for 10 pulses of 2 seconds at 40 kHz. The sonicated samples were then centrifuged at 10,000 *x g* and 4°C to remove cell debris. The protein content was determined using the DC protein assay (Biorad) and protein content was equalised across all samples using 1x citrate synthase assay buffer. A baseline measurement was first taken, containing all relevant reagents and protein, but without oxaloacetate. Then, oxaloacetate was added and changes in absorbance at 412 nm were monitored for 30 minutes with a measurement interval of 24 seconds, using a Synergy HT Multi-detection microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The CS activity was then calculated using the change in absorbance in the linear section of the

reaction according to the manufacturer's protocol, accounting for the sample volume, the pathway length and the protein input. CS activity is expressed as units (µmol/ml/min) per mg protein.

### *Digesta sample analysis*

Intestinal digesta was collected from the distal colon by clamping off the colon between 66% and 80%, and collecting the digesta within the intestinal segment. Dry matter content of feed and freeze dried matter content of digesta were determined according to the International Organization for Standardization (ISO) 6496 [33]. Nitrogen in digesta was determined by using the Kjeldahl method according to ISO 5893 [34]. $TiO_2$ was analysed in freeze-dried digesta samples according to [35]. To determine short chain fatty acid (SCFA) concentrations in the samples, distal colon digesta samples were homogenized, and subsamples were acidified using an equal volume of Ortho-phosphoric acid (0.1M, #100573, Merck), and were then stored at -20 °C until use. For the analysis, samples were thawed and centrifuged for 10 min at 4816 *x g* to obtain the liquid fraction. To this liquid fraction, 15.89 mM 2-methyl valeric acid was added as internal standard, and the mixture was centrifuged for 5 min at 20871 *x g* to remove any remaining debris. The clear supernatant (0.5 µL) was injected onto a gas chromatograph (Trace 1300, Thermo Fisher Scientific, Waltham, MA, USA) with a split/splitless injector operated in split mode (split ratio 1:36) and a temperature of 260 °C, using the HP-FFAP column (30 m x 0.32 mm x 0.25 µm, # 19091F-413, Agilent J&W, Santa Clara, CA, USA) and hydrogen as mobile phase for detection by FID. Using this method, we analysed specific metabolites of primarily protein-fermentation (isobutyrate, isovalerate, valerate and isocaproate) and primarily fibre-fermentation (acetate, propionate, butyrate and caproate). Because we aimed to investigate how diets, through luminal metabolites in the distal colon, affected IEC metabolic function, we expressed metabolite concentration as colonic flux in mmol per hour (see data analysis for calculations). Metabolite concentrations in mmol per kg digesta can be found in Supplementary Figure S5.1.

### *Data analysis and Statistics*

For pig performance parameters, data are presented as mean with pooled standard error (SE). Data of metabolite analysis, metabolic function and isolation parameters are presented using bar-graphs with mean ± SEM. For SCFA and N analysis, respectively two and three samples were missing because they were exhausted. Two pigs were excluded in the viability and metabolic flux analysis because of an inability to accurately normalize the data. An additional eight pigs were absent from the analysis of cell yield because the volume of isolating medium was not recorded. The number

of pigs included for each group is described in the figure and table legends. For pig performance parameters, data for pens were analysed. For metabolite fluxes and IEC isolations, data from individual pigs were analysed, and a random effect of pen was included to account for higher similarity within pens than between pens.

Mean retention time in hours was calculated using the following formula [27]:

$$mean\ retention\ time(h) = 24*(([T]sample*[Q]sample)/([T]diet*Intake/24h))$$

Where $[T]_{diet}$ and $[T]_{sample}$ (g/kg) are $TiO_2$ in the diet and distal colon digesta, $Q_{sample}$ (g) is the tracer pool size in the distal colon, and intake is feed intake per 24h (kg).

Metabolite flow in mmol/hour was calculated using the following formula:

$$metabolite\ flow = [M]sample*[Q]sample/mean\ retention\ time(h)$$

Where $[M]_{sample}$ (mmol/kg digesta) is metabolite concentration in distal colon digesta, and $Q_{sample}$ (g) is the distal colon digesta pool size, obtained between 66 and 80% of the colon.

Net disappearance of nitrogen was calculated using the following formula:

$$net\ N\ disappearance\ colon\ (g/day) = [N]intake*(apparent\ total\ tract\ digestibilityN - apparent\ ileal\ digestibilityN)$$

Where $[N]_{intake}$ is daily nitrogen intake per day (g), ATTD (%) N is apparent total tract digestibility of nitrogen and AID (%) N is apparent ileal digestibility of nitrogen.

Normality of model residuals was checked using the Shapiro-Wilk test, and if assumptions were not met, data were transformed as indicated in the table and Figure legends when applicable. Although model residuals were not normally distributed for isobutyrate and isovalerate due to an outlier, removal of this outlier did not change the conclusions and was therefore not removed for the final analysis. If all assumptions were met, two-way ANOVA was performed with an $\alpha$ of 0.05, using protein source and fibre type as factors and including pen as random effect. In case of significant interaction between factors, a posthoc Tukey test was performed to determine differences between groups. To correlate metabolites to IEC metabolic flux, fermentation metabolites were subclassified as primary protein-fermentation metabolites (ammonia, isobutyrate, isovalerate, valerate and isocaproate) and primarily fibre-fermentation metabolites (acetate, propionate, butyrate and caproate). To ensure that metabolites with high concentrations did not disproportionately influence the overall values of protein- or fibre-fermentation metabolites, all metabolites were rank-scaled before they were

added up. Then, the protein- and fibre- fermentation metabolite fluxes were correlated to IEC metabolic flux parameters using Spearman correlation. All statistical analysis was performed using R version 4.1, and data was visualised using GraphPad v.9 (GraphPad Software, CA, USA).

### *Ethics approval*

All experimental procedures were approved by the Dutch Central Committee of Animal Experiments (the Netherlands) under the authorisation number AVD1040020171667.

### *Data and model availability statement*

All data generated or analysed during this study are available from the corresponding author upon reasonable request. None of the data was deposited in an official repository, but the R-script for brightfield analysis of microscopy image and data normalisation of Seahorse XF Extracellular Flux analysis is available from GitHub (https://github.com/vcjdebo er/seahorse-data-analysis-PIXI).

## Results

### *Effects of the feeds on pig performance*

Nor slaughter weight, nor small and large intestinal length was affected by the dietary treatments (Table 5.2). Collagen-fed pigs had a reduced weight gain compared to whey-fed pigs (Table 5.2, $p<0.01$).



Table 5.2 **Effect of two-weeks of feeding diets differing in protein source and fibre level on performance and intestinal traits in pigs.** Data are presented as mean with pooled SE, n=2 pens per group.

| | Whey | | Collagen | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Low Fibre | High Fibre | Low Fibre | High Fibre | SE | $P_{fibre}$ | $P_{protein}$ | $P_{interaction}$ |
| Start weight (kg) | 23.4 | 22.4 | 22.6 | 22.8 | 0.7 | 0.46 | 0.71 | 0.27 |
| Daily weight gain (g/day) | 355 | 365 | 286 | 258 | 36 | 0.99 | <0.05 | 0.31 |
| Slaughter Weight (kg)[1] | 29 | 28.1 | 27.1 | 26.8 | 0.7 | 0.23 | 0.06 | 0.28 |
| Length Small Intestine (m) | 14.6 | 14 | 14.8 | 13.9 | 0.5 | 0.10 | 0.81 | 0.70 |
| Length Large Intestine (cm) | 265 | 274 | 282 | 248 | 19 | 0.38 | 0.73 | 0.18 |

[1]Slaughter weight data was transformed using exponential transformation approach normal distribution, reported values are back-transformed.

### *Metabolite analysis in distal colon digesta*

Collagen-fed pigs had reduced freeze-dried matter (FDM) content of the distal colon digesta compared to whey-fed pigs, while the inclusion of high fermentable fibre increased distal colon FDM (Supplementary Fig. S5.1A). We found that the net colonic nitrogen disappearance was increased by 135% in the collagen-protein compared to the whey-protein diets (Fig. 5.1A). The dietary treatments did not affect the mean retention time (MRT) in the distal colon (Fig. 5.1B). The collagen-fed pigs had an increased colonic flux of the protein-fermentation metabolites ammonia, isobutyrate, isovalerate and valerate compared to the whey-fed pigs (Fig. 5.1C-G) as well as an increased acetate flux (Fig. 5.1H). Feeding high fermentable fibre increased colonic fluxes of all analysed SCFA's, both fibre fermentation-derived metabolites (acetate, propionate, butyrate and caproate, Fig. 5.1H-K), as well as those related to protein fermentation (ammonia, isobutyrate, isovalerate, valerate and isocaproate, Fig. 5.1C-G).

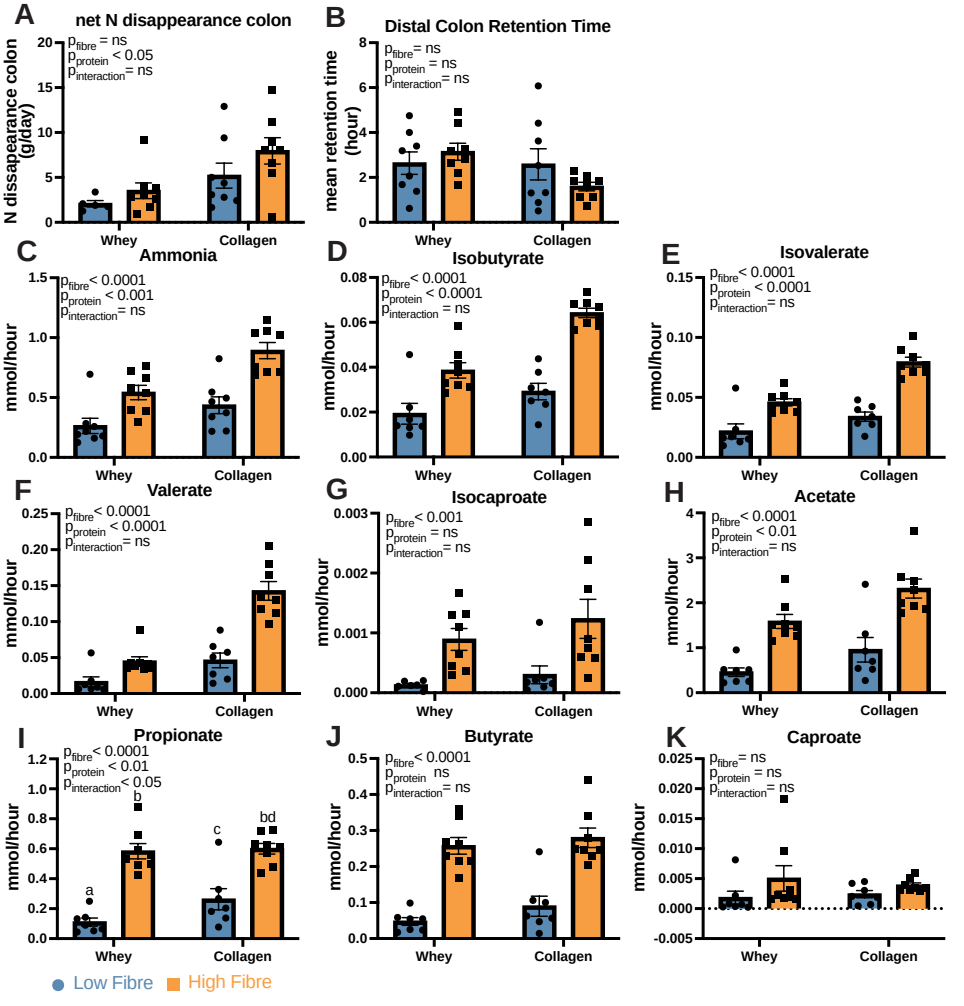


Figure 5.1. **Distal colon digesta metabolite levels after two-weeks of feeding pigs diets varying in dietary protein source and fibre level.** Net nitrogen disappearance in the colon (**A**). Mean retention time (MRT) in distal colon (**B**). Protein fermentation metabolites ammonia (**C**), isobutyrate (**D**), isovalerate (**E**), valerate (**F**) and isocaproate (**G**) and fibre fermentation metabolites acetate (**H**), propionate (**I**), butyrate (**J**) and caproate (**K**) were analysed in distal colon digesta of pigs following two-weeks on the four feeds. The levels were corrected for mean retention time, yielding intestinal fluxes in mmol per hour. Bars represent mean ± SEM. For panel **A**, n=5 for low fibre whey group, n=8 for all other groups. For panel **B**, n=8 for all groups, for panels **C-K**, n=7 for low fibre whey and collagen groups, n=8 for high fibre whey and collagen groups. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis. Data for nitrogen disappearance, butyrate, acetate, propionate, valerate and caproate were log-transformed to approach normal distribution.

### *Intestinal cell isolation parameters*

Regardless of the fibre treatment, feeding pigs with collagen-protein, compared to whey-protein, led to an increase in the viability of isolated colonocytes of 12% (Fig. 5.2A, protein $p<0.01$). In contrast, the yield of cells/cm$^2$ was reduced by 32% in isolations of pigs fed the high fibre diets, regardless of the protein source (Fig. 5.2B, fibre $p<0.05$).

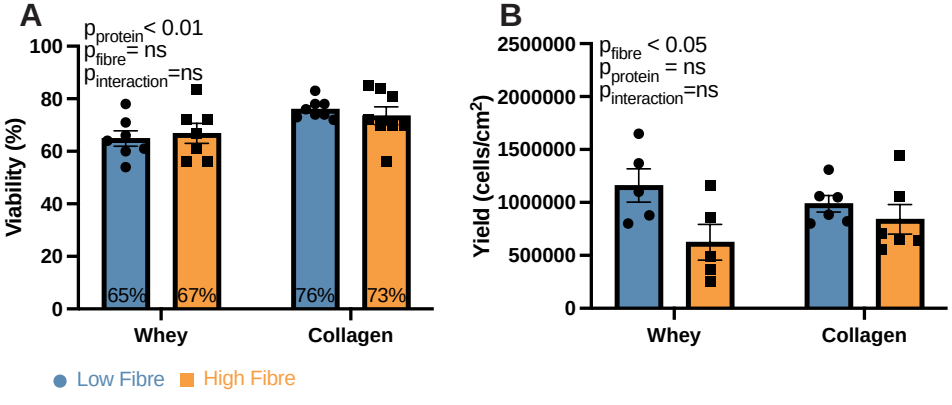



Figure 5.2. **Intestinal epithelial cell isolation parameters after two-weeks of feeding pigs diets varying in dietary protein source and fibre level.** Viability as percentage of dead cells over total cells (**A**; n=7 for whey-protein groups and n=8 for collagen-protein groups) and cell yield expressed as viable cells isolated per cm$^2$ intestinal tissue (**B**; n=5 for whey-protein groups and n=6 for collagen-protein groups) were calculated following isolation from the colonic segments. Bars represent mean ± SEM. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis

### *Extracellular metabolic flux analysis of isolated pig colonocytes*

The dietary interventions did not affect basal mitochondrial respiration of IECs (Fig. 5.3A). Colonocytes of high fermentable fibre fed pigs in the whey-protein diet, but not in the collagen-protein diet had a reduced mitochondrial capacity, as can be seen by

the significant reduction of maximal respiration by 35% (Fig. 5.3B, interaction $p<0.05$) and the significant reduction in spare respiratory capacity by 20% (SRC, Fig. 5.3C, interaction $p< 0.05$). IECs (i.c. colonocytes) from high fermentable fibre fed pigs had a 37% decreased glycoPER compared to IECs of low fermentable fibre fed pigs (Fig. 5.3D, $p<0.001$). The energetic phenotype plots (Fig 5.3E-F) show an overall decrease of metabolic function of IECs in high fermentable fibre upon inclusion of fermentable fibre in a whey-protein-based feed (Fig. 5.3E), while high levels of fermentable fibre in a collagen-protein-based feed mainly reduced glycolytic function of isolated pig colonocytes (Fig 5.3F). Mucosal citrate synthase level in colonocyte scrapings was determined as indicator of mitochondrial density, and this was not affected by the dietary treatments (Fig. 5.4).

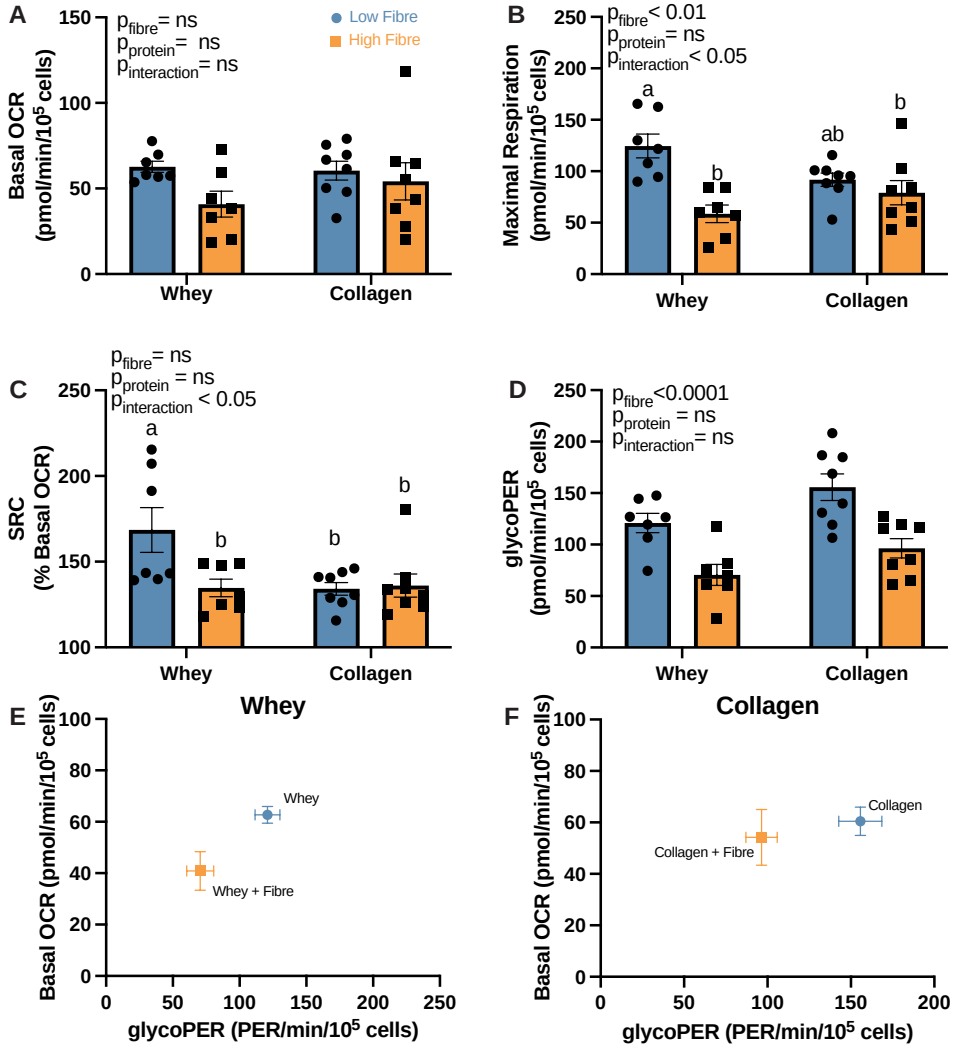


Figure 5.3. **Extracellular metabolic flux analysis of isolated pig colonocytes after two-weeks of feeding pigs diets varying in dietary protein source and fibre level.** Mitochondrial oxygen consumption is represented by basal oxygen consumption (**A**), maximal respiration (**B**) and spare respiratory capacity (**C**). Glycolytic function is measured using glycoPER (**D**). Energetic profile plots for the whey-protein based (**E**) and collagen-protein based (**F**) feeds show the changes in basal metabolic function upon inclusion of fermentable fibre. N=7 for the whey-protein diets and n=8 for the collagen-protein diets. Bars represent mean ± SEM. Significance was analysed using two-way ANOVA, and results are presented in the top left corner of each graph. Similar letters indicate no significant differences between groups in case of significant interaction effects, as analysed using Tukey's posthoc analysis.

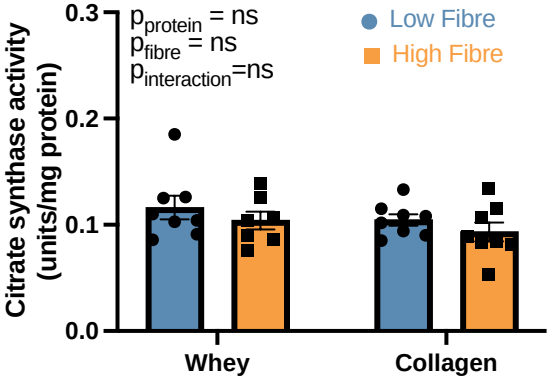



Figure 5.4. **Citrate synthase activity in distal colon mucosal scrapings after two-weeks of feeding pigs diets varying in dietary protein source and fibre level.** Bars represent mean ± SEM, n=7 for low fibre whey, n=6 for high fibre whey and n=8 for both collagen groups. Significance was calculated using two-way ANOVA on log-transformed data.



## Discussion

Intestinal epithelial cells rely on cellular energy production through glycolytic and oxidative pathways to maintain their various functions [36, 37]. Factors that affect epithelial energy production, such as metabolites produced from feedstuffs by the microbiome, may thus have far-reaching consequences for intestinal homeostasis. In this paper, we showed functionally that diet indeed affected the metabolism of isolated pig colonocytes, and observed an interaction between protein source and fibre level. We demonstrated that mitochondrial capacity of isolated IECs was reduced by fermentable fibre only in whey-protein diets. At the same time, glycolytic function of IECs was reduced by high fermentable fibre, irrespective of dietary protein source. Especially in the well digestible whey-protein diets we thus observed an overall reduction of metabolic capacity upon addition of high fermentable fibre to the diet. These findings gave us valuable new insights into the interaction between various dietary components.

### *Possible implications of altered colonocyte metabolic function*

In our study, pigs were growing at expected rates considering the nutritional strategies imposed: a reduction of digestible protein in the collagen diets was reflected in a lower rate of bodyweight gain. Apart from this difference in daily weight gain, no clear differences in pig performance parameters were observed. However, we show that the diets did affect colonocyte oxidative capacity. One of the hallmarks of good intestinal health is a well-functioning intestinal barrier. To maintain this barrier, sufficient ATP needs to be produced to allow for intestinal tight junction assembly and maintenance of intestinal barrier integrity [9, 10], and the decreased metabolic capacity that was observed in pigs fed a high-fibre diet could therefore impact barrier function. Furthermore, it has been shown that elevated oxygenation of the colonic lumen due to lower oxygen consumption in the colonocytes, could allow for higher pathogenic bacterial growth [38, 39]. In our study, we identified lower mitochondrial oxygen consumption capacity, which could translate into higher lumen oxygen levels and possibly higher pathogenic bacterial growth.

### *Effect of dietary interventions on colonocyte metabolic function and microbial metabolite production*

Although dietary fibre is often considered beneficial, partly by providing substrates that increase mitochondrial function and intestinal energy production, some studies indicate a reducing effect of high concentrations of SCFAs on intestinal barrier function and IEC metabolism. For example, *in vivo* studies in rats showed that feeding fructo-oligosaccharides decreased intestinal barrier function [7, 40, 41]. In addition, especially *in vitro* studies have demonstrated that high concentrations of e.g. butyrate can induce apoptosis [42, 43]. These findings challenge the view that dietary fibres are always beneficial for intestinal health and function. In our study, the decrease in metabolic capacity induced by high fibre feeds was especially prominent in the whey-protein feeds. An explanation for this could be that because of the high digestibility of whey-protein, less protein enters the colon which is thus less available as a colonic energy substrate (Fig. 5.1A). To increase the efficient use of the remaining substrates, mitochondrial capacity should be induced to most optimally obtain energy from the limited remaining substrates [7]. This is consistent with our observation of higher metabolic capacity of IECs from pigs fed with low fibre whey-protein diets (Fig. 5.3). Furthermore, we observed a lower spare respiratory capacity of IECs isolated from pigs fed the collagen-protein diets, possibly obscuring the negative impact of high fibre in these diets. How collagen diets decreased spare respiratory capacity could be explained by the increased nitrogen flow into the colon, in addition to increased fluxes of microbial metabolites that could potentially harm mitochondrial function. Protein

fermentation metabolites, such as $H_2S$, ammonia and others, have indeed been shown to impact mitochondria [15, 16, 44].

Interestingly, we did not find an interaction effect of protein source and fibre level for glycoPER, and instead found that high fibre decreased colonocyte glycoPER irrespective of protein source. High fibre diets were reported to increase intestinal proliferation [45], crypt depth [46-48] and mucus layer thickness [49, 50], potentially resulting in increased percentages of differentiated colonocytes in the isolated cell population. Differentiated colonocytes are thought to be less glycolytic, while the transit-amplifying cells, which are their precursors, rely more on glycolysis [36, 51, 52]. Thus, a larger proportion of differentiation colonocytes in the isolated IEC population could underlie the observed decrease glycoPER in high fibre-fed pigs. Although the lowered glycolytic function of differentiated colonocytes was shown to be due to elevated mitochondrial function, we did not observe elevated mitochondrial function, possibly because the mitochondrial oxidative capacity may be driven by fatty acids, or even SCFAs like butyrate, which we did not add extracellularly during our metabolic flux analysis. Since glycolytic function is not dependent on SCFAs, we observed the effects of high fibre feeding irrespective of protein source.

In this study, our primary aim was to investigate whether colonocyte metabolism was altered in response to dietary interventions. An important route for interaction between diet and colonocytes is through microbial metabolites. Since the speed with which metabolites, contained in digesta, travel through the colon could affect the exposure of colonocytes to these metabolites, we calculated colonic fluxes of the metabolites. We expected that the poorly digestible protein would increase the flux of protein-derived metabolites, while including high levels of fermentable fibre would decrease the flux of protein-derived metabolites and increase the fluxes of acetate, butyrate, propionate and caproate. Indeed, we found that high fibre feeds significantly increased the concentration of these fibre fermentation metabolites. However, the high fibre diets also increased the flow of the protein fermentation metabolites ammonia, isobutyrate, isovalerate, valerate and isocaproate. Even though protein fermentation in high fibre diets is not always reported, valerate has been shown to increase upon in high fibre feeding in pigs and other species [48, 53-55]. This is possibly due to an increased microbial richness and stability [56], which could benefit not only fibre-fermenting communities but also the proteolytic ones [57]. Additionally, high fibre feeds could have led to increased mucus production, which consists of protein for about 20%, and could thus also increase flow of protein fermentation products [49, 50, 58].

To investigate whether the colonic metabolite fluxes correlated with IEC metabolic function, we pooled the metabolite fluxes into two groups: primarily protein-fermentation derived metabolites (ammonia, isobutyrate, isovalerate, valerate and isocaproate),

and primarily fibre-fermentation derived metabolites (acetate, propionate, butyrate and caproate). We found comparable negative correlations between the protein or fibre-derived fermentation metabolites and mitochondrial capacity parameters maximal respiration and spare respiratory capacity (r= -0.46 and -0.63; r= -0.40 and -0.40, respectively, Supplementary Table S5.1). In addition, fibre derived metabolites correlated negatively with glycolytic capacity (r= -0.55, Supplementary Table S5.1). Thus, we found that metabolites present in the intestinal lumen negatively correlate to metabolic function of pig colonocytes, suggesting that microbial metabolites could have played a role in the decreased metabolic function of IECs, and potentially affected intestinal health through this route.

### *Methodological improvements and study limitations*

We have taken several steps to ensure accurate measurement of IEC metabolic function. Firstly, citrate synthase was analysed as a marker for mitochondrial density. The observed unaltered citrate synthase levels ensure that the observed changes in energy metabolism were not due to an altered mitochondrial density, but were true alterations in the metabolic function of the IECs (Figure 5.4, [59]). Secondly, IEC viability was measured to investigate whether this was affected by the diets, since a decreased viability has been associated with a reduced mitochondrial metabolism [60]. We did observe lower viability in both groups fed a whey-protein diet, but only in the fibre containing group metabolism was decreased, rendering it unlikely that the metabolic alterations were due to decreased IEC viability. Isolated IEC yields were lower in the high fibre diets. This may be due to the effects of fibre-fermentation metabolites on intestinal proliferation [45], crypt depth [46-48] and thickness of the mucosal layer [49, 50]. It is however unlikely that differences in cell yield could have contributed to the functional metabolic differences that were observed, since equal number of cells were plated for metabolic analysis and on top of this, the cell numbers in the assay wells were quantified and the data were normalised for this. In conclusion, neither difference in viability, yield or mitochondrial density can explain the altered metabolic function of colonocytes in response to the diets.

A limitation of this study is that metabolism of isolated colonocytes is evaluated only in the presence of glutamine, glucose and pyruvate. The use of multiple substrates is important since substrates preference could be altered by the diets, as was shown in several studies in both pigs and rats [61-63]. In our study, it cannot be excluded that IECs from the high fermentable fibre fed pigs have an increased dependency on one of the SCFAs which were not included as substrates in the metabolic analysis. A second limitation is that we did not assess possible alteration in IEC composition. Altered composition of the cell population could impact the results, because each intestinal cell type displays a distinct metabolic phenotype [64], and it would thus be

relevant and interesting to specifically analyse possible alteration in the composition of the isolated IEC population to better interpret the results.

## Conclusions

In conclusion, we found that diets affect the metabolism isolated pig colonocytes, and that there was an interaction between protein source and fibre level. Glycolytic activity was reduced in colonocytes isolated from high fibre fed pigs, irrespective of protein source, while metabolic capacity was reduced in only in high fibre-fed pigs in a whey-protein diet. Metabolic analysis of isolated pig colonocytes could be a sensitive tool to further assess functional metabolic effects of dietary interventions. This is important because intestinal epithelial energy metabolism is tightly linked to cell function and may impact intestinal health and function.

**Acknowledgements:** We would like to acknowledge Arjen van Dolderen and Adri Pater for their help during the animal experiment, and Karthikeyan Gunasekaran for the technical support.

**Author contributions: A.FB.**: Methodology, Investigation, Formal analysis, Writing – Original draft, Writing – Review & Editing, Formal analysis, Data Curation, Writing–- Original Draft, Visualization. **L.N.**: Investigation, Writing–- Review & Editing. J.K.: Conceptualization, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **V.C.J.d.B.**: Conceptualization, Methodology, Software, Writing–- Review & Editing, Supervision, Funding acquisition. **W.J.J.G.**: Conceptualization, Formal analysis, Writing–- Review & Editing, Supervision, Project administration, Funding acquisition.

## References

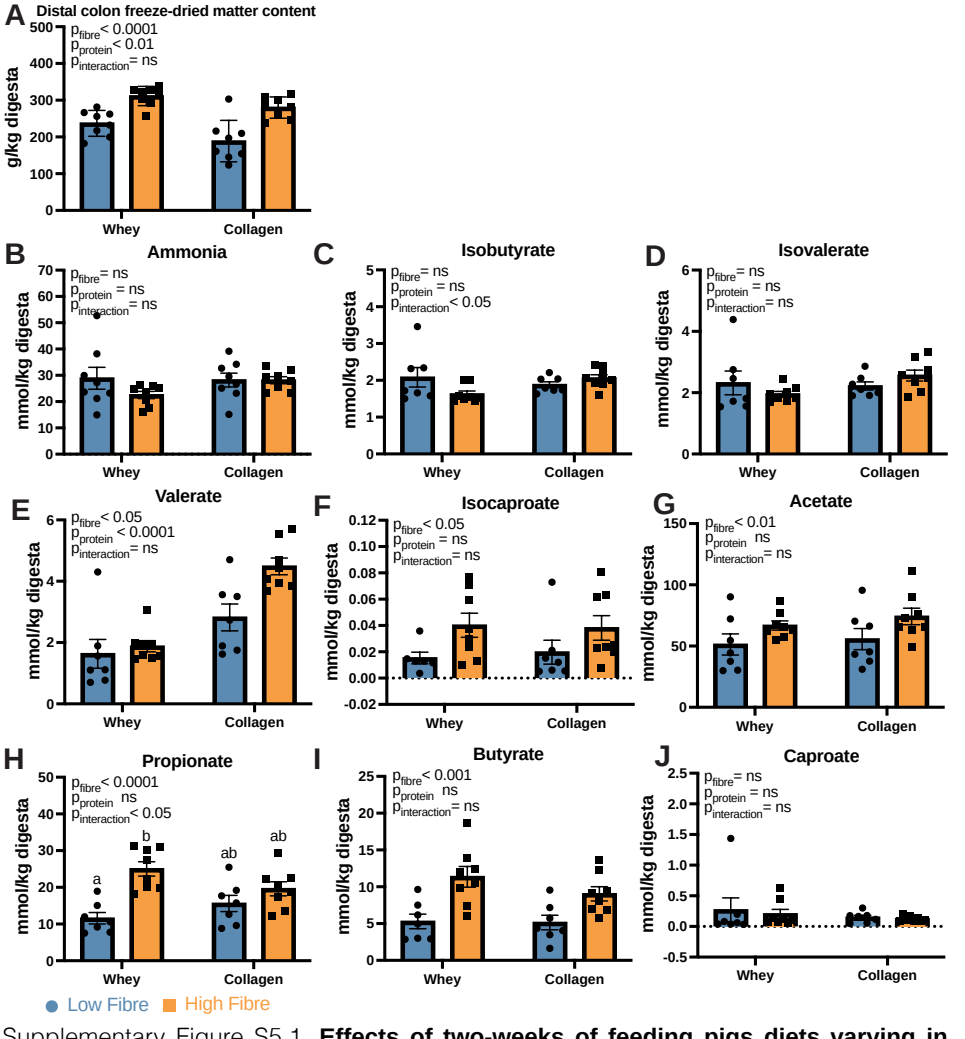

1. Potten, C.S., C. Booth, and D.M. Pritchard, *The intestinal epithelial stem cell: the mucosal governor.* International Journal of Experimental Pathology, 1997. **78**(4): p. 219-243.
2. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nature Reviews Immunology, 2014. **14**(3): p. 141-153.
3. Rolfe, D.F. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals.* Physiological Reviews, 1997. **77**(3): p. 731-758.
4. van Erp, R.J.J., et al., *Reduced Feed Intake, Rather than Increased Energy Losses, Explains Variation in Growth Rates of Normal-Birth-Weight Piglets.* The Journal of Nutrition, 2018. **148**(11): p. 1794-1803.
5. Peng, L., et al., *Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers.* The Journal of Nutrition, 2009. **139**(9): p. 1619-1625.
6. Miao, W., et al., *Sodium Butyrate Promotes Reassembly of Tight Junctions in Caco-2 Monolayers Involving Inhibition of MLCK/MLC2 Pathway and Phosphorylation of PKC2.* International Journal of Molecular Sciences, 2016. **17**(10): p. 1696.
7. Rodenburg, W., et al., *Impaired barrier function by dietary fructo-oligosaccharides (FOS) in rats is accompanied by increased colonic mitochondrial gene expression.* BMC Genomics, 2008. **9**(1): p. 144.
8. Donohoe, D.R., et al., *The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon.* Cell Metabolism, 2011. **13**(5): p. 517-526.
9. Bjarnason, I. and K. Takeuchi, *Intestinal permeability in the pathogenesis of NSAID-induced enteropathy.* Journal of Gastroenterology, 2009. **44**(19): p. 23-29.
10. JanssenDuijghuijsen, L.M., et al., *Mitochondrial ATP Depletion Disrupts Caco-2 Monolayer Integrity and Internalizes Claudin 7.* Frontiers in Physiology, 2017. **8**: p. 794.
11. Drochner, W., *Digestion of carbohydrates in the pig.* Archiv für Tierernaehrung, 1993. **43**(2): p. 95-116.
12. Gilbert, M.S., et al., *Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2018. **315**(2): p. G159-g170.
13. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-798.
14. Pieper, R., et al., *Health relevance of intestinal protein fermentation in young pigs.* Animal Health Research Reviews, 2016. **17**(2): p. 137-147.
15. Blachier, F., et al., *Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences.* Amino Acids, 2007. **33**(4): p. 547-62.
16. Darcy-Vrillon, B., et al., *Short chain fatty acid and glucose metabolism in isolated pig colonocytes: modulation by NH4+.* Molecular and Cellular Biochemistry, 1996. **156**(2): p. 145-151.
17. Bekebrede, A.F., et al., *Mitochondrial and glycolytic extracellular flux analysis optimization for isolated pig intestinal epithelial cells.* Scientific Reports, 2021. **11**(1): p. 19961.
18. Zijlstra, R.T. and E. Beltranena, *Chapter 11: Co-products in swine nutrition and feed formulation*, in *Poultry and pig nutrition: Challenges of the 21st century*, L. Babinszky, M.W.A. Verstegen, and W.H. Hendriks, Editors. 2019, Wageningen Academic Publishers: Wageningen, The Netherlands. p. 245-262.
19. Bikker, P., et al., *The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets.* Journal of Animal Science, 2006. **84**(12): p. 3337-3345.
20. Jha, R. and J.F.D. Berrocoso, *Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review.* Animal Feed Science and Technology, 2016. **212**: p. 18-26.

21. Kim, J.C., et al., *Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract.* British Journal of Nutrition, 2008. **99**(6): p. 1217-25.
22. Jeaurond, E.A., et al., *Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs.* Canadian Journal of Animal Science, 2008. **88**(2): p. 271-281.
23. Schulthess, J., et al., *The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages.* Immunity, 2019. **50**(2): p. 432-445.e7.
24. Donohoe, Dallas R., et al., *The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation.* Molecular Cell, 2012. **48**(4): p. 612-626.
25. Li, Q., et al., *Butyrate Suppresses the Proliferation of Colorectal Cancer Cells via Targeting Pyruvate Kinase M2 and Metabolic Reprogramming.* Molecular & Cellular Proteomics, 2018. **17**(8): p. 1531-1545.
26. Everts, H., *Energy requirement for maintenance in growing pigs*, in *CVB-Documentation report*. 2015, WUR Livestock Research, Dept. Animal Nutrition: Wageningen, The Netherlands.
27. de Vries, S. and W.J.J. Gerrits, *Chapter 10: The use of tracers or markers in digestion studies*. 2018, Wageningen: Wageningen Acedemic Publishers.
28. Martens, B.M.J., et al., *Whole digesta properties as influenced by feed processing explain variation in gastrointestinal transit times in pigs.* British Journal of Nutrition, 2019. **122**(11): p. 1242-1254.
29. Schop, M., *Modelling digestion kinetics in pigs Predicting nutrient absorption based on diet and ingredient properties*. 2020, Wageningen University: Wageningen, The Netherlands.
30. Centraal Veevoeder Bureau (CVB) *Chemische samenstellingen en nutritionele waarden van voedermiddelen.* Federatie Nederlandse Diervoederketen. Centraal Veevoederbureau, Wageningen, The Netherlands., 2018.
31. Pau, G., et al., *EBImage--an R package for image processing with applications to cellular phenotypes.* Bioinformatics (Oxford, England), 2010. **26**(7): p. 979-981.
32. Janssen, J.J.E., et al., *Novel standardized method for extracellular flux analysis of oxidative and glycolytic metabolism in peripheral blood mononuclear cells.* Scientific Reports, 2021. **11**(1): p. 1662-1662.
33. International Organization for Standardization *ISO 6496: animal feeding stuffs—determination of moisture and other volatile matter content.* 1999, International Organization for Standardization: Geneva, Switzerland.
34. International Organization for Standardization *ISO 5983: animal feeding stuffs—determination of N content and calculation of crude protein content. Part 1: Kjeldahl method.* . 2005, International Organization for Standardization: Geneva, Switzerland.
35. Myers, W.D., et al., *Technical note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide.* J Anim Sci, 2004. **82**(1): p. 179-83.
36. Yang, H., et al., *Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis.* Scientific Reports, 2016. **6**: p. 31917.
37. Zhou, W., et al., *Glucose stimulates intestinal epithelial crypt proliferation by modulating cellular energy metabolism.* Journal of Cellular Physiology, 2018. **233**(4): p. 3465-3475.
38. Byndloss, M.X., et al., *Microbiota-activated PPARγ- signaling inhibits dysbiotic Enterobacteriaceae expansion.* Science, 2017. **357**(6351): p. 570-575.
39. Cevallos, S.A., et al., *Increased Epithelial Oxygenation Links Colitis to an Expansion of Tumorigenic Bacteria.* mBio, 2019. **10**(5): p. e02244-19.
40. Ten Bruggencate, S.J.M., et al., *Dietary Fructooligosaccharides Increase Intestinal Permeability in Rats.* The Journal of Nutrition, 2005. **135**(4): p. 837-842.
41. Genda, T., et al., *Fructo-oligosaccharide-Induced Transient Increases in Cecal Immunoglobulin A Concentrations in Rats Are Associated with Mucosal Inflammation*

*in Response to Increased Gut Permeability.* The Journal of Nutrition, 2017. **147**(10): p. 1900-1908.
42. Matthews, G.M., G.S. Howarth, and R.N. Butler, *Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism.* Chemotherapy, 2012. **58**(2): p. 102-109.
43. Peng, L., et al., *Effects of Butyrate on Intestinal Barrier Function in a Caco-2 Cell Monolayer Model of Intestinal Barrier.* Pediatric Research, 2007. **61**(1): p. 37-41.
44. Libiad, M., et al., *Hydrogen sulfide perturbs mitochondrial bioenergetics and triggers metabolic reprogramming in colon cells.* Journal of Biological Chemistry, 2019. **294**(32): p. 12077-12090.
45. Jin, L., et al., *Effects of dietary fiber on intestinal growth, cell proliferation, and morphology in growing pigs.* Journal of Animal Science, 1994. **72**(9): p. 2270-2278.
46. Mentschel, J. and R. Claus, *Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment.* Metabolism, 2003. **52**(11): p. 1400-1405.
47. Serena, A., M.S. Hedemann, and K.E. Bach Knudsen, *Feeding high fibre diets changes luminal environment and morphology in the intestine of sows.* Livestock Science, 2007. **109**(1): p. 115-117.
48. Zaworska-Zakrzewska, A., et al., *Growth Performance, Gut Environment and Physiology of the Gastrointestinal Tract in Weaned Piglets Fed a Diet Supplemented with Raw and Fermented Narrow-Leafed Lupine Seeds.* Animals, 2020. **10**(11): p. 2084.
49. Willemsen, L.E.M., et al., *Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts.* Gut, 2003. **52**(10): p. 1442-1447.
50. Barcelo, A., et al., *Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon.* Gut, 2000. **46**(2): p. 218-224.
51. Fan, Y.-Y., et al., *A bioassay to measure energy metabolism in mouse colonic crypts, organoids, and sorted stem cells.* American Journal of Physiology - Gastrointestinal and Liver Physiology, 2015. **309**(1): p. G1-G9.
52. Litvak, Y., M.X. Byndloss, and A.J. Bäumler, *Colonocyte metabolism shapes the gut microbiota.* Science (New York, N.Y.), 2018. **362**(6418): p. eaat9076.
53. Hao, Y., et al., *Effects of Total Dietary Fiber on Cecal Microbial Community and Intestinal Morphology of Growing White Pekin Duck.* Frontiers in Microbiology, 2021. **12**(2462): p. Article 727200.
54. Tunio, A., et al., *Large intestine morphology and microflora activity in piglets fed diets with two levels of raw or micronized blue sweet lupin seeds.* Livestock Science, 2020. **240**: p. 104137.
55. Zhao, J.B., et al., *Effect of wheat bran on apparent total tract digestibility, growth performance, fecal microbiota and their metabolites in growing pigs.* Animal Feed Science and Technology, 2018. **239**: p. 14-26.
56. Tap, J., et al., *Gut microbiota richness promotes its stability upon increased dietary fibre intake in healthy adults.* Environmental Microbiology, 2015. **17**(12): p. 4954-4964.
57. Xu, J., et al., *Metatranscriptomic analysis of colonic microbiota's functional response to different dietary fibers in growing pigs.* Animal Microbiome, 2021. **3**(1): p. 45.
58. Raimondi, S., et al., *Identification of mucin degraders of the human gut microbiota.* Scientific Reports, 2021. **11**(1): p. 11094.
59. Vigelsø, A., N.B. Andersen, and F. Dela, *The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training.* International Journal of Physiology, Pathophysiology and Pharmacology, 2014. **6**(2): p. 84-101.
60. Osellame, L.D., T.S. Blacker, and M.R. Duchen, *Cellular and molecular mechanisms of mitochondrial function.* Best Practice & Research Clinical Endocrinology & Metabolism, 2012. **26**(6): p. 711-723.

61. Darcy-Vrillon, B., et al., *Metabolic Characteristics of Pig Colonocytes after Adaptation to a High Fiber Diet.* The Journal of Nutrition, 1993. **123**(2): p. 234-243.
62. Marsman, K.E. and M.I. McBurney, *Dietary Fiber Increases Oxidative Metabolism in Colonocytes but Not in Distal Small Intestinal Enterocytes Isolated from Rats.* The Journal of Nutrition, 1995. **125**(2): p. 273-282.
63. Fleming, S.E. and R. Gill, *Aging Stimulates Fatty Acid Oxidation in Rat Colonocytes But Does Not Influence the Response to Dietary Fiber.* The Journals of Gerontology: Series A, 1997. **52A**(6): p. B318-B330.
64. Ho, G.-t. and A.L. Theiss, *Mitochondria and Inflammatory Bowel Diseases: Toward a Stratified Therapeutic Intervention.* Annual Review of Physiology, 2022. **84**(1): p. 435-459.

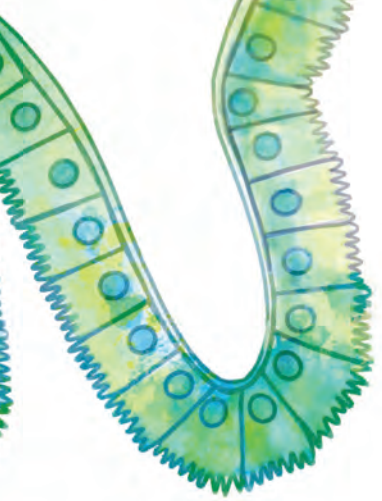
## Supplementary material




Supplementary Figure S5.1. **Effects of two-weeks of feeding pigs diets varying in dietary protein source and fibre level on intestinal metabolite production.** Freeze-dry matter (FDM) content was analysed in the distal colon (**A**). Protein fermentation metabolites ammonia (**B**), isobutyrate (**C**), isovalerate (**D**), valerate (**E**) and isocaproate (**F**) and fibre fermentation metabolites acetate (**G**), propionate (**H**), butyrate (**I**) and caproate (**J**) were analysed in distal colon digesta of pigs following two-weeks on the four feeds. SCFAs are shown as mmol/kg fresh digesta. Bars represent mean ± SEM, n=7 for low fibre whey and collagen groups, n=8 for high fibre whey and collagen groups. Similar letters indicate no significant differences between groups as was calculated using two-way ANOVA. Data for acetate, isobutyrate, isovalerate, valerate and isocaproate were log-transformed and caproate was inverse-transformed to approach normal distribution.

Supplementary Table S5.1. **Correlation of metabolites and functional metabolic analysis in distal colon following two-weeks of feeding pigs diets varying in dietary protein source and fibre level.** Protein fermentation metabolites are the sum of the scaled metabolites ammonia, isobutyrate, isovalerate, valerate and isocaproate. Fibre fermentation metabolites are the sum of the scaled metabolites acetate, propionate, butyrate and caproate.

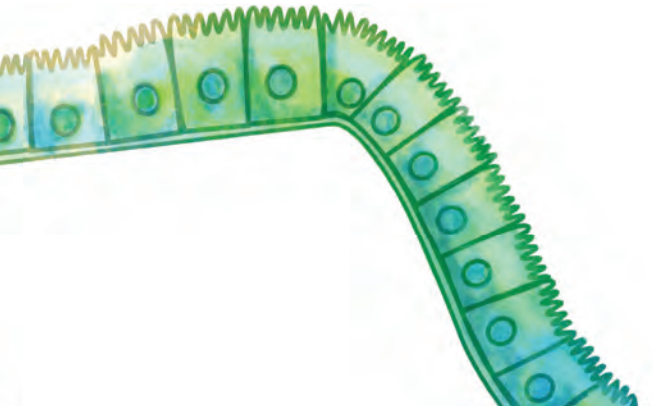
| | Basal OCR | | Maximal respiration | | spare respiratory capcacity (% of basal OCR) | | glycoPER | |
|---|---|---|---|---|---|---|---|---|
| | spearman r | p-value | spearman r | p-value | spearman r | p-value | spearman r | p-value |
| Colonic flux of protein fermentation metabolites | -0.24 | 0.23 | -0.46 | <0.05 | -0.4 | <0.05 | -0.31 | 0.12 |
| Colonic flux of fibre fermentation metabolites | -0.35 | 0.07 | -0.63 | <0.0001 | -0.4 | <0.05 | -0.55 | <0.0001 |



# Chapter 6

## Functional and Molecular Profiling of Fasted Piglets Reveals Decreased Energy Metabolic Function and Cell Proliferation in the Small Intestine

Anna F. Bekebrede
Vincent C.J. de Boer
Walter J.J. Gerrits
Jaap Keijer



*Submitted*

## Abstract

The small intestine requires energy to exert its important role in nutrient uptake and barrier function. Pigs are an important source of food and a model for humans. Young piglets and infants can suffer from periods of insufficient food intake. Whether this functionally affects the small intestinal epithelial cell (IEC) metabolic capacity, and how this may be associated with an increased vulnerability to intestinal disease is unknown. We therefore performed a 48h fasting intervention in young piglets. After feeding a standard weaning diet for 2 weeks, six-week-old piglets (n=16 per group) were fasted for 48h, and mid-jejunal IECs were collected upon slaughter. Functional metabolism of isolated IECs was analysed with the Seahorse XF analyser and gene expression was assessed using RNA-sequencing. Fasting decreased the mitochondrial and glycolytic function of the IECs by 50% and 45%, respectively ($p < 0.0001$), signifying that overall metabolic function was decreased. The RNA-sequencing results corroborated our functional metabolic measurements, showing that particularly pathways related to mitochondrial energy production were decreased. Besides oxidative metabolic pathways, decreased cell-cycle progression pathways were most regulated in the fasted piglets, which was confirmed by 43% reduction of cells stained with the proliferation marker Ki67 ($p < 0.05$). Finally, the expression of barrier function genes was reduced upon fasting. In conclusion, we found that the decrease in IEC energy metabolic function in response to fasting is supported by a decreased gene expression of mitochondrial pathways and is likely linked to the observed decreased intestinal cell proliferation and barrier function, providing insight in the vulnerability of piglets, and infants, to decreased food intake.



**Keywords:** intestine; fasting; metabolic function; mitochondria; cell proliferation

## Introduction

The gastrointestinal tract is the gateway to the body: it enables digestion and selective uptake of nutrients, while simultaneously maintaining a barrier against the outside milieu. The intestine's absorptive and barrier functions are actualized by intestinal epithelial cells (IECs), a type of cell that is continuously generated from stem cells in the crypts, which migrate to the villus tip as they mature, in a 5–7-day cycle [1]. The intestinal epithelium forms a physical barrier to protect the host, with IECs comprising a tight layer with actively maintained cell-cell contact and specialized cells that secrete mucus to create a protective layer on top of the IECs [2]. To perform its complex role, the intestine constitutes an enormous surface area, which is divided into various longitudinal regions with distinct functions. Fermentation of poorly digestible nutrients occurs distally, in the colon, which is also important for water and electrolyte transport [3]. Digestion and uptake of nutrients mainly occurs in the small intestine [3]. Much of digestion takes place in the first part of the small intestine, the duodenum, while nutrient uptake primarily happens in the jejunum, the middle part of the small intestine, and this is therefore an important site to study functional effects of nutritional interventions [3].

Sustaining intestinal functions requires an enormous amount of energy, which is mainly produced in the mitochondria. Mitochondria consume oxygen in the oxidative phosphorylation (OXPHOS) pathway to oxidize reducing equivalents that are generated from substrates, to ultimately generate the cellular energy carrier adenosine triphosphate (ATP). Energy demand in the intestine doubles upon intake of a meal, as can be seen from the 25% increase in resting metabolic rate for humans [4], and 15-20% for growing pigs [5]. Inability to produce sufficient energy to answer to intestinal demands can impair intestinal barrier function. For example, inhibition of mitochondrial ATP production was shown to induce internalization of the tight junction protein claudin 7, resulting in decreased barrier function [6]. Intestinal permeability was also shown to be increased by the widely used drugs NSAIDs, as a likely consequence of induced mitochondrial uncoupling, decreasing energy efficiency of ATP production per mole substrate [7-9]. Furthermore, based on gene expression profiling, an increased ATP utilization has also been proposed to explain a decreased intestinal barrier integrity [10]. Together this indicates that an adequate mitochondrial energy production is essential to maintain intestinal barrier and IEC function.

To produce energy, mitochondria require substrates that in the intestine can originate either from the arterial blood supply or directly from the diet [11]. That luminal substrates are essential for intestinal mitochondrial energy production is evidenced by prolonged fasting in mice, which was found to decrease expression of electron transport chain (ETC) and tricarboxylic acid cycle (TCA) genes in the intestine [12]. Reduced availability of luminal substrates may impair intestinal function by reducing

the ability of IEC mitochondria to produce sufficient ATP, which is needed for nutrient uptake and barrier integrity. In animal husbandry, a limited availability of substrates from the diet is frequent in weaning, because it mostly associated with a check in food intake [13, 14]. This is accompanied with an increased susceptibility for intestinal disease, as evidenced by the high incidence of diarrhea at this time [15]. Globally, pig diarrhea accounts for large losses of lives and industrial profits each year, as indicated by several local studies [16-18]. Similarly in humans, childhood diarrhea accounts for nearly 1.7 billion cases every year, leading to over half a million deaths in children under 5 years of age [19]. Malnutrition is identified as one of the underlying causes potentiating diarrhea development [19, 20]. However, it is unclear whether limited luminal substate availability, and consequent decreased mitochondrial ATP production, causally contributes to the increased susceptibility of piglets and infants to develop intestinal diseases, because the effects of weaning and malnutrition are mostly studied as a whole, without isolating the specific contribution of fasting to these complex phenomena. In addition, the evidence for decreased mitochondrial function upon fasting is currently primarily obtained using indirect measurements of mitochondrial function in the intestine, using gene expression analysis as a tool. However, gene expression does not necessarily accurately reflect altered mitochondrial oxidative capacity and metabolic flux. Furthermore, it is not clear whether fasting impacts IEC ATP production as a whole, or whether mainly mitochondrial ATP production is impacted, and glycolytic ATP production largely compensates for this. Finally, it is unclear which IEC functions are primarily altered in association with decreased mitochondrial function and whether these may explain an increased vulnerability of the intestine to infection and diarrhoea. Thus, we studied whether and how fasting functionally affects IEC energy metabolism *in vivo* and with which altered molecular processes this is primarily associated. For this, we weaned piglets and fed them a standard weaning diet for two weeks, and then exposed them to a 48h fasting challenge, in order to isolate the effects of fasting from other weaning-associated challenges. Mid-jejunal IECs were isolated to measure mitochondrial respiration and glycolytic flux, using a previously optimized method [21]. In addition, molecular processes affected by the 48h fast were investigated in mid-jejunal scrapings using RNA-sequencing and confirmed using immunohistochemistry.



## Methods

### *Animals*

All experiments and methods were performed in accordance local and (inter) national guidelines. The Central Authority for Scientific Procedures on Animals (CCD), and the Animal Welfare Body (IvD) approved the protocol of the experiment (AVD1040020209884), which was in accordance with the Dutch law on animal

experimentation and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The experiment was carried out in compliance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). The study described here was started together with another study, which ran in parallel until weaning. Thirty-two gilts (Topigs Tempo x TN20) were selected from fourteen litters on a commercial farm, from sows with a parity range between one and seven (parity 1, 2 sows; parity 3, 4 sows; parity 4, 3 sows; parity 5, 2 sows; parity 6, 2 sows; parity 7, 1 sow). Cross-fostering was minimized, and if needed only applied to male piglets. Apart from Lianol basdiar (Ardol BV, Susteren, The Netherlands) in the first post-natal week, the piglets did not receive creep feed. Piglets were weaned between post-natal day 22 and 26, and were transferred to CARUS, the research facility of Wageningen University. To select gilts, all female piglets in the litter were weighed, and only the piglets with ± 1 standard deviation (SD) from the mean were selected. At CARUS, piglets were allocated to the pens based on the sow's parity, the farrowing date, the genetic background and their body weight at weaning, to minimize variation between pens and treatments. Piglets were pair-housed in 2 $m^2$ partially slated pens, and temperature was kept between 24-26.5°C with a humidity of 65%, lights and radio were switched on between 7:00 and 19:00h. Lights were dimmed to 5% and radio was off from 19:00 till 7:00h. Health and welfare were assessed visually thrice a day during feeding, and faecal consistency score was assessed (score of 1-5, 1 = liquid diarrhoea, 5 = hard faeces). The feeds were produced by Research Diet Services (RDS BV, Wijk bij Duurstede, The Netherlands) in a single batch in the form of pellets (Table 6.1). Piglets were fed at 2.4x the maintenance energy requirement (NEm = 0.29 MJ NE/kg $BW^{0.75}$), divided over three equally portioned meals a day at 7:30h, 12:30 and 17:00h. Piglets were weighed at the start of the experiment and then every week to adjust feed intake to their current weight. Water was available *ad libitum* throughout the entire study period. 48h before the end of the experiment piglets either continued to receive feed thrice a day at 2.4x NEm, or were fasted until the end of the study period. At the end of the study period, piglets were sedated using intramuscular injection of Zoletil+Xylazine (5:2 ratio, 1 mg/10 kg BW), and euthanized by lethal injection with pentobarbital (24 mg/kg BW).

Table 6.1 **Ingredient and calculated nutrient composition of the starter diet**[1].

| components | |
|---|---|
| *Ingredient composition (%)* | |
| Soybean meal | 16.5 |
| Sunflower meal | 6 |
| Wheat | 27.1 |
| Barley | 25 |
| Corn | 20 |

| | |
|---|---|
| Soya oil | 1.3 |
| L-lysine HCl | 0.52 |
| L-threonine | 0.16 |
| DL-Methionine | 0.15 |
| L-tryptophan | 0.035 |
| L-valine | 0.02 |
| Premix[2] | 0.5 |
| $CaCO_3$ | 1.3 |
| $Ca(H_2PO_4)_2$ | 0.9 |
| NaCl | 0.4 |
| citric acid | 0.1 |
| Phytase | 0.0025 |
| total | 100 |
| *Nutrient composition (g/kg)* | |
| Dry Matter | 871.6 |
| Crude Protein | 181 |
| Crude Fat | 39.3 |
| Crude Ash | 52.9 |
| Starch | 433.6 |
| Sugars | 35.7 |
| NE (MJ/kg) | 10.1 |

Abbreviations: NE = net energy.
[1] Nutrient composition was calculated based on ingredient composition and table values for the composition of the ingredients [22].
[2] Supplied per kilogram of feed: retinyl acetate, 10,000 IU; cholecalciferol, 2,000 IU; DL-a-tocopherol, 40 mg: menadione, 1.5 mg; thiamine 1.0 mg; riboflavin, 4 mg; pyridoxin-HCl, 1.5 mg; cyanocobalamin, 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; Choline chloride, 150 mg; Folic acid, 0.4 mg; Biotin, 0.05 mg; iron(II)sulphate monohydrate, 331 mg; copper(II)sulphate pentahydrate, 80 mg; manganese(II)oxide, 49 mg; zinc sulphate monohydrate, 194 mg, potassium iodate, 1 mg; sodium selenite, 0.56 mg.



### *Small intestinal cell isolation*

The method for IEC isolation was previously optimized for colonocyte isolation [21], but here successfully applied to small intestinal IEC isolation. Following excision from the abdominal cavity, the entire intestine was separated from the mesentery, and the small intestine was identified. The small intestine was spread out and 50% of the intestinal length was identified, which corresponds to mid-jejunum. A 16 cm segment distal of the 50% point was used for histological sample preparation and mucosal scrapings. A 30 cm segment proximal of the 50% point was removed and placed in aerated modified Krebs Henseleit Buffer (KHB) containing 5 mM glucose (#K3753, Sigma

Aldrich, St. Louis, MO, USA, hereafter referred to as modified-KHB), supplemented with 2.5 g/L Bovine serum albumin (BSA, #A7906, Sigma-Aldrich). When all samples were obtained, intestines were rinsed thoroughly with modified KHB and inverted. Using dialysis clamps (#Z371092, Sigma Aldrich) a sac was created by filling the inverted intestine with modified-KHB, which was then placed in 118 mM NaCl, 4.7 mM KCl , 1.2 mM $MgSO_4$, 1.2 $KH_2PO_4$, 10 mM HEPES ($Ca^{2+}$-free KHB, pH 7.4), supplemented with 10 mM Dithiothreitol (DTT), 20 mM Ethylenediaminetetraacetic acid (EDTA) and 2.5 g/L fatty-acid free BSA (#3117057001, Sigma-Aldrich) to further wash the mucus away. The intestines were incubated in this wash buffer for 20 minutes in an oscillating water bath at 37 °C. Then, the buffer was discarded and the jejunal sacs were reverted and filled with $Ca^{2+}$-free KHB buffer containing, 10 mM DTT, 400 U/mL hyaluronidase type IV (#3884, Sigma-Aldrich) and 2.5 g/L fatty acid-free BSA. After a fifteen-minute incubation, the intestinal sacs were gently massaged for 15 seconds, after which the content of the sacs was collected in 50 mL tubes. The cells were then passed through a 40 μM cellulose filter top remove debris and large tissue pieces. The collected cells were washed twice using modified-KHB containing 2.5 g/L BSA and once with pH balanced XF DMEM assay medium supplemented with pH balanced 10 mM XF glucose, 2 mM XF glutamine and 1 mM XF pyruvate. Cells were spun down at 400 *x g* for five minutes. Then, cells were counted using the Bürker chamber, and cell viability was assessed by staining cells with ViaStain (#CS2-0106, Nexcelom Bioscience, Lawrence, MA, USA) and imaging them using the Cellometer K4 (Nexcelom Bioscience). Cell yield did not differ between groups, and cell viability was above 80% for all isolated cell populations (Supplementary Figure S1).

### *Metabolic flux analysis*

Isolated IECs were plated in a XF96 cell plates that were coated with Cell-Tak (#354240, Corning, New York, NY, USA) according to manufacturer's protocol, no longer than one week prior to the assay. Per XF Extracellular Flux analysis run, IECs isolated from eight piglets were included. A standard curve was included on every plate to allow better normalization within and between plates, according to a previously published protocol [23]. To generate the standard curve, IECs from all eight pigs were pooled and plated at concentration ranging from 75,000-300,000 cells/well in 50 µL pH 7.4 balanced XF DMEM assay medium supplemented with 10 mM XF glucose, 2 mM XF glutamine and 1 mM XF pyruvate. The IECs isolated from individual pigs were plated at 140,000 cells/well in eight replicates in the same medium. Cells were then left to settle for 5 min prior to spin-down (200 x g for 2' with zero break). After spin-down, cell plates were imaged as described below, while kept at 37 °C. Following imaging, an additional volume of 130 µL assay medium was added and cell plates were incubated for another 20 minutes in a non-$CO_2$ 37 °C incubator. Extracellular flux analyses (XF assays) were performed

using the Seahorse XFe96 (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA, USA). XF assays were performed using serial injections of first a combination of 1.5 µM Oligomycin (#O4875, Sigma-Aldrich) and 1 µM FCCP (carbonyl cyanide-p-trifl uoromethoxyphenylhydrazone, #C2920, Sigma-Aldrich), followed by a combination of 1.25 µM Rotenone (#R8875, Sigma-Aldrich) and 2.5 µM Antimycin A (#A8674, Sigma-Aldrich) and finally 50 mM 2-deoxyglucose (2-DG, #D8375, Sigma-Aldrich). The XF assay protocol consisted of 12 measurement cycles of 3 minutes, with 2 minutes of mixing in between measurements.

### *Cell number quantification for metabolic Flux assay*

Brightfield images of the inner probe area of each well in the XF96 cell plates were obtained prior to the XF assay run using a 37 °C equilibrated Cytation 1 Cell Imaging Multi-Mode Reader (BioTek, Winooski, Vermont, USA) using a 4x objective. LED intensity was kept at 5 and integration time at 80 milliseconds for all cell plates. To ensure optimal image quality, focus height was adjusted by visual inspection for each cell plate. The brightfield images obtained prior to the XF assay run were then processed and quantified using an in-house generated R-script [23], that uses the EBImage package available for Bioconductor [24]. A low-pass Gaussian blur filter was applied to generate a background image, which was subsequently subtracted from the original image. The background corrected image was then inverted and cropped by 5% to remove potential boundary noise from the XF assay plate molded stops. The final processed images were used to calculate the total pixel intensity for each image. To convert pixel intensities back to cell numbers, the values of the internal plate standard curves were fitted with a second order polynomial regression. The obtained cell numbers were finally used for normalization of the Seahorse XF assays. Per pig, eight replicate wells were included during Seahorse XF analysis and normalisation, and mean of all eight replicates was then used for further processing.



### *Tissue collection and RNA isolation*

Mucosal scraping samples were obtained from mid-jejunal section. Mucosal scrapings were snap frozen in liquid nitrogen and stored at -80°C until use. In order to isolate RNA, mucosal scrapings were first crushed to powder in liquid nitrogen with pestle and mortar. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. For each sample, 5-10 mg of powdered tissue was dissolved in a mixture of RLT buffer (Qiagen, Hilden, Germany) and -mercaptoethanol (M6250, Sigma Aldrich). Tissue samples were subsequently lysed and homogenized by shaking at maximum speed in a thermomixer (Eppendorf, Hamburg, Germany) at room temperature for at least 45 minutes. Then, 70% ethanol was added, and total RNA was bound on the RNeasy spin column. After DNA

digestion with DNase I treatment, the column was washed three times with RW1 and RPE buffer, and total RNA was eluted in RNase free water. Quality of the RNA was assessed using Nanodrop spectrophotometer (IsoGen Life Science, de Meern, the Netherlands) and Agilent 2200 Tapestation (Agilent Technologies Inc., Santa Clara, CA, USA). Samples met the criteria with a ratio higher than 1.8 for both 260/280 and 260/230 or an RNA integrity number (RIN) above 7.

### *RNA-seq and data analysis*

RNA preparation, library construction, sequencing on the DNB-sequencing platform and read clean-up were performed at Beijing Genomics Institute (BGI, Hongkong). Quality check of the clean reads was preformed using FASTQC [25]. Reads were aligned to the pig genome (Sscrofa11.1.104) using STAR2.7 [26], and counts were quantified using HTSeq [27]. Average sequencing depth was 24M paired end reads, of which at least 92.9% were uniquely mapped. After read alignment and counting, data analysis and statistical testing was performed in R 4.1, using appropriate Bioconductor packages.

Genes with a total sum of less than 10 counts were removed. Then, differentially expressed genes (DEGs) between fed and fasted piglets were identified using the DE2seq package, using Benjamini-Hochberg correction for multiple testing [28]. An adjusted *p*-value of below 0.05 was considered significantly regulated. Principal component analysis was performed using variance stabilizing transformed (VST) data. Gene set enrichment analysis (GSEA) [29] was performed using clusterProfiler [30] for the Reactome annotated gene sets [31] that were extracted using the Molecular Signatures Database (MSigDB [32]). Gene sets were considered enriched with a Benjamini–Hochberg (BH) adjusted *p*-value < 0.05. Only the pathways with a *p*-value < $3.5 \times 10^{-3}$ were included for further analysis and clustering. The 25 pathways that remained were then clustered based on semantic similarities in the Gene Ontology (GO) description of the genes in each pathway, using the GoSEMSim Package [33]. The human MitoCarta 3.0 gene set was used as a reference inventory for mitochondrial genes [34]. The gene set for barrier genes as assembled by Vancamelbeke *et al.* was used to identify significantly regulated barrier genes [35]. Volcano plots and heatmaps were generated using the EnhancedVolcano and ComplexHeatmap packages [36, 37]

### *Immunohistochemistry*

Mid-jejunal tissue sections were cut longitudinally, washed in cold PBS, rolled up proximally to distally, and placed inside a histology cassette. Tissue was then fixed in 4% Paraformaldehyde (1.040.051.000, Merck, Kenilworth, NJ, USA) and stored at 4˚C for 24h, after which tissues were dehydrated as follows; 4h 70% EtOH, 4h 80% EtOH,

4h 90% EtOH, 4h 100% EtOH, 4h 100% EtOH, 4h 100% EtOH, 3h xylene, 2h xylene, 2h xylene and 4h paraffin, and embedded in paraffin blocks.

Tissue sections of 5 µm were cut using a Leica microtome, and subsequently placed at ~42 ˚C to stretch the samples, before mounting them on a glass slide, and drying them overnight at 37˚C. Prior to staining, slides were deparaffinised (2x5' Xylene, 2x3' 100% EtOH, 3' 96% EtOH, 3' 70% EtOH, 2x3' demi water), followed by a ten-minute heat-mediated antigen retrieval in 0.1M sodium citrate buffer (pH 6.0) at sub-boiling temperatures by microwaving the slides. Slides were then cooled to room temperatures and rinsed in Tris-buffered saline (TBS) (pH 7.4). To block aldehyde residues, slides were then incubated in 0.75% glycine in TBS for 20 minutes. After rinsing with TBS, sections were pre-incubated with 5% (v/v) normal goat serum (Vector Laboratories, Peterborough, UK) in TBS for 60 min at room temperature in a humidifying box. Slides were subsequently incubated overnight at 4°C in a humidifying box with primary rabbit polyclonal anti-Ki67 (1:500, ab15580, Abcam, Cambridge, UK) anti-body diluted in TBS-BSA-c (Aurion, Wageningen, The Netherlands). Slides were then rinsed with TBS and treated with a fluorochrome labelled goat-anti-rabbit (Alexa Fluor 546, Invitrogen, CA, USA) diluted 1:200 (v/v) in TBS-BSA-c for 1 hour at room temperature. Finally, nuclear counterstaining was performed for 10 minutes using DAPI (1 µg/ml; Sigma-Aldrich, Saint Louis, MO, USA). Sections were imaged at 10 times magnification using fluorescence microscopy (Leica DM6B), a digital camera (DFC365 FX) and imaging software (LasX; all Leica Microsystems, Amsterdam, The Netherlands). Per slide, 10 representative images were obtained and included in the analysis. Images were analysed according to a previously published protocol [38]. First, images were loaded into CellProfiler software (Broad institute) to select the appropriate objects. Briefly, DAPI objects were identified that ranged between 2 and 20 pixels. Then, only Ki67 stained objects that overlapped with the previously identified DAPI stained objects were selected. Data for the identified objects was then exported both as a spreadsheet and as images, and those files further processed using the FCS express software (De Novo software, Pasadena, CA). First, large DAPI objects, often due to artifacts in the tissue during processing, were removed. Then, low intensity and high intensity DAPI images were excluded. Lower bound of intensity was identified as median image intensity – 2SD, and upper bound of image intensity was identified as median image intensity + 3SD. Subsequently, the low intensity Ki67 stained object, which could be artifacts or background staining, were removed by taking median image intensity +1 SD as the lower bound, and median image intensity + 10 SD as the upper bound. The percentage of cells in the Ki67 gate over the total amount of cells in the DAPI gate was taken as the final ratio of Ki67 over DAPI stained cells. See Choudhury *et al.* for further details about image processing [38].

### *Data availability*

The RNA-sequencing data has been deposited in the GEO database under ascension number GSE203439. The R-script for image analysis is available from GitHub.

### *Statistical data analysis*

Data are presented as mean ± standard deviation. Piglets were pair-housed for the duration of the experiment, but due to the nature of the intervention and analysis performed, individual pigs were analysed as experimental units. For the body-weight measurements, eight datapoints were missing because they were not assessed, while all other analysis were performed with n=16 per group. Normality of model residuals was checked using Shapiro-Wilk test, and if assumptions were not met, data was transformed as indicated in the figure legends. If all assumptions for normality were met, statistical testing was performed using student's t-test or repeated measures ANOVA, as indicated in figure legends. A $p$-value of $< 0.05$, or a BH adjusted $p$-value of $<0.05$ was considered statistically significant. Repeated measures ANOVA was performed in SPSS Version 25.0 (IBM Corp, Armonk, NY), all other statistical analyses and data visualisations were performed using GraphPad Prism v.9 (GraphPad Software, CA, USA) and R version 4.1.

## Results

All piglets received an equal amount of a standard weaning diet throughout the two-week period following weaning, resulting in equal growth in both fed and fasted groups in the two weeks preceding the fasting intervention (Fig. 6.1A). As expected, the 48h fasting intervention led to a significant decrease in weight in the fasted group (Fig. 6.1A-B, $p < 0.001$), while the fed group continued to eat and gain weight throughout the 48h intervention period (Fig. 6.1B). Fasting also significantly decreased small intestinal (SI) weight, both absolute SI weight and SI weight corrected for bodyweight (Fig. 6.1C-D, $p < 0.0001$). Absolute SI length was also decreased in response to fasting (Fig. 6.1E, $p < 0.01$), but no significant effect of fasting on SI length relative to bodyweight was observed (Fig. 6.1E-F).

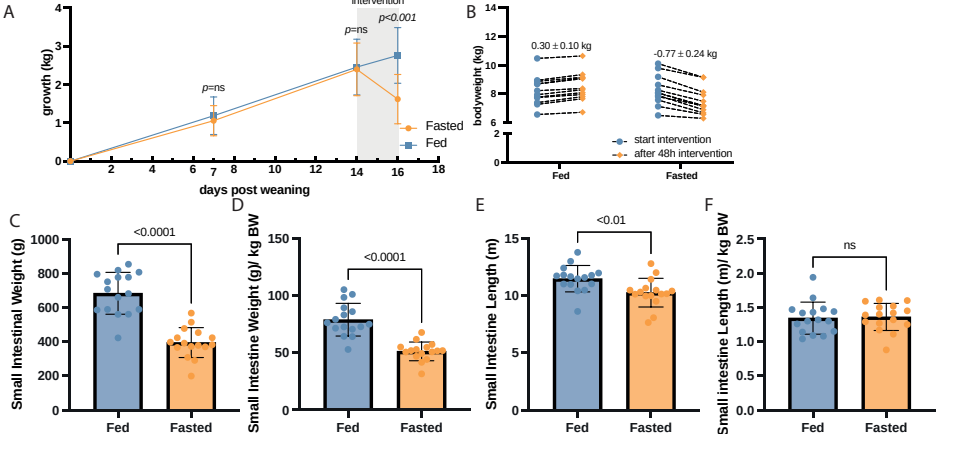


Figure 6.1 **The macroscopic effects of 48h fasting in 6-week-old piglets.** (**A**) Piglet growth during the study-period, n=12 per group. (**B**) Piglet weight for the fed and fasted groups before and after the start of the 48h fasting period, n=12 per group. (**C-D**) Small intestinal weight at the end of the study period, raw values and corrected for bodyweight, n=16 per group. (**E-F**) Small intestinal length at the end of the study period, raw values and corrected for bodyweight, n=16 per group. Data are presented as mean ± SD and each dot in the bar-graphs represent an individual piglet. Significance was determined using repeated measures ANOVA (piglet weight), or Student's t-test (intestinal weight and length).

Metabolism of isolated IECs was functionally assessed using seahorse XF analysis. Fasting decreased basal mitochondrial OCR by 51±9.9% (Fig. 6.2A, $p < 0.0001$), and likewise reduced mitochondrial capacity, as was shown by a reduction of maximal mitochondrial OCR by 43±9.8% (Fig. 6.2B, $p = 0.0001$). In addition, fasting decreased basal glycolytic function by 45±10.3% (basal glycolytic proton efflux rate (glycoPER), Fig. 6.2C, $p < 0.0001$) and compensatory glycolytic function by 35±14.8% (compensatory glycoPER, Fig. 6.2D, $p < 0.05$). Overall, fasting thus simultaneously decreased oxidative and glycolytic IEC extracellular flux, leading to decreased metabolic oxidative capacity during fasting (Fig. 6.2E).

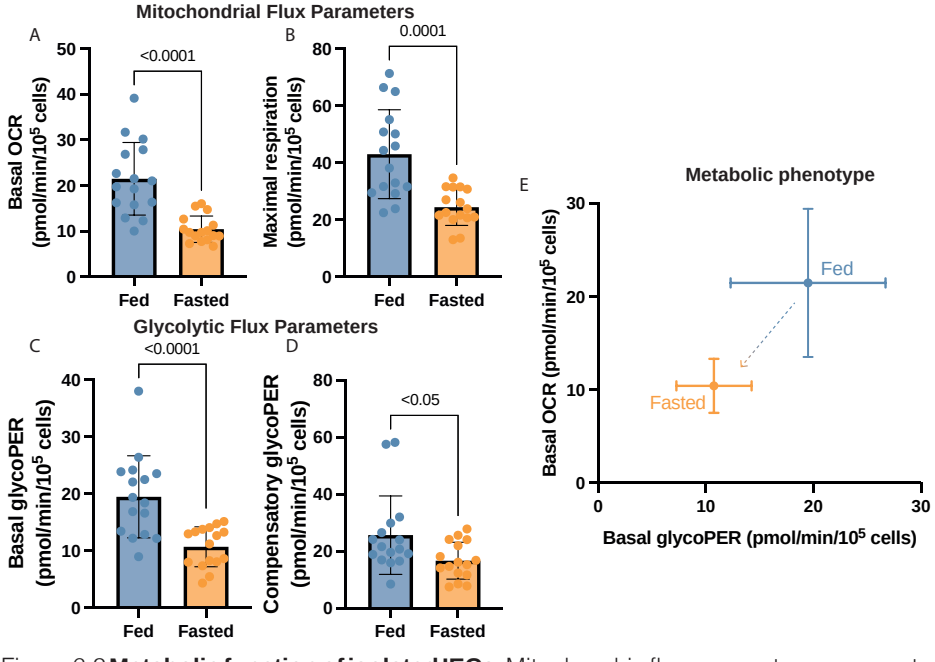



Figure 6.2 **Metabolic function of isolated IECs**. Mitochondria flux parameters represented by: (**A**) Basal OCR and (**B**) Maximal respiration. Glycolytic flux parameters represented by: (**C**) Basal glycoPER and (**D**) Compensatory glycoPER. (**E**) Metabolic phenotype graph, showing basal OCR versus basal glycoPER. Data are presented as mean ± SD, n=16 per group. Significance was determined using Student's t-test. Maximal respiration and Compensatory glycoPER were log-transformed to meet normality assumptions.

Intestinal epithelial gene expression was analysed using RNAseq analysis. PCA analysis on transformed counts from RNAseq data using variance stabilizing transformation revealed a clear separation between the fed and fasted six-week-old piglets; principal component 1 explained 33% of the variation in the data (Fig. 6.3A). Gene set enrichment analysis (GSEA) using the Reactome curated gene sets showed that 34 pathways were significantly regulated upon fasting. To select the most regulated pathways, we only included the pathways with a *p*-value below $3.5x10^{-3}$, which left us with 25 pathways. These 25 most regulated pathways were grouped in five clusters, using an algorithm based on semantic similarities in the gene ontology (GO) description of the genes in the pathways (Fig. 6.3B; [33]). Fasting downregulated the pathways in clusters 2 through 5, while the pathways in cluster 1 were upregulated upon fasting. Cluster 1 consisted of five pathways primarily related to protein translation. The top 10 genes in the cluster were involved in protein translation initiation (*EIF3A*, *EIF3G*, *EIF4B* and *EIF3H*) or were part of the ribosome machinery (*RPL30*, *RPS16*, *RPS11*, *RPS28*, *RPS8* and *RPL11*) (Fig. 6.3C). Cluster 2 consisted of ten pathways that were primarily related to cell-cycle. The top 10 genes in the cluster were related to cell-cycle progression (*ANAPC7* and *HSP90AA1*), microtubular organization (*TUBB4B*, *TUBA4A*, *CETN2* and *DYNC1LI1*), chromatin structuring (*WAPL*) and organelle assembly (*GORASP2*). Cluster 3 consisted of three pathways related to mitochondrial function. Top 10 genes in the cluster were mainly TCA-cycle enzyme genes (*DLTS*, *SUCL2A*, *IDH3A*) and ETC complex genes (*COX6B*, *NDUFS1*, *NDUFS3*, *NDUFA6* and *NDUFB5*), but also other metabolically important genes such as malic enzyme (*ME1,* converts malate to pyruvate) and basigin (*BSG,* plays a role in correct localization of glucose transporters on the cell-surface). Cluster 4 consisted of three pathways related to stress response. The top 10 genes in the cluster were involved in proteosome assembly and stability (*PSME3*, *PSMD14*, *PSMD11* and *HSPA8*), signal transduction (*MAP2K3* and *HSP90AA1*), nuclear protein import (*KPNA4*) and protein translation initiation and quality control via protein (un)folding (*EIF4E, CANX* and *HSP90B1*). Cluster 5 was the least well-defined cluster, containing four pathways related to protein folding, extracellular matrix organization and transporters. The top 10 regulated DEGs in cluster 5 are involved in protein quality control and extracellular matrix organization (*COL8A1*, *CANR*, *CANX*), lipid metabolism (*SCD* and *ELOVL6*), glycoprotein and glycolipid production (*GFPT1*, *GMPPB* and *GNPNAT1*) or are transporters (*SLC10A2* and *VDR*).

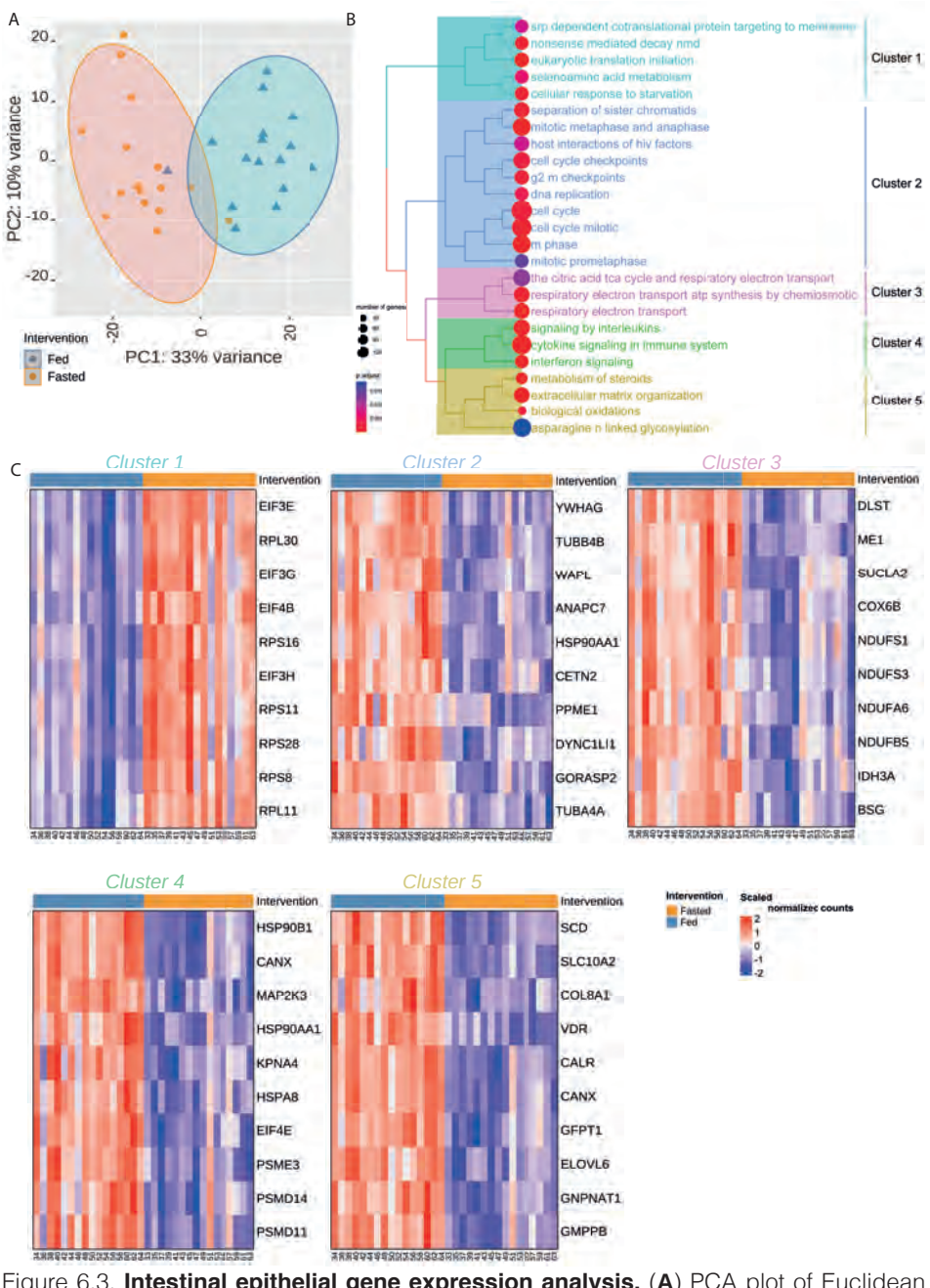
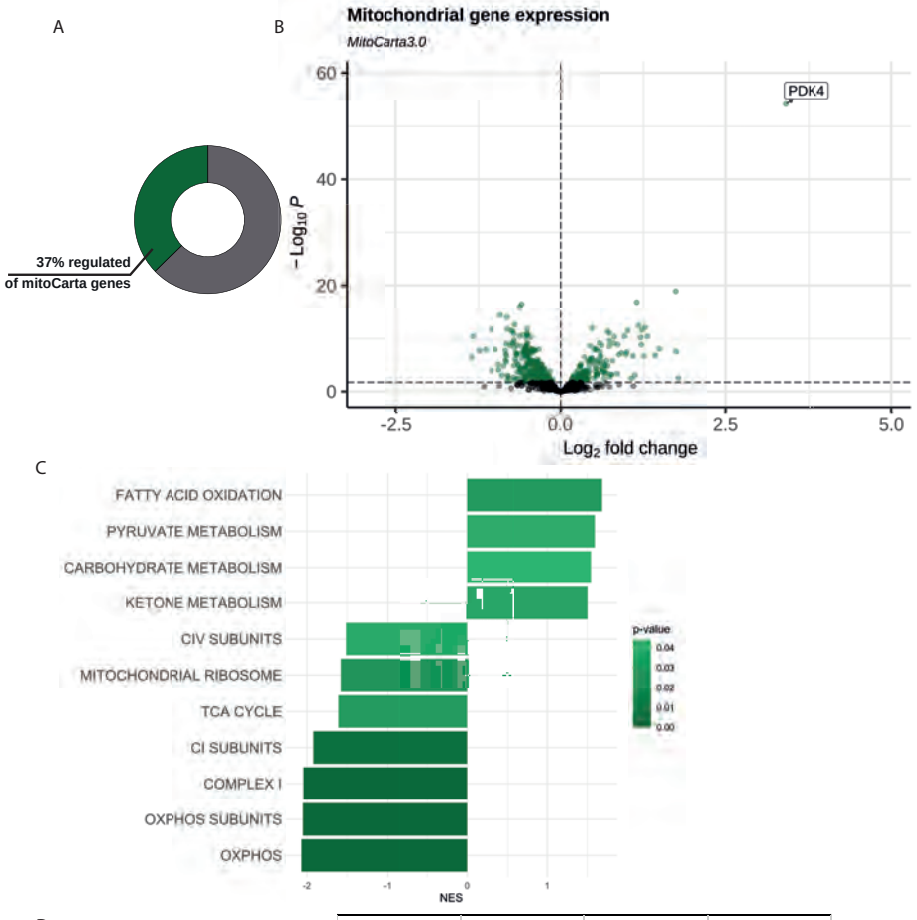



Figure 6.3. **Intestinal epithelial gene expression analysis.** (**A**) PCA plot of Euclidean distance between samples on variance stabilizing transformed data. Samples were coloured based on group. (**B**) Treeplot of Reactome pathway analysis of the differentially expressed genes (DEGs), clustered on similarity using the Jaccard similarity coefficient. (**C**) Heatmaps of the top 10 significantly regulated genes in each cluster. Scaled normalized counts were plotted for each individual gene and piglet, n=16 per group.

One of the primary regulated gene expression clusters consisted of the downregulated mitochondrial energy metabolism pathways in cluster 3, which is in full accordance with the downregulation of basal and maximal mitochondrial OCR upon fasting in our functional metabolic analysis in freshly isolated pig IECs (Fig. 6.2). To gain an even deeper understanding of the molecular changes that occur within the mitochondria upon fasting, we specifically analysed mitochondrial gene expression. We used the MitoCarta 3.0 annotated gene set: a gene set containing 1139 genes with strong support for mitochondrial localization [34]. Fasting piglets led to altered expression of 424 of the MitoCarta genes, which was 37% of all MitoCarta genes (Fig. 6.4A). Of those, 147 DEGs were upregulated, while 278 DEGs were downregulated (Fig. 6.4B, Supplementary Table S6.1). Especially the expression of *PDK4*, a critical regulator of the pyruvate dehydrogenase complex, stood out as being highly upregulated (Fig. 6.4B). We then performed GSEA analysis using the pathway annotation of MitoCarta 3.0 (Fig. 6.4C). Fasting of the piglets resulted in a significant regulation of eleven mitochondrial pathways, four of which were upregulated and seven that were downregulated. The genes with the highest $\log_2$FC for each pathway are listed in Figure 6.4D, and are given below in brackets after each pathway. Upregulated pathways were fatty acid oxidation (*ACAD11* and *ACAA2*), ketone metabolism (*BDH1* and *ACAA2*), carbohydrate metabolism (*PDK4* and *GLYCTK*) and pyruvate metabolism (*PDK4* and *PDK2*). When looking specifically at the genes regulated within the carbohydrate and pyruvate metabolic pathways, we observed that mainly the gluconeogenic genes were upregulated, while overall carbohydrate metabolism was downregulated (Supplementary Fig. S6.2). Downregulated pathways were mainly related to expression of TCA cycle enzymes (*SUCLA2* and *IDH3A*) and electron transport chain complex I (*NDUFAB1* and *NDUFA5*) and complex IV (*COX7A2* and *NDUFA4*). In addition, fasting led to decreased expression of mitochondrial ribosomal protein expression (*MRPS28* and *MRPS27*).




D

| | gene 1 | $log_2$FC | gene 2 | $log_2$FC |
|---|---|---|---|---|
| fatty acid oxidation | ACAD11 | 1.74 | ACAA2 | 0.88 |
| pyruvate metabolism | PDK4 | 3.4 | PDK2 | 1.17 |
| carbohydrate metabolism | PDK4 | 3.4 | GLYCTK | 1.23 |
| ketone metabolism | BDH1 | 0.92 | ACAA2 | 0.88 |
| CIV subunits | COX7A2 | -0.55 | NDUFA4 | -0.52 |
| mitochondrial ribosome | MRPS28 | -0.71 | MRPS27 | -0.66 |
| TCA cycle | SUCLA2 | -0.72 | IDH3A | -0.7 |
| CI subunits | NDUFAB1 | -0.65 | NDUFA5 | -0.58 |
| Complex I | NDUFAF4 | -0.86 | NDUFAF5 | -0.67 |
| OXPHOS subunits | NDUFAB1 | -0.65 | NDUFA5 | -0.58 |
| OXPHOS | HIGD1A | -1.15 | NDUFAF4 | -0.86 |

Figure 6.4 **MitoCarta analysis of differentially expressed genes (DEGs).** (**A**) Pie-chart showing that 37% of the 1126 genes in the MitoCarta 3.0 set were significantly regulated upon 48h fasting in piglets. (**B**) Volcano plot displaying the DEGs of the MitoCarta 3.0 gene set. 886 of the 1126 genes in the MitoCarta 3.0 set were present in our dataset and plotted in the volcano-plot. Of those 886 genes, 424 genes were differentially regulated between fed and fasted piglets. (**C**) Gene Set Enrichment Analysis (GSEA) of the MitoCarta 3.0 annotated mitochondrial pathways, showing which mitochondrial pathways were significantly regulated with a nominal *p*-value < 0.05. (**D**) List of the two genes in each pathway with the highest Log2FC upon 48h fasting in piglets.

In addition to regulation of mitochondrial genes, we found that fasting had the most profound effect on cell cycle, since the largest of the five clusters, cluster 2, consisted of ten pathways that were all related to cell cycle and proliferation (Fig. 6.3B). This suggests that small intestinal jejunal cell turnover is lower in fasted piglets. To verify this, we analysed cell proliferation immunohistochemically using the marker Ki67, which is expressed only in the nucleus of dividing cells. Indeed, immunostaining showed that fasting decreased the ratio of the proliferating cells over total number of cells by 43±5.3% (Ki67/DAPI, Fig. 6.5A and B, $p < 0.05$), confirming the finding that fasting significantly decreased cell proliferation in the jejunum of six-week-old piglets.

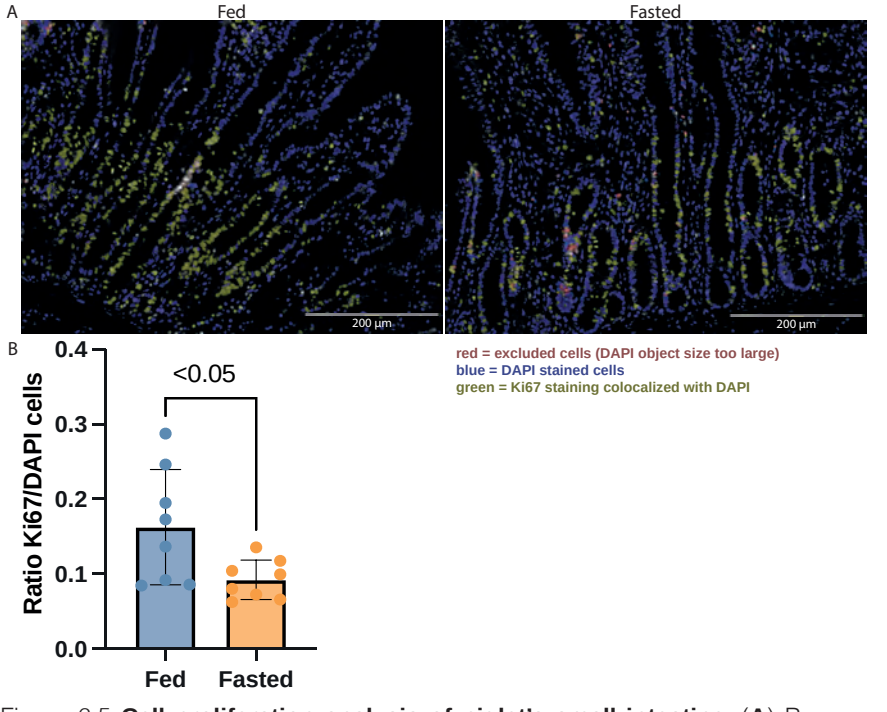



Figure 6.5 **Cell proliferation analysis of piglet's small intestine.** (**A**) Representative images of processed immunohistochemical analysis of fed and fasted piglets stained with Ki67. Red objects are DAPI stained objects that are larger than the defined size. Scalebar is 200 mM. (**B**) Bar-graph of the Ki67 stained cells corrected for the total number of cells per crypt. Data is presented as mean ± SD, n=16 per group. Significance was determined using Student's t-test.

Decreased cell proliferation has been linked to a decreased barrier function [39]. We hypothesized that the fasting intervention, and the resulting decreased intestinal metabolic function and cell proliferation, contributes to decreased intestinal barrier function. Therefore, we investigated the effect of fasting on intestinal barrier function related genes, using a gene set described by Vancamelbeke *et al.* [35], that includes genes classified as being involved in one of eight categories: mucus layer, tight junctions, adherence junctions, desmosomes, hemidesmosomes, cytoskeleton, extracellular

matrix and regulating proteins. We found that of the 128 genes in this gene set, 93 were present in our dataset (Fig. 6.6A). Out of these 93 genes, 26 were significantly regulated, the majority of which were downregulated (Fig. 6.6B). The most significantly regulated genes were mucus genes (*TFF1, TFF2* and *EMCN*), tight junction genes (*CLDN2*, *MARVELD3*), an adherence junction gene (*CDH1*, also known as E-cadherin), regulating genes (*MAGI3* and *GNA12*), and a cytoskeleton gene (*MYL9*). These data suggest an overall decreased intestinal barrier function in response to fasting.

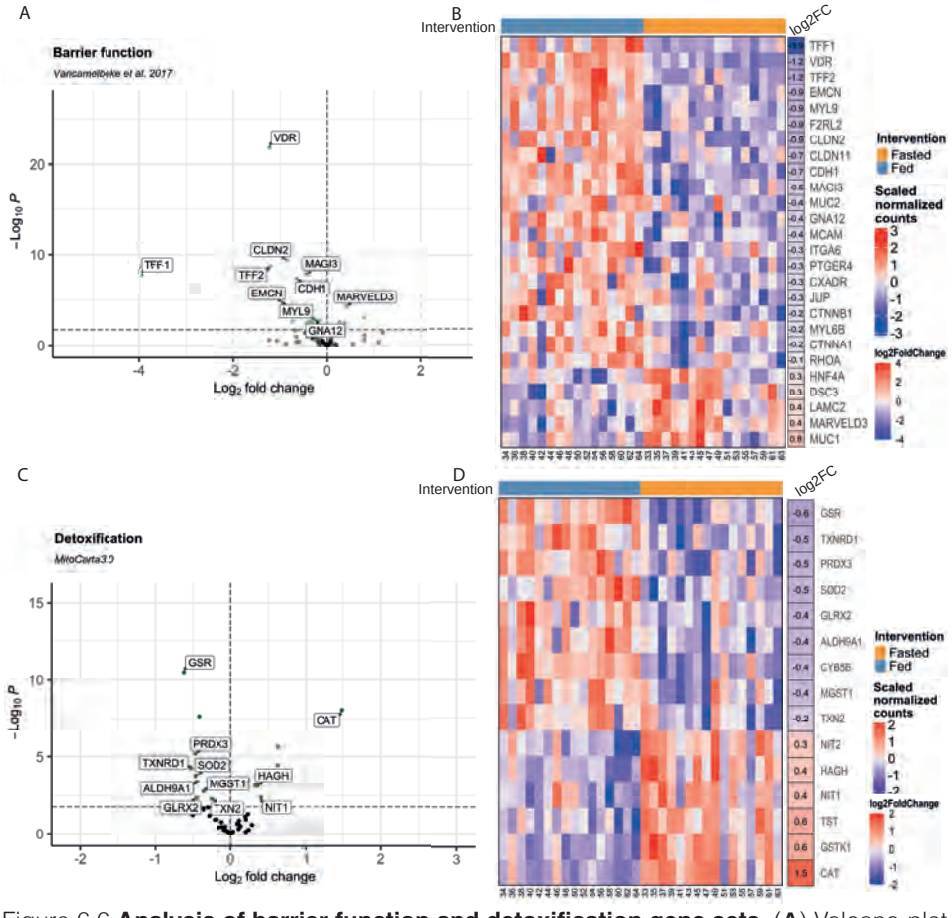



Figure 6.6 **Analysis of barrier function and detoxification gene sets.** (**A**) Volcano-plot of barrier function genes as annotated by [35]; 93 of 128 genes are present and plotted in the volcano-plot, and 26 are significantly regulated. The top ten significantly regulated genes are labelled. (**B**) Heatmap of the 26 significantly regulated genes in the barrier function gene-set, with scaled normalized counts per pig and an additional column depicting the Log2FC of each gene in response to fasting (log2FC value plotted in the square). (**C**) Volcano-plot of mitochondrial detoxification genes [34]. 42 of the 51 genes are present and plotted in the volcano-plot, and 15 are significantly regulated. The eleven redox detoxification specific genes are labelled. (**D**) Heatmap of the 15 significantly regulated genes in the detox gene-set, with scaled normalized counts per pig and an additional column depicting the Log2FC of each gene in response to fasting (log2FC value plotted in the square).

Fasting is common during the weaning period in pigs. During the weaning transition, increased oxidative stress has been observed in the intestine [40-44]. Because mitochondria are a major source of reactive oxygen species (ROS) [45], we specifically analysed the MitoCarta 3.0 annotated mitochondrial detoxification pathway. Out of the 42 genes present in the dataset, fifteen were regulated upon fasting (Fig. 6.6C and D), nine of which were downregulated and six were upregulated. Out of the fifteen significantly regulated detoxification genes, eleven were specific redox detoxification genes (see labelled genes in Fig. 6.6C). Eight redox genes were downregulated (most prominently *GSR*, *TXNRD1*, *PRDX3* and *SOD2*), and three were upregulated (*CAT*, *HAGH* and *NIT1*). Thus, we observe both an up and downregulation of mitochondrial redox genes, suggesting an adaptation of redox homeostasis rather than a change in a specific direction.

## Discussion

Intestinal tissues rely on sufficient energy production to maintain homeostasis. A decreased expression of key energy-producing genes has been observed previously upon fasting in mice and weaned in piglets, which is accompanied by reduced food intake [12, 43], but concomitant functional data were lacking. In this study, we showed that 48h fasting decreased both the mitochondrial and the glycolytic extracellular flux of isolated jejunal IECs in six-week-old piglets. Since metabolic extracellular flux analysis of the isolated IECs was performed in the presence of sufficient energy substrates (pyruvate, glucose and glutamine), this indicated that metabolic function of IECs is not only decreased because of a lack of substrates, but due to long-lasting changes in mitochondrial function. We not only substantiate literature findings by providing functional data, but also validated our own transcriptome analysis where mitochondrial pathways were among the top regulated Reactome gene sets upon fasting. Focussed analysis of mitochondrial gene expression indeed revealed that especially the expression of TCA-cycle enzymes and ETC complex I and complex IV were downregulated. Isolation of IECs and assessment of their metabolic flux is thus a viable tool to assess IEC functionality in response to a physiological intervention. This tool can be particularly useful to explore the contribution of fasting to the overall decreased intestinal resilience observed upon weaning in piglets, by isolating the feed-deprivation response from all other physiological challenges occurring during this transitionary phase. We were therefore able to show that decreased metabolic function upon fasting coincided and possibly underlies alterations in e.g. cell proliferation and barrier function. Decreased or absent feed intake could thereby make the intestine more prone to develop disease

In this study, 48h fasting induced significant metabolic changes in the intestine of

young, six-week-old piglets, with *PDK4* standing out. *PDK4* was increased 3.4-fold and was previously shown to be induced by fasting in the intestine of mice [12, 46]. *PDK4* is a well-known metabolic regulator that drives cells away from glucose oxidation and towards fatty acid oxidation [47]. Indeed, higher PDK4 expression has been suggested as marker of increased fatty acid oxidation [48]. The higher *PDK4* expression is thus expected to induce fatty acid oxidation, which was indeed the most upregulated mitochondrial pathway in our data (Fig. 6.4). Increased fatty acid oxidation, and likely lipid breakdown, is strengthened by the simultaneous decreased expression of key lipogenic genes such as *ACLY* and *FASN* (Supplementary Table S6.1). Mechanistically, the increased expression of *PDK4* may be also linked to the observed upregulation of ketogenesis. The ketone body ß-hydroxybutyrate has been identified as a post-translational histone lysine modification [49], and histone ß-hydroxybutyrylation induced *PDK4* expression in the intestine of fasted mice [50]. Via ß-hydroxybutyrylation, ketogenesis could thus potentially contribute to the induction of lipolysis in the intestine of fasted piglets. This, however, requires experimental confirmation. Because *PDK4* is expected to increase lipid oxidation and simultaneously decrease carbohydrate metabolism, the apparent upregulation of carbohydrate metabolism was unexpected (Fig. 6.4). Close inspection however, revealed that the key gluconeogenic genes were upregulated, while overall carbohydrate metabolism was indeed decreased, as expected (Supplementary Fig. S6.1). Increased gluconeogenesis is concomitant with increased *PDK4* expression [51], and is in line with the increased recognition that intestinal gluconeogenesis upon fasting is important for maintaining whole-body glucose levels [52-54]. The importance of gluconeogenesis for piglet health is furthermore highlighted by the finding that decreased gluconeogenesis contributed to postnatal growth retardation in pigs [55]. Together, our data underpin the role of *PDK4* as a central regulator of metabolism upon fasting, also in the small intestinal epithelium.



Even though the intestine plays a key role during fasting by providing ketone bodies and glucose to support whole-body metabolic function, this metabolic shift appears insufficient to maintain nutrient flux in the IECs, as is apparent from the overall decreased mitochondrial and glycolytic function of isolated IECs upon fasting as measured using extracellular flux analysis in freshly isolated IECs. This can possibly be explained by the fact that other organs could be prioritized over the intestine, as occurs physiologically during intensive exercise to particularly support muscles [56] or during starvation to ensure brain energy supply [57]. In addition, mitochondria are key regulators of apoptosis [58]. Lowering mitochondrial metabolism could serve to prevent induction of apoptosis [59]. Our gene expression data do not indicate that apoptosis is regulated or that cell death is a prevalent response to fasting, possibly because of the decreased mitochondrial metabolism.

The decreased overall mitochondrial metabolic capacity may impact other key functions of the intestine, either as a direct consequence of energy shortage or otherwise. We therefore investigated which key intestinal functions are further affected by fasting in piglets, and found that proliferation is markedly affected by 48h fasting. This has also been described in the intestine of mice [12], and agrees with the decreased cell proliferation that is often observed in response to the weaning associated decrease in feed intake [60]. The IECs typically have a very high turnover rate, with renewal every 5-7 days [1] and as many as 300 cells are being produced in a single adult mouse crypt [61]. This high level of proliferation and renewal is needed to withstand the constant exposure to the abrasive luminal environment. Failure to maintain intestinal proliferation could contribute to intestinal dysfunction. Thus, a decreased proliferation could possibly result in a decreased barrier function. That this may be the case is supported by our data, because we observed an overall decreased expression of barrier function genes (Fig. 6.6). Not only tight junction genes and adherence genes (e.g., *CLDN2* and *CDH1),* but also mucus genes were downregulated (e.g., *TFF1, TFF2* and *EMCN*). Barrier function maintenance, upheld by, among others, tight junction gene expression and mucus production, is a crucial, but energy-demanding process, that is often reported to be decreased upon insufficient feed intake upon weaning [62, 63]. These findings highlight the importance of maintaining sufficient feed intake throughout the post-weaning period, to support key intestinal processes such as barrier function and cell proliferation.

A striking finding in our study was that translation initiation factors and ribosomal gene expression was increased in response to fasting (Fig. 6.3). Possibly, this is an example of “translation on demand”, meaning that mRNA transcription is maintained to ensure rapid translation upon reintroduction of feed [64]. Indicative of the “translation on demand” mechanism is increased mRNA abundance observed in the intestine of fasted young rats to prepare for elevated protein synthesis upon acutely increased energy availability [65]. Alternatively, the initiation factors that are upregulated may be involved in translation of mitochondrial genes, as was recently found for EIF3 [66]. Nonetheless, the increased expression of the eukaryotic initiation factors and the ribosomal proteins appears inadequate to maintain critical intestinal functions such as maintaining metabolic function, cell proliferation and barrier function upon 48h fasting in piglets.

Several studies have found that weaning induced oxidative stress in piglets. For example, ROS levels were found to be increased in serum and jejunal mucosa of weaned piglets [41, 42]. It is often hypothesized that weaning is additionally accompanied by decreased antioxidant capacity, further exacerbating oxidative stress. However, the results in literature vary greatly. For example, the expression of antioxidant genes

such as *CAT* and *GPX2* [41], *GPX2* and *GPX4* [43], and *GPX2*, *SOD3* and *CAT* [44] were found to be decreased upon fasting. But some of those studies simultaneously report antioxidant genes that are not regulated upon weaning, e.g. *SOD1* and *SOD2* [41]. In addition, and in contrast to the previously mentioned studies, Novais *et al.* reported increased expression of antioxidant defence genes such as *TXNRD2* and *GPX2* [40]. Interpretations of these findings is further complicated by the fact that studies often employ very targeted analysis of only a small set of selected genes. Because mitochondria are a major source of ROS [45], especially when they become dysfunctional, we investigated the impact of fasting on specifically on mitochondrial redox defence, and found no evident regulation in one direction (Fig. 6.4 and Fig. 6.6). Thus, although there is both significant up and downregulation of specific genes, there is no clear pattern in the mitochondrial redox homeostasis alterations. There may still be increased ROS formation upon fasting. However, additional pathways could be employed to mitigate them, such as decreased mitochondrial oxidation to prevent ROS formation [67]. Thus, the observed decreased mitochondrial function may be a way to alleviate redox stress.

The findings presented in this study are of obvious relevance for pigs, but also have potential ramifications for humans. Malnutrition is a frequently in lower income countries, where food availability is not guaranteed [68]. Because research in infants is not ethically justified, alternative animal models with close similarity to humans are needed to broaden our understanding of how the intestine of young individuals responds to periods of fasting. Pigs are a good model for humans, especially for large organs systems like the intestine [69]. In addition, pigs have similar responses as humans upon nutritional interventions [70]. This makes the findings of our study interesting to also translate to infants, highlighting the importance of adequate enteral feed intake to prevent onset of disease.



We found that 48h fasting reduced the capacity of the intestinal epithelium to generate sufficient ATP and that this coincides with decreased cell proliferation and intestinal barrier function. Insufficient intestinal epithelial substrate availability may thus contribute to enhanced susceptibility to infection and diarrhea. Our functional analysis of metabolic function of IECs provides a tool to further study and substantiate this. In addition, specific gene expression sets related to metabolic responses in the intestinal epithelium can be developed using the tools presented in this study. However, this will require further studies to develop sets that are robust to other influences than fasting. Finally, our data indicate that targeted support of mitochondrial energy metabolism may provide a strategy to prevent negative consequences of limited intestinal epithelial substate availability. This may help to combat disease susceptibility in the weaning period in pigs, but it may also have a role in preventing or alleviating infant diarrhea.

**Acknowledgements:** We would like to thank Bart Lagerwaard, Bert Beukers, Arjan van Dolderen, Jorden Uiterwijk, Carla Boersma and Ruiyu Zhang for skilful technical assistance technical assistance.

**Authors contributions: A.F.B.:** Conceptualization, Data curation, Formal analysis, Investigation, Visualisation, Writing – Original draft and Writing – Review & Editing; **V.C.J.d.B.:** Supervision, Funding Acquisition, Conceptualization, Methodology and Writing – Review & Editing; **W.J.J.G.:** Supervision, Funding Acquisition, Conceptualization and Writing – Review & Editing; **J.K.:** Supervision, Funding Acquisition, Conceptualization, Validation and Writing – Review & Editing

## References

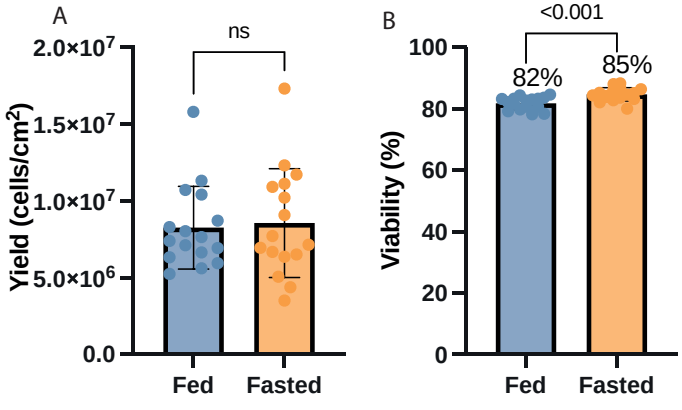

1. Potten, C.S., C. Booth, and D.M. Pritchard, *The intestinal epithelial stem cell: the mucosal governor.* International Journal of Experimental Pathology, 1997. **78**(4): p. 219-243.
2. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nature Reviews Immunology, 2014. **14**(3): p. 141-153.
3. Kiela, P.R. and F.K. Ghishan, *Physiology of Intestinal Absorption and Secretion.* Best practice & research. Clinical gastroenterology, 2016. **30**(2): p. 145-159.
4. Rolfe, D.F. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals.* Physiological Reviews, 1997. **77**(3): p. 731-758.
5. van Erp, R.J.J., et al., *Reduced Feed Intake, Rather than Increased Energy Losses, Explains Variation in Growth Rates of Normal-Birth-Weight Piglets.* The Journal of Nutrition, 2018. **148**(11): p. 1794-1803.
6. JanssenDuijghuijsen, L.M., et al., *Mitochondrial ATP Depletion Disrupts Caco-2 Monolayer Integrity and Internalizes Claudin 7.* Frontiers in Physiology, 2017. **8**: p. 794.
7. Bjarnason, I. and K. Takeuchi, *Intestinal permeability in the pathogenesis of NSAID-induced enteropathy.* Journal of Gastroenterology, 2009. **44**(19): p. 23-29.
8. Mahmud, T., et al., *Nonsteroidal antiinflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation.* Arthritis & Rheumatism, 1996. **39**(12): p. 1998-2003.
9. Somasundaram, S., et al., *Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat.* Alimentary Pharmacology & Therapeutics, 2000. **14**(5): p. 639-650.
10. Rodenburg, W., et al., *Impaired barrier function by dietary fructo-oligosaccharides (FOS) in rats is accompanied by increased colonic mitochondrial gene expression.* BMC Genomics, 2008. **9**(1): p. 144.
11. Stoll, B., et al., *Substrate oxidation by the portal drained viscera of fed piglets.* American Journal of Physiology, 1999. **277**(1 Pt 1): p. E168-75.
12. Sokolović, M., et al., *Fasting induces a biphasic adaptive metabolic response in murine small intestine.* BMC genomics, 2007. **8**: p. 361-361.
13. Bruininx, E.M.A.M., et al., *Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs1.* Journal of Animal Science, 2002. **80**(6): p. 1413-1418.
14. Campbell, J.M., J.D. Crenshaw, and J. Polo, *The biological stress of early weaned piglets.* Journal of Animal Science and Biotechnology, 2013. **4**(1): p. 19-19.
15. Pluske, J.R., D.L. Turpin, and J.-C. Kim, *Gastrointestinal tract (gut) health in the young pig.* Animal Nutrition, 2018. **4**(2): p. 187-196.
16. Van Breda, L.K., et al., *Pre- and post-weaning scours in southeastern Australia: A survey of 22 commercial pig herds and characterisation of Escherichia coli isolates.* PLOS ONE, 2017. **12**(3): p. e0172528.
17. Alvarez, J., et al., *Impact of Porcine Epidemic Diarrhea on Performance of Growing Pigs.* PLOS ONE, 2015. **10**(3): p. e0120532.
18. Hong, T.T.T., et al., *Survey on the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam.* Tropical Animal Health and Production, 2006. **38**(5): p. 397-405.
19. World Health Organization *Diarrhoeal disease*. 2017; Available from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease.
20. Pelletier, D.L., et al., *The effects of malnutrition on child mortality in developing countries.* Bulletin of the World Health Organization, 1995. **73**(4): p. 443-448.
21. Bekebrede, A.F., et al., *Mitochondrial and glycolytic extracellular flux analysis optimization for isolated pig intestinal epithelial cells.* Scientific Reports, 2021. **11**(1): p. 19961.
22. Blok, M.C., et al., *A new Dutch net energy formula for feed and feedstuffs for*

*growing and fattening pigs*. 2015, Wageningen Livestock Research: Wageningen.
23. Janssen, J.J.E., et al., *Novel standardized method for extracellular flux analysis of oxidative and glycolytic metabolism in peripheral blood mononuclear cells.* Scientific Reports, 2021. **11**(1): p. 1662-1662.
24. Pau, G., et al., *EBImage--an R package for image processing with applications to cellular phenotypes.* Bioinformatics (Oxford, England), 2010. **26**(7): p. 979-981.
25. Andrews, S., *FastQC: a quality control tool for high throughput sequence data*. 2010, Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.
26. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
27. Anders, S., P.T. Pyl, and W. Huber, *HTSeq--a Python framework to work with high-throughput sequencing data.* Bioinformatics, 2015. **31**(2): p. 166-9.
28. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
29. Subramanian, A., et al., *Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles.* Proceedings of the National Academy of Sciences, 2005. **102**(43): p. 15545-15550.
30. Wu, T., et al., *clusterProfiler 4.0: A universal enrichment tool for interpreting omics data.* The Innovation, 2021. **2**(3): p. 100141.
31. Gillespie, M., et al., *The reactome pathway knowledgebase 2022.* Nucleic Acids Research, 2021. **50**(D1): p. D687-D692.
32. Liberzon, A., et al., *The Molecular Signatures Database (MSigDB) hallmark gene set collection.* Cell systems, 2015. **1**(6): p. 417-425.
33. Yu, G., et al., *GOSemSim: an R package for measuring semantic similarity among GO terms and gene products.* Bioinformatics, 2010. **26**(7): p. 976-8.
34. Rath, S., et al., *MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations.* Nucleic acids research, 2021. **49**(D1): p. D1541-D1547.
35. Vancamelbeke, M., et al., *Genetic and Transcriptomic Bases of Intestinal Epithelial Barrier Dysfunction in Inflammatory Bowel Disease.* Inflammatory bowel diseases, 2017. **23**(10): p. 1718-1729.
36. Blighe K, R.S., Lewis M, *EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling*. 2021.
37. Gu, Z., R. Eils, and M. Schlesner, *Complex heatmaps reveal patterns and correlations in multidimensional genomic data.* Bioinformatics, 2016. **32**(18): p. 2847-9.
38. Choudhury, R., et al., *Impact of early-life feeding on local intestinal microbiota and digestive system development in piglets.* Scientific Reports, 2021. **11**(1): p. 4213.
39. Subramanian, S., H. Geng, and X.-D. Tan, *Cell death of intestinal epithelial cells in intestinal diseases.* Sheng li xue bao : [Acta physiologica Sinica], 2020. **72**(3): p. 308-324.
40. Novais, A.K., et al., *Weaning differentially affects mitochondrial function, oxidative stress, inflammation and apoptosis in normal and low birth weight piglets.* PLOS ONE, 2021. **16**(2): p. e0247188.
41. Zhu, L.H., et al., *Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs.* Journal of Animal Science, 2012. **90**(8): p. 2581-2589.
42. Wei, H.K., et al., *A carvacrol–thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets.* animal, 2017. **11**(2): p. 193-201.
43. Cao, S.T., et al., *Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets.* Journal of Animal Science, 2018. **96**(3): p. 1073-1083.
44. Meng, Q., et al., *Weaning Alters Intestinal Gene Expression Involved in Nutrient Metabolism by Shaping Gut Microbiota in Pigs.* Frontiers in Microbiology, 2020. **11**: p. 694-694.

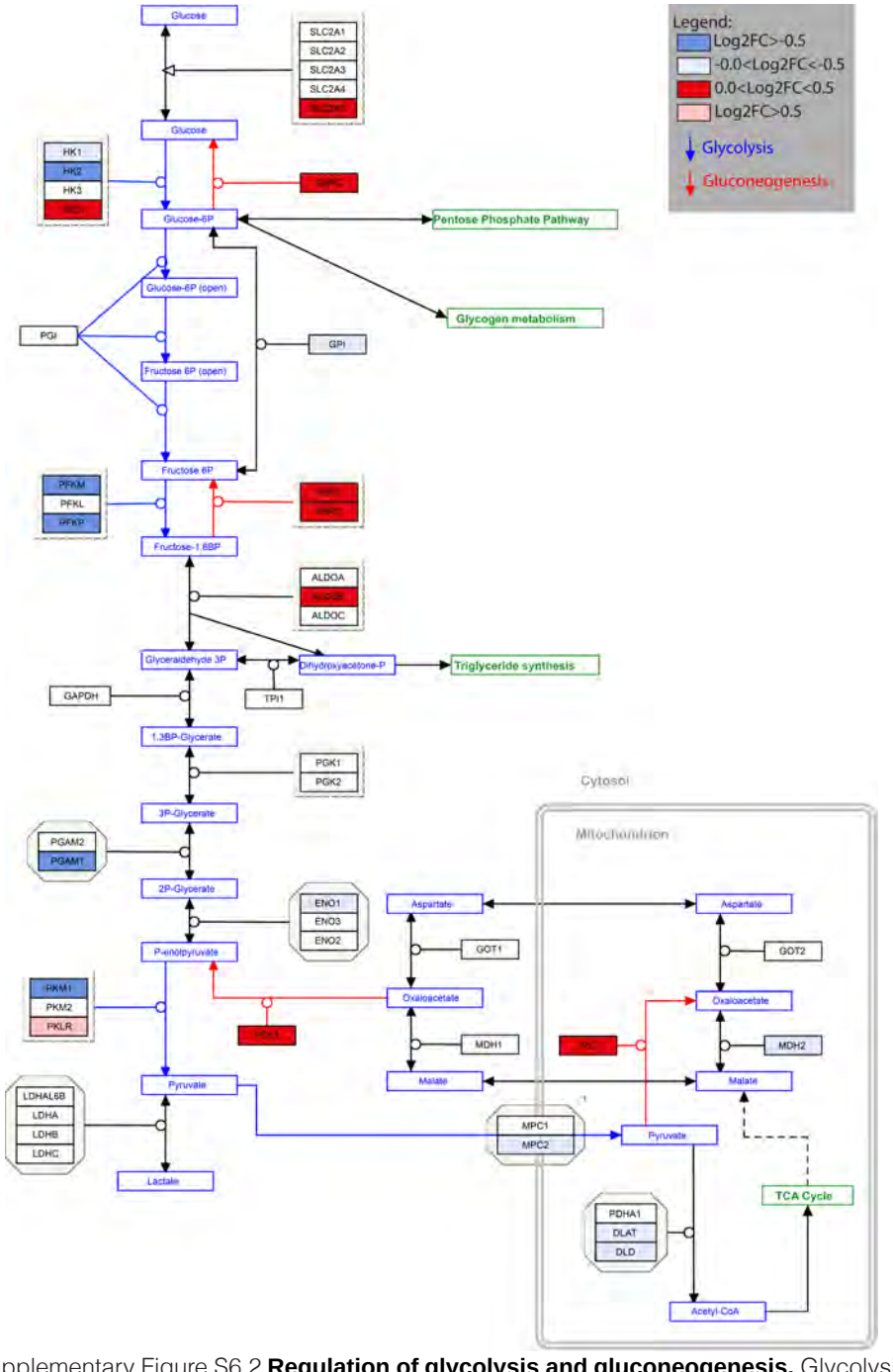
45. Murphy, M.P., *How mitochondria produce reactive oxygen species.* The Biochemical journal, 2009. **417**(1): p. 1-13.
46. Mihaylova, M.M., et al., *Fasting Activates Fatty Acid Oxidation to Enhance Intestinal Stem Cell Function during Homeostasis and Aging.* Cell Stem Cell, 2018. **22**(5): p. 769-778.e4.
47. Zhang, S., et al., *The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility.* Nutrition & metabolism, 2014. **11**(1): p. 10-10.
48. Pettersen, I.K.N., et al., *Upregulated PDK4 expression is a sensitive marker of increased fatty acid oxidation.* Mitochondrion, 2019. **49**: p. 97-110.
49. Xie, Z., et al., *Metabolic Regulation of Gene Expression by Histone Lysine β-Hydroxybutyrylation.* Molecular Cell, 2016. **62**(2): p. 194-206.
50. Terranova, C.J., et al., *Reprogramming of H3K9bhb at regulatory elements is a key feature of fasting in the small intestine.* Cell Reports, 2021. **37**(8): p. 110044.
51. Jeoung, N.H., et al., *Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homoeostasis during starvation.* The Biochemical journal, 2006. **397**(3): p. 417-425.
52. Mithieux, G., et al., *Induction of control genes in intestinal gluconeogenesis is sequential during fasting and maximal in diabetes.* American Journal of Physiology-Endocrinology and Metabolism, 2004. **286**(3): p. E370-E375.
53. Soty, M., et al., *A gut–brain neural circuit controlled by intestinal gluconeogenesis is crucial in metabolic health.* Molecular Metabolism, 2015. **4**(2): p. 106-117.
54. TeSlaa, T., et al., *The Source of Glycolytic Intermediates in Mammalian Tissues.* Cell Metab, 2021. **33**(2): p. 367-378.e5.
55. Qi, M., et al., *Small intestinal transcriptome analysis revealed changes of genes involved in nutrition metabolism and immune responses in growth retardation piglets.* Journal of Animal Science, 2019. **97**(9): p. 3795-3808.
56. JanssenDuijghuijsen, L.M., et al., *Endurance Exercise Increases Intestinal Uptake of the Peanut Allergen Ara h 6 after Peanut Consumption in Humans.* Nutrients, 2017. **9**(1).
57. Peters, A., et al., *The selfish brain: competition for energy resources.* Neuroscience & Biobehavioral Reviews, 2004. **28**(2): p. 143-180.
58. Bock, F.J. and S.W.G. Tait, *Mitochondria as multifaceted regulators of cell death.* Nature Reviews Molecular Cell Biology, 2020. **21**(2): p. 85-100.
59. Márquez-Jurado, S., et al., *Mitochondrial levels determine variability in cell death by modulating apoptotic gene expression.* Nature Communications, 2018. **9**(1): p. 389.
60. Wang, J., et al., *Gene Expression Is Altered in Piglet Small Intestine by Weaning and Dietary Glutamine Supplementation.* The Journal of Nutrition, 2008. **138**(6): p. 1025-1032.
61. Marshman, E., C. Booth, and C.S. Potten, *The intestinal epithelial stem cell.* BioEssays, 2002. **24**(1): p. 91-98.
62. Wijtten, P.J., J. van der Meulen, and M.W. Verstegen, *Intestinal barrier function and absorption in pigs after weaning: a review.* Br J Nutr, 2011. **105**(7): p. 967-81.
63. Verdonk, J.M.A.J., *Nutritional strategy affects gut wall integrity in weaned piglets*. 2006, Wageningen University Wageningen.
64. Liu, Y., A. Beyer, and R. Aebersold, *On the Dependency of Cellular Protein Levels on mRNA Abundance.* Cell, 2016. **165**(3): p. 535-550.
65. Burrin, D.G., et al., *Stage of Development and Fasting Affect Protein Synthetic Activity in the Gastrointestinal Tissues of Suckling Rats.* The Journal of Nutrition, 1991. **121**(7): p. 1099-1108.
66. Shah, M., et al., *A Transcript-Specific eIF3 Complex Mediates Global Translational Control of Energy Metabolism.* Cell Reports, 2016. **16**(7): p. 1891-902.
67. Cortassa, S., B. O'Rourke, and M.A. Aon, *Redox-Optimized ROS Balance and the relationship between mitochondrial respiration and ROS.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2014. **1837**(2): p. 287-295.

68. GBD 2019 Diseases and Injuries Collaborators *Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019.* The Lancet, 2020. **396**(10258): p. 1204-1222.
69. Ziegler, A., L. Gonzalez, and A. Blikslager, *Large Animal Models: The Key to Translational Discovery in Digestive Disease Research.* Cellular and Molecular Gastroenterology and Hepatology, 2016. **2**(6): p. 716-724.
70. Sciascia, Q., G. Daş, and C.C. Metges, *REVIEW: The pig as a model for humans: Effects of nutritional factors on intestinal function and health.* Journal of Animal Science, 2016. **94**(suppl_3): p. 441-452.

## Supplementary material




Supplementary Figure S6.1 **Isolation parameters of primary mid-jejunal epithelial cells.** (**A**) Yield in cells per $cm^2$ tissue and (**B**). viability were analysed to assess isolation success. Values for yield were log-transformed to reach normality, and significance was determined using student's t-test. Data are presented as individual datapoint, together with mean and standard deviation.

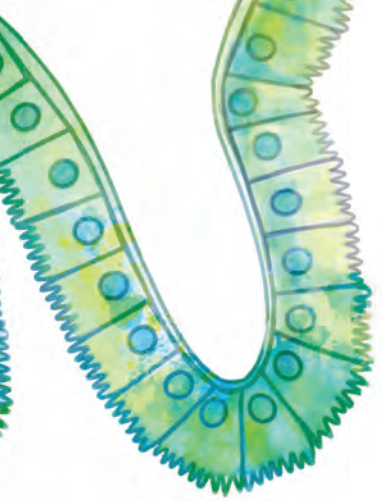



Supplementary Figure S6.2 **Regulation of glycolysis and gluconeogenesis.** Glycolysis genes are primarily downregulated (in blue), while gluconeogenesis genes are upregulated (in red).

Supplementary Table S6.1 **Top 20 down- and upregulated MitoCarta 3.0 genes with gene symbol, Log2FC and Benjamin-Hoch adjusted p-value**.

| Genes | $Log_2FC$ | padj |
|---|---|---|
| *Downregulated* | | |
| AIFM1 | -0.59 | 9.4E-15 |
| DLST | -0.61 | 2.5E-14 |
| SLC25A12 | -0.91 | 5.9E-13 |
| SERHL2 | -0.81 | 1.3E-12 |
| GHITM | -0.69 | 2.3E-11 |
| ACLY | -0.51 | 1.2E-10 |
| TIMM17A | -0.81 | 1.3E-10 |
| FASN | -1.05 | 1.4E-10 |
| AGPAT5 | -0.75 | 2.2E-10 |
| SUCLA2 | -0.72 | 2.6E-10 |
| IDE | -0.51 | 6.0E-10 |
| APOO | -0.71 | 7.5E-10 |
| NDUFS1 | -0.51 | 1.1E-09 |
| NDUFS3 | -0.56 | 1.1E-09 |
| NDUFA6 | -0.50 | 1.2E-09 |
| GSR | -0.62 | 1.8E-09 |
| ACOT7 | -1.33 | 2.0E-09 |
| LETM1 | -0.45 | 2.5E-09 |
| NDUFB5 | -0.51 | 4.6E-09 |
| IDH3A | -0.70 | 7.7E-09 |
| *Upregulated* | | |
| PDK4 | 3.40 | 1.0E-50 |
| ACAD11 | 1.74 | 6.4E-17 |
| CHPT1 | 1.15 | 4.8E-15 |
| PDK2 | 1.17 | 3.6E-11 |
| OAT | 1.28 | 6.9E-11 |
| LACTB | 0.47 | 8.7E-11 |
| GLYCTK | 1.23 | 1.8E-10 |
| TOMM20L | 1.01 | 2.2E-10 |
| WARS2 | 0.71 | 3.1E-10 |
| CROT | 0.73 | 5.5E-10 |

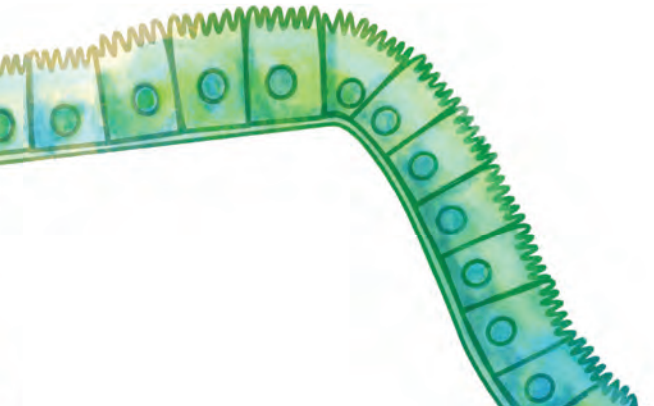
| | | |
|---|---|---|
| DHRS4 | 0.96 | 1.8E-09 |
| BCL2L11 | 1.31 | 1.9E-09 |
| SLC25A38 | 0.81 | 2.3E-09 |
| TOP1MT | 1.21 | 2.9E-09 |
| CMC4 | 0.74 | 1.0E-08 |
| METTL5 | 0.59 | 2.4E-08 |



# Chapter 7

## Small Intestinal Fasting Responses of Suckling and Feed Habituated Piglets

Anna F. Bekebrede
Vincent C.J. de Boer
Walter J.J. Gerrits
Jaap Keijer



*In preparation*

## Abstract

The small intestine requires large amounts of energy to maintain its function. Especially in young individuals, absence of enteral nutrition may enhance susceptibility to intestinal diseases by limiting energy availability. This may interact with the developmental, feed habituation stage of the small intestine We therefore investigated whether the molecular response of the small intestine to 48h of fasting is different between four-week-old suckling piglets and six-week-old feed habituated piglets. Using RNA-sequencing in mid-jejunal intestinal scrapings, we identified molecular pathways affected similarly, pointing towards robust regulation, and oppositely, indicative of differential regulation, upon fasting in suckling and feed habituated piglets. In general, gene expression was more strongly altered in suckling than in feed habituated piglets. Among the five pathways similarly affected by fasting were two mitochondrial pathways that were both downregulated, indicating the consistent downregulation of mitochondrial metabolism upon fasting. Other similarly affected pathways include the downregulated pathways “extracellular matrix organisation” and “asparagine N-linked glycosylation”, and the upregulated pathway “eukaryotic translation initiation”. Eleven pathways were oppositely regulated upon fasting, and could roughly be classified as either belonging to cell cycle progression or immune function. All of these pathways were upregulated in suckling piglets, while being downregulated in feed habituated piglets. Our results provide mechanistic information on the effects of temporary unavailability of nutrients inside the intestine on intestinal health at various stages of development, relevant for pig and infants.

**Keywords**; Small intestine, nutrient availability, mitochondria, cell cycle, immune function.

## Introduction

The small intestine is the body's main site for nutrient absorption, while it simultaneously maintains a barrier against the outside milieu. These processes are primarily performed by the most abundant cell type, the intestinal epithelial cells (IECs) [1]. Maintaining the small intestinal functions requires large amounts of energy [2, 3]. Like most differentiated cells, IECs predominantly produce energy in the mitochondria. Mitochondria utilize various energy substrates to generate reducing equivalents, that are oxidised in the oxidative phosphorylation pathway to generate cellular energy in the form of ATP. IEC obtain these energy substrates from both the systemic circulation via the basolateral membrane, and directly from the intestinal lumen via the brush border membrane [4]. Low, or even absent, food intake makes the intestine more susceptible to gastrointestinal disorders in especially young individuals. For instance, diarrhoea is a common occurrence during the weaning-transition phase in pig husbandry [5-8], and gastrointestinal diseases are also prevalent among children in especially low-income countries, where it still ranks third in disability-adjusted life-years (DALYs) lost [9].

The maturation state of the intestine contributes to the increased susceptibility of young individuals to periods of food deprivation. In pig husbandry, weaning mostly occurs at a time when the intestine is not yet fully developed. It has been demonstrated that increased solid feed intake in the preweaning period contributes to intestinal development, alleviating post-weaning health problems [10, 11]. Through exposure to plant proteins, starch and fibres, a solid diet induces structural changes in the intestine of piglets that stimulates intestinal maturation to maintain proper nutrient digestion, absorption and barrier integrity [12-15]. In human healthcare, the requirement for enteral nutrition to support intestinal development is also recognised, and is especially advised for preterm and low birth-weight infants [16, 17]. Prior exposure to solid feed thus stimulates intestinal development and maturation, and this likely contributes to the difference in the response to fasting that is observed between the developing compared to the developed intestine. Indeed, a primarily beneficial effect is attributed to fasting in adult vertebrates [18-20], while fasting has detrimental effects in young piglets and mice [21, 22].

In addition, weaning-stress is not only caused by a low feed intake, but is also associated with other stressors, such as maternal separation and a new environment [23, 24]. Incomplete development of the intestine at time of weaning may further exacerbate these stressors. Therefore, fasting at time of weaning is one of multiple stressors. To better distinguish the effect of fasting, we weaned and feed habituated piglets for two weeks before performing a 48h fasting intervention. We found that 48h fasting decreased IEC metabolic function in the jejunum of feed habituated, six-week-old piglets, which was confirmed by decreased expression of metabolic, and especially

mitochondrial, pathways [**chapter 6**]. In addition, we found that fasting feed habituated piglets reduced the crucial molecular processes of cell proliferation [**chapter 6**]. Here, we compare the molecular response of piglets to fasting at time of weaning (four-week-old) to that of the feed habituated piglets (six-week-old), to investigate how the developmental, feed habituation stage and additional stressors associated with weaning impacted the fasting response.

## Methods

### *Animals*

All experiments and methods were performed in accordance local and (inter) national guidelines. The Central Authority for Scientific Procedures on Animals (CCD), and the Animal Welfare Body (IvD) approved the protocol of the experiment (AVD1040020209884), which was in accordance with the Dutch law on animal experimentation and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The experiment was carried out in compliance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Sixty-four gilts (Topigs Tempo x TN20) were selected from fifteen litters on a commercial farm. Gilts were from sixteen litters of sows with a parity range between one and seven (parity 1, 2 sows; parity 3, 4 sows; parity 4, 4 sows; parity 5, 2 sows; parity 6, 2 sows; parity 7, 1 sow). Cross-fostering was minimised, and if needed only applied to male piglets. Apart from a nutritional supplement (Lianol basdiar , Ardol BV, Susteren, The Netherlands) in the first post-natal week, the piglets did not receive creep feed. Piglets were weaned between post-natal day 22 and 26. First, all female piglets in the litter were weighed, and only the piglets with ± 1 standard deviation (SD) from the mean were selected, to ensure minimal variation in body weight. Female piglets were then allotted to one of four experimental groups, based on sow's parity, farrowing date and genetic background. The four experimental groups were as follows: suckling fed, suckling fasted, feed habituated fed and feed habituated fasted, n=16 per group (Fig. 7.1). Suckling piglets remained at the commercial farm for the duration of the experiment, while feed habituated piglets were transferred to the research facility of Wageningen University (CARUS) at weaning. Suckling piglets either remained with the sows for the duration of the experiment, or were transferred to a separate pen and housed in groups of four, independent of litter, for the 48h duration of the fasting period. During this time, piglets had *ad libitum* access to water. The comparison between fasted and fed piglets after feed habituation has been reported previously [**chapter 6**].

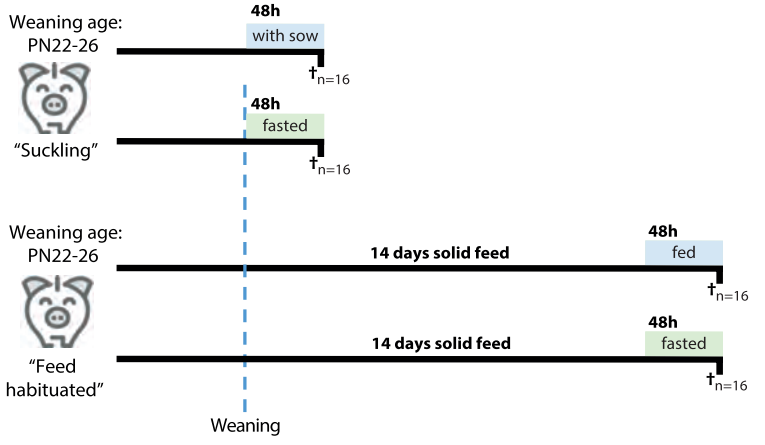



Figure 7.1. **Visual representation of the study setup.** Suckling piglets, ~four weeks old, either remained with the sow throughout the study period, or were taken away from the sow and fasted for 48h. Feed habituated piglets, weaned at ~four weeks, were fed a solid feed for two weeks, after which one group of piglets continued to receive feed, while another group was fasted for 48h. Sixteen female piglets were included per group.

At CARUS, piglets were allocated to the pens based on their initial body weight, to minimize variation in BW between pens and treatments, and were pair-housed in 2 $m^2$ partially slatted pens. At the research facility, temperature was kept between 24 and 26.5°C with a humidity of 65%, lights and radio were switched on between 7:00 and 19:00h. Lights were dimmed to 5% and radio was off from 19:00 till 7:00h. Health and welfare were assessed visually thrice a day during feeding, and faecal consistency score was assessed (score of 1-5, 1 = liquid diarrhoea, 5 = hard faeces). The feeds were produced by Research Diet Services (RDS BV, Wijk bij Duurstede, The Netherlands) in a single batch in the form of pellets (Table 7.1). Piglets were fed at 2.4x maintenance energy requirement (NEm = 0.29 MJ NE/kg $BW^{0.75}$), divided equally over three portioned meals a day at 7:30h, 12:30 and 17:00h. Piglets were weight at the start of the experiment and then every week to correct feed intake for their current weight. Water was available *ad libitum* for all piglets throughout the entire study period. 48h before the end of the experiment, piglets either continued to receive feed thrice a day at 2.4x NEm, or were fasted until the end of the study period. At the end of the study period, piglets were sedated using intramuscular injection of Zoletil+Xylazine (5:2 ratio, 1 mg/10 kg BW), and euthanised by lethal injection with pentobarbital (24 mg/kg BW).

Table 7.1 **Ingredient and calculated nutrient composition of the starter diet**[1].

| components | |
|---|---|
| *Ingredient composition (%)* | |
| Soybean meal | 16.5 |
| Sunflower meal | 6 |
| Wheat | 27.1 |
| Barley | 25 |
| Corn | 20 |
| Soya oil | 1.3 |
| L-lysine HCl | 0.52 |
| L-threonine | 0.16 |
| DL-Methionine | 0.15 |
| L-tryptophan | 0.035 |
| L-valine | 0.02 |
| Premix[2] | 0.5 |
| CaCO3 | 1.3 |
| $Ca(H_2PO_4)_2$ | 0.9 |
| NaCl | 0.4 |
| citric acid | 0.1 |
| Phytase | 0.0025 |
| total | 100 |
| *Nutrient composition (g/kg)* | |
| Dry Matter | 871.6 |
| Crude Protein | 181 |
| Crude Fat | 39.3 |
| Crude Ash | 52.9 |
| Starch | 433.6 |
| Sugars | 35.7 |
| NE (MJ/kg) | 10.1 |

Abbreviations: NE = net energy.

[1] Nutrient composition was calculated based on ingredient composition and table values for the composition of the ingredients [25].

[2] Supplied per kilogram of feed: retinyl acetate, 10,000 IU; cholecalciferol, 2,000 IU; DL-a-tocopherol, 40 mg: menadione, 1.5 mg; thiamine 1.0 mg; riboflavin, 4 mg; pyridoxin-HCl, 1.5 mg; cyanocobalamin, 20 µg; niacin, 30 mg; D-pantothenic acid, 15 mg; Choline chloride, 150 mg; Folic acid, 0.4 mg; Biotin, 0.05 mg; iron(II)sulphate monohydrate, 331 mg; copper(II)sulphate pentahydrate, 80 mg; manganese(II)oxide, 49 mg; zinc sulphate monohydrate, 194 mg, potassium iodate, 1 mg; sodium selenite, 0.56 mg.

### *Tissue collection and RNA isolation*

Following excision from the abdominal cavity, the entire intestine was separated from the mesentery, and the small intestine was identified. The small intestine was spread out and 50% of intestinal length was identified, which corresponds to mid-jejunum. A 16 cm segment distal of the 50% mark was used for mucosal scrapings. Mucosal scrapings were snap frozen in liquid nitrogen and stored at -80°C until use. To isolate RNA, mucosal scrapings were first crushed to powder with pestle and mortar in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. For each sample, 5-10 mg of powdered tissue was dissolved in a mixture of RLT buffer (Qiagen) and -mercaptoethanol (M6250, Sigma Aldrich, St. Louis, MO, USA). Tissue samples were subsequently lysed and homogenised by shaking them in a thermomixer at room temperature at maximal speed for at least 45 minutes. Then, 70% ethanol was added, and total RNA was bound to the filter on the RNeasy spin column. After DNase I treatment to digest DNA, the column was washed three times with RW1 and RPE buffer, and total RNA was eluted in RNase free water. RNA quality and integrity was assessed using Nanodrop spectrophotometer (IsoGen Life Science, de Meern, the Netherlands) and Agilent 2200 Tapestation (Agilent Technologies Inc., Santa Clara, CA, USA). Samples met the criteria with a ratio higher than 1.8 for both 260/280 and 260/230 or a RNA integrity number (RIN) above 7.

### *RNA-seq and data analysis*

RNA preparation, library construction, DNB-sequencing and read clean-up were performed at Beijing Genomics Institute (BGI, Hongkong). Quality check of the clean reads was preformed using FASTQC [26]. Reads were aligned to the pig genome (Sscrofa11.1.104) using STAR2.7 [27], and counts were quantified using HTSeq [28]ordImport. Average sequencing depth was 24M paired end reads, of which at least 92.9% were uniquely mapped. read alignment and counting, data analysis and statistical testing was performed in R 4.1, using appropriate Bioconductor packages. The raw and gene-count data can be found in the GEO repository under ascension number GSE203439.

Genes with a total sum of less than 10 counts were removed. Then, differentially expressed genes (DEGs) between fed and fasted piglets were identified with the DE2seq package, using Benjamini-Hochberg correction for multiple testing [29]. An adjusted p-value of below 0.05 was considered significantly regulated. Principal component analysis was performed on variance stabilised transformed (VST) data. Gene overlap to create the VENN diagram was calculated using the GeneOverlap package [30]. Gene set enrichment analysis (GSEA) [31] was performed using clusterProfiler [32] for the Reactome annotated gene sets [33] that were extracted

using the Molecular Signatures Database (MSigDB [34]). Gene sets were considered enriched with a Benjamini–Hochberg (BH) adjusted *p*-value < 0.05. To only include the most significantly regulated pathways, the median adjusted p-value was determined and only the gene sets with a BH adjusted p-value below the median were included in further analysis. Volcano plots and heatmaps were generated using the EnhancedVolcano and ComplexHeatmap packages [35, 36].

### *Statistical data analysis and sample inclusion*

Statistical analyses and data visualizations were performed using R version 4.1. The tight junction pathway was visualised using the online KEGG mapper tool [37].

## Results

### *PCA analysis of transcriptome data*

We performed a 48h fasting intervention in sucking piglets (aged four weeks) and two-weeks feed habituated (aged six weeks; Fig. 7.1). RNA-sequencing was performed on jejunal scrapings to identify the differentially expressed genes (DEGs) in response to the fasting intervention at both developmental stages. Normalised gene counts from RNAseq were transformed using variance stabilizing transformation, and principal component analysis (PCA) revealed a clear separation between gene expression data in intestinal scrapings of fed vs. fasted suckling piglets, as well as a clear separation between feed habituated vs. suckling piglets (Fig. 7.2A). Overall, the separation between fed and fasted in feed habituated piglets appeared smaller than in the suckling piglets. This indicated that fasting at the suckling stage had a more severe impact on jejunal gene expression (Fig. 7.2A). This is also reflected in the total amount of DEGs. Fasting suckling piglets induced differential expression of 8460 genes, while fasting feed habituated piglets showed a differential expression of 5275 genes. In addition, more genes were uniquely regulated in response to fasting suckling piglets (5078) compared to fasting feed habituated piglets (1893; Fig. 7.2B).

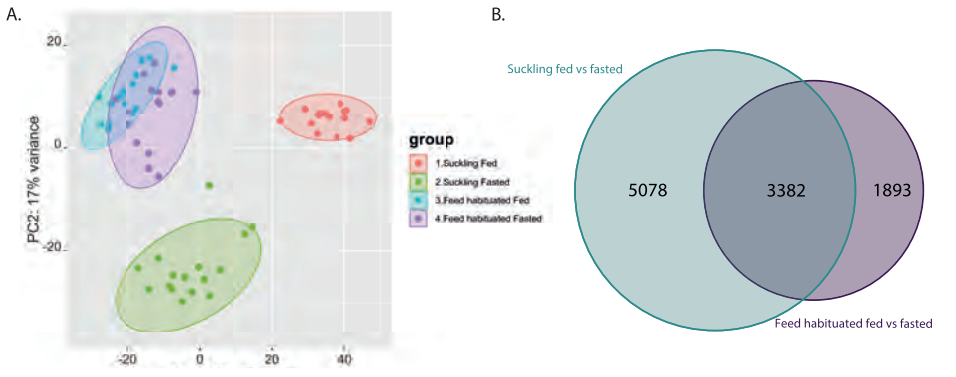



Figure 7.2 **Jejunal effects of 48h fasting in suckling and feed habituated piglets** (**A**) PCA plot of Euclidean distance between jejunal samples (corresponding to individual piglets) using variance stabilizing transformed data. Samples are coloured based on group. (**B**) VENN diagram showing the effect of fasting suckling and feed habituated piglets. Fasting of suckling piglets resulted in differential expression of 8460 genes, while fasting of feed habituated piglets resulted in differential expression of 5275 genes. In total, 3382 genes were commonly differentially regulated in fasting of suckling and feed habituated piglets.

## *Pathway analysis of fasting response in suckling and feed habituated piglets*

We investigated which pathways are regulated by 48h fasting in suckling and feed habituated piglets, using Reactome annotated pathways [33]. To focus on the most significantly regulated pathways, only pathways with an adjusted *p*-value below the median *p*-value of all the pathways were included in the analysis (Fig. 7.3). Five pathways were regulated in a similar direction in both suckling and feed habituated piglets in a similar direction, of which four were downregulated and one was upregulated. Two of those five pathways are related to mitochondrial function: the more comprehensive “TCA cycle and respiratory electron transport” pathway that includes a lot of mitochondrial genes and pathways, and the more specific “respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins” pathway, which is focussed on respiratory electron transport. There were also pathways only regulated in suckling or in feed habituated piglets. Among those that were downregulated are “muscle contraction” and “neuronal system” in suckling piglets, and “mitotic G2-G2 M phases” and “separation of sister chromosomes” in feed habituated piglets (Fig. 7.3). Among those that were upregulated are “metabolism of RNA” and “mitotic spindle checkpoint” in suckling piglets, and “cellular response to starvation” and “biological oxidations” in feed habituated piglets. Finally, there were eleven pathways that were regulated in the opposite direction in suckling compared to feed habituated piglets. Among those were “M phase”, “mitotic metaphase and anaphase”, but also “signalling by interleukins” and “interferon signalling. Combined, the pathways that were oppositely regulated can be classified as belonging to two categories: cell cycle regulation and immune or stress responses.

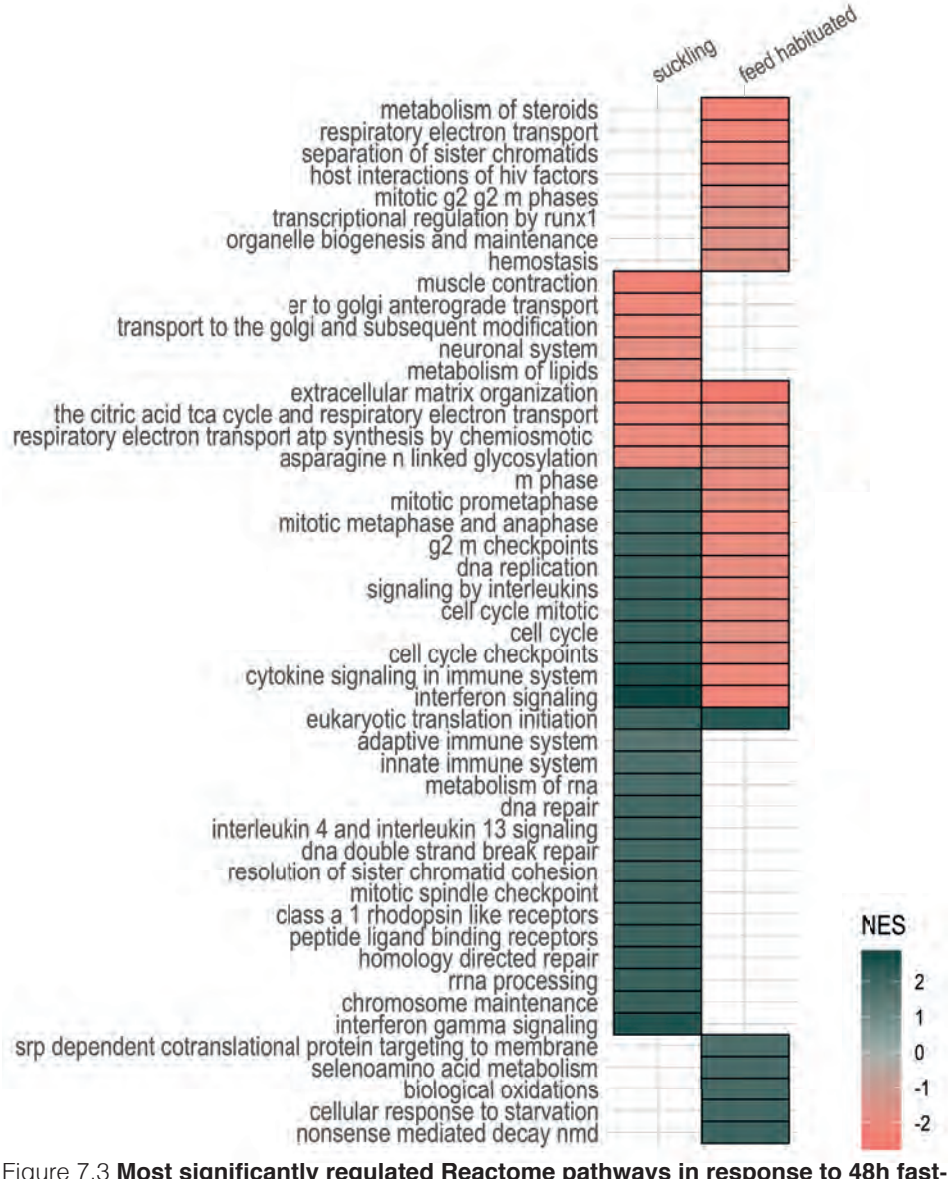



Figure 7.3 **Most significantly regulated Reactome pathways in response to 48h fasting in jejunum of suckling and feed habituated piglets.** NES = normalised enrichments score.

### *Similarly regulated molecular pathways in suckling and feed habituated piglets*

The pathways that are similarly regulated between suckling and feed habituated piglets, are likely pathways that respond robustly to fasting. These were studied in more detail.

For the mitochondrial Reactome pathways, we selected “TCA cycle and respiratory transport”, as this pathway also includes all the genes present in the “respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins” pathway. More genes responded respond to fasting in the suckling piglets than in the feed habituated piglets (89 versus 70, respectively), and the higher $Log_2FC$ and $Log_{10}$ p values imply that the pathway was also regulated more strongly in suckling piglets (Fig. 7.4A). Of the top five most significantly regulated genes in suckling piglets, only one gene was also regulated in feed habituation piglets (*SUCLA2*), while the four other genes (*SLC16A3, NDUFV3, ND5* and *Loc100517855*) were not regulated in the feed habituated piglets (Fig. 7.4A). In total, 44 genes were regulated in both suckling and feed habituated piglets, which shows that, although the pathway is more strongly regulated in suckling piglets, there is a large overlap in the regulation of the pathway “TCA cycle and respiratory electron transport” in both groups (Fig. 7.4A and supplementary Fig. S7.1A).

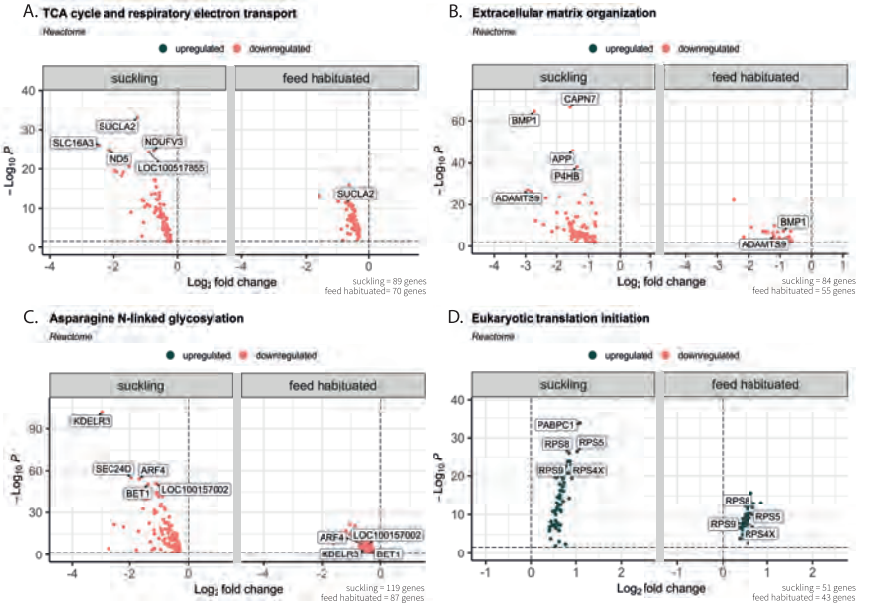



Figure 7.4 **Similarly regulated jejunal pathways upon 48h fasting in suckling and feed habituated piglets.** Volcano plots of the differentially expressed genes (DEGs) in the Reactome ‘TCA cycle and respiratory electron transport’ (**A**), ‘extracellular matrix organization’(**B**) ‘asparagine N-linked glycosylation’ (**C**), and ‘eukaryotic translation initiation’(**D**) pathways.

The pathway “extracellular matrix organization” is also downregulated in both suckling and feed habituated piglets (Fig. 7.4B). More genes respond to fasting in suckling then in feed habituated piglets (84 versus 55 genes), and those differentially expressed were downregulated with a higher $log_2FC$ and higher $-log_{10}$ p value. Out of the top five most significantly regulated genes in suckling piglets, two (*BMP1* and *ADAMTS9*) were

also regulated in feed habituated piglets, while three (*CAPN7*, *APP* and *P4HB*) were only regulated in suckling piglets. Because tight junction proteins are key components of the extracellular matrix in the intestinal epithelium, we used the KEGG annotated "tight junctions" pathway to visualise the effect of 48h fasting, and found that indeed more genes were regulated in the sucking piglets, and they were regulated with a higher $log_2$FC (Fig. 7.5). Together, these data suggest that the intestinal barrier integrity is decreased in both suckling and feed habituated piglets, but the suckling piglets are more severely impacted by the 48h fasting intervention that the feed habituated piglets.

The pathway "asparagine N-linked glycosylation" describes the process of attaching sugar-moieties to the asparagine amino acids of newly synthesised polypeptides in the endoplasmic reticulum (ER), and is crucial for protein folding sorting and secretion [38]. The pathway was downregulated in both suckling and feed habituated piglets (Fig. 7.4C). Again, more genes were regulated in suckling than in feed habituated piglets (119 versus 87 genes, respectively), and the regulation was more significant and stronger in the suckling piglets, indicated by higher $-log_{10}$p values and $log_2$FC. Of the top five most significantly regulated genes in suckling piglets, four (*ARF*, *LOC100157002*, *BET1*, and *KDELR3*) overlapped with feed habituated piglets, while one (*SEC24D*) was only regulated in suckling piglets (Fig. 7.4C). In total, 49 genes in the pathway were upregulated in both suckling and feed habituated piglets (Supplementary Fig. S7.1C), which demonstrates the large similarity in regulation of this pathway upon 48h fasting in both suckling and feed habituated piglets.

The pathway "Eukaryotic translation initiation" was the only common pathway upregulated in suckling and feed habituated piglets (Fig. 7.4D). This pathway includes genes that code for ribosomal and eukaryotic translation initiation factors (EIF), that together enable mRNA to protein translation [39]. More genes were regulated in suckling than in feed habituated piglets (51 versus 43 genes, respectively). Although the regulation was more significant in the suckling piglets, indicated by higher $-log_{10}$ p values, the $log_2$FC values were similar in suckling and feed habituated piglets. Of the top five most significantly regulated genes in suckling piglets, four (*RPS8*, *RPS5*, *RPS9*, and *RPS4X*) overlapped with feed habituated piglets, while one (*PABPC1*) was only regulated in suckling piglets (Fig. 7.4D). In total, 41 genes in the pathway were upregulated in both suckling and feed habituated piglets (Supplementary Fig. S7.1D), which again shows the large similarity in regulation of this pathway upon 48h fasting of both suckling and feed habituated piglets.

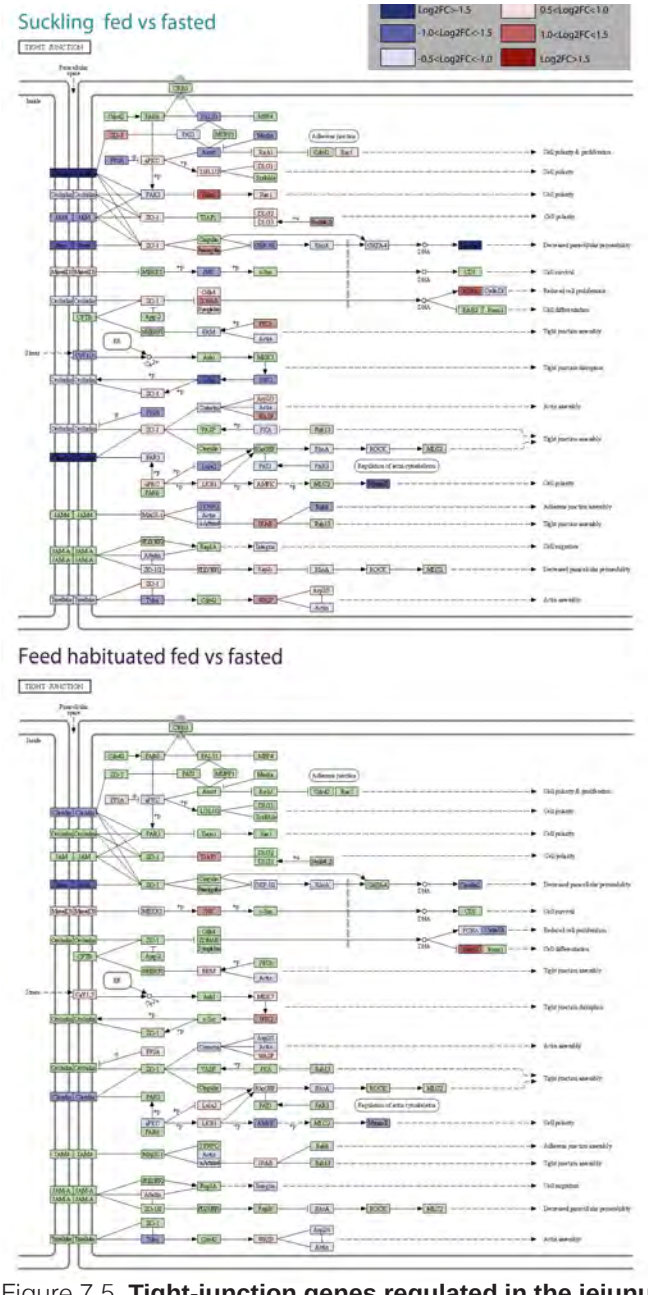



Figure 7.5. **Tight-junction genes regulated in the jejunum in response to fasting of suckling and feed habituated piglets**. Genes are coloured according to log2FC (see legend, blue = downregulated, red = upregulated, green = not regulated) and mapped using the KEGG mapper tool.

### *Oppositely regulated molecular pathways in suckling and feed habituated piglets*

The developmental feed habituation stage of the intestine may impact how the intestine responds to fasting. We found that eleven pathways were regulated in opposite direction in suckling vs. feed habituated piglets, many of which belong either to cell cycle regulation or to the immune or stress response. We selected the pathway "cell-cycle checkpoints" because of its key involvement in cell cycle regulation, and the pathway "cytokine signalling in immune system" because this pathway includes also all the genes present in the remaining two immune system related pathways.

All significantly regulated genes in the "cell-cycle checkpoints" pathways were upregulated in suckling piglets, while they were downregulated in feed habituated piglets (Fig. 7.6A). More genes in this pathway were regulated in suckling than in feed habituated piglets (80 versus 69 genes, respectively), and the regulation was stronger and more significant in suckling piglets, as can be seen from the higher fold-changes and higher $-\log_{10}$ p values in the suckling piglets as compared to the feed-habituated piglets (Fig. 7.6A). Of the top five most regulated genes in suckling piglets, two (*RMI1* and *NBN*) were also regulated in feed habituated piglets, whereas the other three (*PSMB8*, *PSMB9* and *CENP*) were not regulated in the feed habituated piglets (Fig. 7.6A). In total, there were 30 genes differentially expressed in both suckling and feed habituated piglets, but in the opposite direction (Supplementary Fig. S7.1E).

For the "cytokine signalling in immune system" pathway, the genes were upregulated in suckling piglets and downregulated in feed habituated piglets (Fig. 7.6B). More genes were regulated in suckling compared to feed habituated piglets (144 versus 106, respectively), while the genes also had a higher $\log_2$FC and were more significantly regulated in the suckling piglets. Of the top five most regulated genes in suckling piglets, two (*OASL* and *BCL6*) were also regulated in feed habituated piglets, while three other genes (*CXCL10, IRS1* and *TNFSF13B*) were not regulated in feed habituated piglets. In total, 44 genes were regulated in both suckling and feed habituated piglets, but in the opposite direction (Supplementary Fig. S7.1F), demonstrating the difference between the jejunal fasting response in piglets in different developmental feed habituation stages.

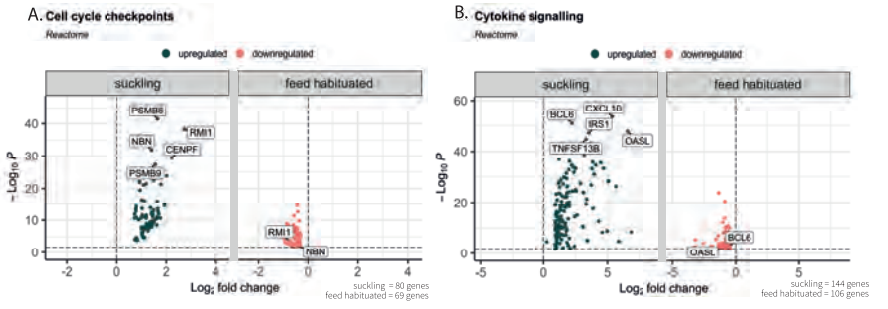



Figure 7.6. **Oppositely regulated jejunal pathways upon 48h fasting in suckling and feed habituated piglets.** Volcano plots of the differentially expressed genes (DEGs) in the Reactome 'cell cycle checkpoints' (**A**) and 'Cytokine signalling in immune system' (**B**) pathways.

## Discussion

Fasting is a common consequence of weaning, with up to 10% of piglets not consuming their first meal up to 48h after weaning [40, 41], and this contributes to increased intestinal susceptibility to gastrointestinal diseases [8]. Solid feed intake induces intestinal maturation [13, 42], and may thereby exert a protective effect on the intestine during times of feed deprivation. In this study, we therefore aimed to investigate whether the fasting response of the jejunum was different between suckling piglets and piglets two weeks habituated to solid feed, and which molecular pathways were differentially regulated. We observed that in general, regulation upon fasting was stronger in suckling piglets. Some molecular pathways were similarly regulated, indicative of a robust response to the fasting intervention, even though the response to fasting generally was more pronounced in the suckling piglets. These common robustly regulated pathways are mitochondrial function (two times), extracellular matrix organization, asparagine N-linked glycosylation and eukaryotic translation initiation. We also observed key processes, mainly involved in cell cycle progression and immune signalling, that were regulated in the opposite direction upon 48h fasting in suckling compared to feed habituated piglets. Together, these results provide us with insight in the response to fasting of the small intestine, and in developmental food habituation related responses.

Apart from differences in age and development, suckling and feed habituated piglets differed in the type and amount of stressors they experienced during the fasting intervention, which potentially impacts the molecular response to fasting. To be able to fast suckling piglets, they were effectively weaned at the start of the fasting period. This means that they experienced additional stress caused by maternal separation and a new physical and social environment [23, 24]. In addition, prior to the fasting challenge, suckling piglets were exclusively fed sows milk; a liquid that contains lactose, dairy

proteins and is high in fat (~7-8%) [43, 44]. In contrast, the feed habituated piglets were fed a solid diet, based primarily on starch and plant based protein, containing fibres and lower amounts of fat (4%) (Table 7.1). While the jejunal pathways commonly regulated in sucking and feed habituated piglets can be clearly attributed to fasting, the pathways that respond differently between the suckling and the feed habituated piglets may be due to differences in developmental feed habituation stage, but they may also result from environmental stress or the different dietary contrasts.

In this study, we showed that mitochondrial function is a key process that is regulated upon fasting in early life, independent of prior exposure to solid feed. Previously, we functionally confirmed these transcriptome results by demonstrating that metabolic flux of IECs isolated from feed habituated piglets was indeed decreased after 48h fasting. This underpins the validity of our data. A decrease  was seen in glycolytic and oxidative fluxes, supporting a decrease in IECs energy generating capacity. Our gene expression data shows that this is a robust fasting response common to suckling and feed habituated piglets. As sufficient energy supply is needed to maintain a IEC function (e.g. [2, 3].), the decreased IEC metabolic function may, at least in part, explain the predisposition to intestinal diseases that is associated with malnutrition and fasting in young piglets and infants [8, 24, 45-47].

Maintaining a barrier against the outside milieu is one of the key functions of the intestine, and this is actualised in part by extracellular matrix proteins [48]. Fasting decreased expression of extracellular matrix genes, irrespective of prior exposure to solid feed. However, the extracellular matrix was more severely impacted by fasting in suckling piglets, suggesting that the impact of energy substrate depletion during this period is more critical then at a later age. Indeed, later weaning age was found to less severely decrease intestinal permeability [49]. A component of the extracellular matrix that is important to maintain the intestinal barrier function is the mucus layer, which was previously also reported to be decreased in starved rat intestine [50]. In our visual observations, we observed decreased thickness of the mucus layer in fasted piglets, most prominently in suckling piglets (data not shown). In addition, the significantly downregulated pathway “asparagine N-linked glycosylation” is not only crucial for the highly energy demanding processes of protein folding, sorting and secretion [38], but was also shown to be involved in protein production of mucus [51]. Combined, these results suggest a compromised intestinal barrier function upon fasting in both suckling and feed habituated piglets, with more prominent effects in the younger animals.



Eukaryotic translation initiation factors (EIFs) and ribosomal gene expression was upregulated irrespective of prior exposure to feed, possibly indicating increased mRNA translation. Increased mRNA levels were previously reported as a result of fasting in the intestine of fasted young rats [52]. In this context, it is important that

expression of EIFs and ribosomal genes does not equal increased protein synthesis, because the function of EIFs and ribosomal genes are primarily regulated on a post-translational level. Increased translation may be a mechanism to prepare for increased protein synthesis upon renewed energy availability, and is known as "translation on demand" [53]. This response may be especially prominent in developing tissues, because protein translation in liver and muscle of neonatal piglets was found to be more affected by fasting and refeeding than in older piglets [54]. Thus, the increased EIF and ribosomal gene expression observed in response to fasting rather seems to be a conserved 'priming' of the intestine in anticipation reintroduction of the luminal nutrient supply.

We also found that fasting regulated certain pathways in the opposite direction at different developmental feed habituated stages of the intestine. While cell cycle pathways were upregulated in suckling piglets, these pathways were downregulated in feed habituated piglets. Similar to our findings for the feed habituated piglets, decreased cell cycle progression has been reported in piglets upon weaning [55-58]. We hypothesize that the increased expression of cell cycle genes in suckling piglets does not necessarily induce increased proliferation of the intestine, but instead indicates a severe stress response. For example, among the top regulated genes upon fasting in suckling piglets are *PSMB8* and *PSMB9* that are also regulated in response to inflammation [59]. Additionally, the gene *NBN,* which is part of the MRN complex involved in double-stranded DNA break repair [60], and the gene *RMI1,* which is involved in maintaining genome stability [61] were among the most prominently regulated genes, and are indicative of a stress response. The increased stress response to fasting in suckling piglets can stem from the impactful separation from the sow occurring at time of fasting, but also from the altered developmental stage of the intestine. At birth, young piglets have an immature intestine, and rely on maternal factors to complete maturation [62]. Increased maturation during this time is reflected in high proliferation occurring in the first few weeks of life [58]. As the intestine matures, the effects of fasting seem to become less severe, as was observed in chickens and pigs [49, 63, 64]. Possibly, this is because the intestine becomes less dependent on turnover and can therefore better deal with a short-term decreased metabolic function. For future research, it would be interesting to separate age from developmental effects, to better understand what causes the divergent cell cycle regulation in suckling and feed habituated piglets.

Finally, we observed that pathways related to immune and stress signalling were differentially regulated upon fasting suckling or feed habituated piglets. While fasting increased inflammation in suckling piglets, it resulted in decreased immune function in feed habituated piglets. This divergent response may be caused by several factors.

Suckling piglets are removed from the sow at time of weaning, which also causes an abrupt loss of maternal antibodies provided through sows milk. In addition, the piglets are placed in a new pen and are mixed with piglets from other litters, causing increased immunological stress. In contrast, feed habituated piglets have had time to adapt to loss of maternal immunity, and were kept in the same pen during the fasting intervention. Similar to our findings for the suckling piglets, weaning was previously shown to induce a transient inflammatory state [65, 66]. However, in accordance with our observation in feed habituated piglets, fasting was previously found to decrease immune function and interferon signalling due to the decreased nutrient availability, resulting in lower activation of mTORC1, a central regulator of metabolic signals [67, 68]. Thus, our findings imply that the increased feed intake upon weaning is not the primary cause for increased inflammation observed upon weaning, but instead is a result of concurrent immunological stressors.

We found that fasting resulted in a stronger effects in the suckling compared to the feed habituated piglets. As argued, a less complete intestinal development as well as additional stressors may play a role in the more severe response in the sucking piglets. However, all our data were obtained in young piglets. These responses may differ from adult or older individuals. While we show that IEC metabolic function is decreased in the suckling and still young feed habituated piglets, this response seems in contrast to the fasting responses observed in older or high caloric diet exposed individuals, where mitochondrial function and fatty acid oxidation were increased [18, 69, 70]. This is thought to favour stem cells because of their dependency on mitochondrial fatty acid oxidation [71]. Indeed, fasting or calorie restriction induced increased mitochondrial function was found to translated into a better regenerative capacity of stem cells, thereby ameliorating the age-related decline in stem cell function [72]. The differences between our data obtained in piglets, compared to data obtained at a later age may possibly be related to related to differences in growth, cell turnover and regenerating capacity between young and old individuals. It would be of interest to analyse the small intestinal fasting response in young feed habituated piglets of slightly different age, to exclude the impact of other stressors and fully focus on intestinal maturation state and include adult pigs and old pigs, preforming transcriptome analysis together with functional metabolic analysis and analysis of the IEC maturation, proliferation and turnover rate, to better understand whether and how the fasting response depends on age.



In conclusion, we observed that fasting induced both similar and differential molecular pathways in the small intestine of suckling and feed habituated piglets, with gene expression being most strongly affected in suckling piglets. Together, our findings provide mechanistic information on the consequence of feed deprivation at various

stages of development, which enables us to identify which pathways are most likely caused by cellular energy stress. Further analysis of especially the robustly regulated pathways upon weaning can aid in the development of strategies to specifically support intestinal energy production during periods of reduced feed intake. This is important to improve piglet welfare and resilience in the pig rearing industry, which suffers from losses due to intestinal diseases, especially during the weaning period. It is equally important for infants, especially for those that suffer from an insufficient nutrient supply.

**Acknowledgements:** We would like to thank Bart Lagerwaard, Bert Beukers, Arjan van Dolderen, Jorden Uiterwijk, Carla Boersma and Ruiyu Zhang for skilful technical assistance technical assistance.

**Authors contributions: A.F.B.:** Conceptualization, Data curation, Formal analysis, Investigation, Visualisation, Writing – Original draft and Writing – Review & Editing; **V.C.J.d.B.:** Supervision, Funding Acquisition, Conceptualization, Methodology and Writing – Review & Editing; **W.J.J.G.:** Supervision, Funding Acquisition, Conceptualization and Writing – Review & Editing; **J.K.:** Supervision, Funding Acquisition, Conceptualization, Validation and Writing – Review & Editing

## References

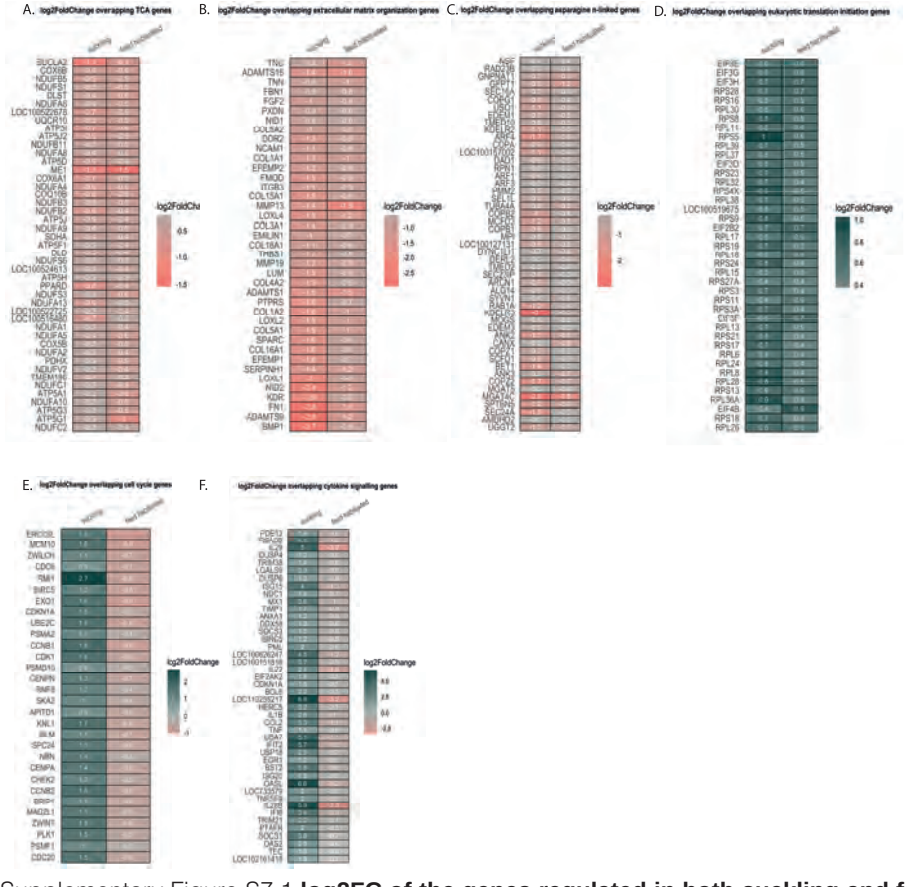

1. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nature Reviews Immunology, 2014. **14**(3): p. 141-153.
2. Rolfe, D.F. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals.* Physiological Reviews, 1997. **77**(3): p. 731-758.
3. Cant, J.P., B.W. McBride, and W.J. Croom, Jr., *The regulation of intestinal metabolism and its impact on whole animal energetics.* J Anim Sci, 1996. **74**(10): p. 2541-53.
4. Stoll, B., et al., *Substrate oxidation by the portal drained viscera of fed piglets.* American Journal of Physiology, 1999. **277**(1 Pt 1): p. E168-75.
5. Pluske, J.R., D.L. Turpin, and J.-C. Kim, *Gastrointestinal tract (gut) health in the young pig.* Animal Nutrition, 2018. **4**(2): p. 187-196.
6. Lallès, J.-P., et al., *Gut function and dysfunction in young pigs: physiology.* Anim. Res., 2004. **53**(4): p. 301-316.
7. van Beers-Schreurs, H.M.G., et al., *Weaning and the Weanling Diet Influence the Villous Height and Crypt Depth in the Small Intestine of Pigs and Alter the Concentrations of Short-Chain Fatty Acids in the Large Intestine and Blood.* The Journal of Nutrition, 1998. **128**(6): p. 947-953.
8. Le Dividich, J. and B. Sève, *Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet.* Domestic Animal Endocrinology, 2000. **19**(2): p. 63-74.
9. GBD 2019 Diseases and Injuries Collaborators *Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019.* The Lancet, 2020. **396**(10258): p. 1204-1222.
10. Choudhury, R., et al., *Impact of early-life feeding on local intestinal microbiota and digestive system development in piglets.* Scientific Reports, 2021. **11**(1): p. 4213.
11. Choudhury, R., et al., *Early life feeding accelerates gut microbiome maturation and suppresses acute post-weaning stress in piglets.* Environ Microbiol, 2021. **23**(11): p. 7201-7213.
12. Montagne, L., et al., *Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning.* British Journal of Nutrition, 2007. **97**(1): p. 45-57.
13. Pluske, J.R., D.J. Hampson, and I.H. Williams, *Factors influencing the structure and function of the small intestine in the weaned pig: a review.* Livestock Production Science, 1997. **51**(1): p. 215-236.
14. Meng, Q., et al., *Weaning Alters Intestinal Gene Expression Involved in Nutrient Metabolism by Shaping Gut Microbiota in Pigs.* Frontiers in Microbiology, 2020. **11**: p. 694-694.
15. Boudry, G.l., et al., *Weaning Induces Both Transient and Long-Lasting Modifications of Absorptive, Secretory, and Barrier Properties of Piglet Intestine.* The Journal of Nutrition, 2004. **134**(9): p. 2256-2262.
16. Burrin, D.G., et al., *Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough?* The American Journal of Clinical Nutrition, 2000. **71**(6): p. 1603-10.
17. Senterre, T., *Practice of enteral nutrition in very low birth weight and extremely low birth weight infants.* World Rev Nutr Diet, 2014. **110**: p. 201-14.
18. Mihaylova, M.M., et al., *Fasting Activates Fatty Acid Oxidation to Enhance Intestinal Stem Cell Function during Homeostasis and Aging.* Cell Stem Cell, 2018. **22**(5): p. 769-778.e4.
19. Ensminger, D.C., et al., *Fasting ameliorates oxidative stress: A review of physiological strategies across life history events in wild vertebrates.* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 2021. **256**: p. 110929.
20. Teruya, T., et al., *Diverse metabolic reactions activated during 58-hr fasting are revealed by non-targeted metabolomic analysis of human blood.* Scientific reports,

2019. **9**(1): p. 854-854.
21. Lalles, J.P. and J.C. David, *Fasting and refeeding modulate the expression of stress proteins along the gastrointestinal tract of weaned pigs.* J Anim Physiol Anim Nutr (Berl), 2011. **95**(4): p. 478-88.
22. Mazurais, D., et al., *Fasting and refeeding impacts on piglet jejunal transcriptome during weaning period.* Livestock Science, 2007. **108**(1): p. 13-16.
23. Moeser, A.J., C.S. Pohl, and M. Rajput, *Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs.* Animal Nutrition, 2017. **3**(4): p. 313-321.
24. Campbell, J.M., J.D. Crenshaw, and J. Polo, *The biological stress of early weaned piglets.* Journal of Animal Science and Biotechnology, 2013. **4**(1): p. 19-19.
25. Blok, M.C., et al., *A new Dutch net energy formula for feed and feedstuffs for growing and fattening pigs*. 2015, Wageningen Livestock Research: Wageningen.
26. Andrews, S., *FastQC: a quality control tool for high throughput sequence data*. 2010, Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.
27. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
28. Anders, S., P.T. Pyl, and W. Huber, *HTSeq--a Python framework to work with high-throughput sequencing data.* Bioinformatics, 2015. **31**(2): p. 166-9.
29. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
30. Shen, L., *GeneOverlap: An R package to test and visualize gene overlaps*. 2021.
31. Subramanian, A., et al., *Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles.* Proceedings of the National Academy of Sciences, 2005. **102**(43): p. 15545-15550.
32. Wu, T., et al., *clusterProfiler 4.0: A universal enrichment tool for interpreting omics data.* The Innovation, 2021. **2**(3): p. 100141.
33. Gillespie, M., et al., *The reactome pathway knowledgebase 2022.* Nucleic Acids Research, 2021. **50**(D1): p. D687-D692.
34. Liberzon, A., et al., *The Molecular Signatures Database (MSigDB) hallmark gene set collection.* Cell systems, 2015. **1**(6): p. 417-425.
35. Blighe K, R.S., Lewis M, *EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling.* 2021.
36. Gu, Z., R. Eils, and M. Schlesner, *Complex heatmaps reveal patterns and correlations in multidimensional genomic data.* Bioinformatics, 2016. **32**(18): p. 2847-9.
37. Kanehisa, M., Y. Sato, and M. Kawashima, *KEGG mapping tools for uncovering hidden features in biological data.* Protein Science, 2022. **31**(1): p. 47-53.
38. Helenius, A., Aebi, and Markus, *Intracellular Functions of N-Linked Glycans.* Science, 2001. **291**(5512): p. 2364-2369.
39. Jackson, R.J., C.U.T. Hellen, and T.V. Pestova, *The mechanism of eukaryotic translation initiation and principles of its regulation.* Nature reviews. Molecular cell biology, 2010. **11**(2): p. 113-127.
40. Bruininx, E.M.A.M., et al., *Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs1.* Journal of Animal Science, 2002. **80**(6): p. 1413-1418.
41. Dybkjær, L., et al., *Eating and drinking activity of newly weaned piglets: Effects of individual characteristics, social mixing, and addition of extra zinc to the feed1.* Journal of Animal Science, 2006. **84**(3): p. 702-711.
42. Moeser, A.J., et al., *Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2007. **293**(2): p. G413-G421.
43. Costermans, N.G.J., et al., *Influence of the metabolic state during lactation on milk production in modern sows.* Animal, 2020. **14**(12): p. 2543-2553.
44. Hojgaard, C.K., T.S. Bruun, and P.K. Theil, *Impact of milk and nutrient intake of*

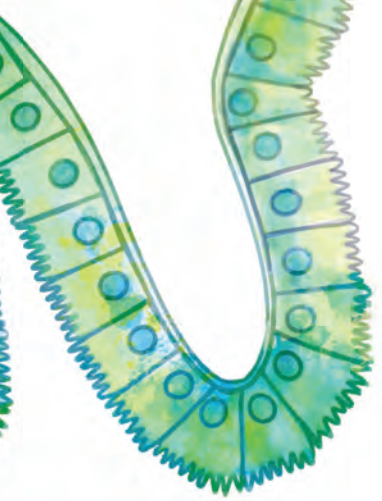
*piglets and sow milk composition on piglet growth and body composition at weaning.* Journal of animal science, 2020. **98**(3): p. skaa060.
45. Madec, F., et al., *Measurement of digestive disorders in the piglet at weaning and related risk factors.* Preventive Veterinary Medicine, 1998. **35**(1): p. 53-72.
46. Pelletier, D.L., et al., *The effects of malnutrition on child mortality in developing countries.* Bulletin of the World Health Organization, 1995. **73**(4): p. 443-448.
47. World Health Organization *Diarrhoeal disease.* 2017; Available from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease.
48. Vancamelbeke, M., et al., *Genetic and Transcriptomic Bases of Intestinal Epithelial Barrier Dysfunction in Inflammatory Bowel Disease.* Inflammatory bowel diseases, 2017. **23**(10): p. 1718-1729.
49. Wijtten, P.J., J. van der Meulen, and M.W. Verstegen, *Intestinal barrier function and absorption in pigs after weaning: a review.* Br J Nutr, 2011. **105**(7): p. 967-81.
50. Sherman, P., et al., *Mucin depletion in the intestine of malnourished rats.* Am J Physiol, 1985. **248**(4 Pt 1): p. G418-23.
51. González-Morelo, K.J., M. Vega-Sagardía, and D. Garrido, *Molecular Insights Into O-Linked Glycan Utilization by Gut Microbes.* Frontiers in Microbiology, 2020. **11**.
52. Burrin, D.G., et al., *Stage of Development and Fasting Affect Protein Synthetic Activity in the Gastrointestinal Tissues of Suckling Rats.* The Journal of Nutrition, 1991. **121**(7): p. 1099-1108.
53. Liu, Y., A. Beyer, and R. Aebersold, *On the Dependency of Cellular Protein Levels on mRNA Abundance.* Cell, 2016. **165**(3): p. 535-550.
54. Davis, T.A., et al., *Developmental changes in the feeding-induced stimulation of translation initiation in muscle of neonatal pigs.* American Journal of Physiology-Endocrinology and Metabolism, 2000. **279**(6): p. E1226-E1234.
55. Yang, H., et al., *Effects of weaning on intestinal crypt epithelial cells in piglets.* Scientific Reports, 2016. **6**: p. 36939.
56. Wang, J., et al., *Gene Expression Is Altered in Piglet Small Intestine by Weaning and Dietary Glutamine Supplementation.* The Journal of Nutrition, 2008. **138**(6): p. 1025-1032.
57. Zhu, L.H., et al., *Gene expression profiling analysis reveals weaning-induced cell cycle arrest and apoptosis in the small intestine of pigs1.* Journal of Animal Science, 2014. **92**(3): p. 996-1006.
58. Verdile, N., et al., *Evolution of pig intestinal stem cells from birth to weaning.* Animal, 2019. **13**(12): p. 2830-2839.
59. Yang, Z., et al., *Cardiovascular inflammation and lesion cell apoptosis: a novel connection via the interferon-inducible immunoproteasome.* Arteriosclerosis, thrombosis, and vascular biology, 2009. **29**(8): p. 1213-1219.
60. Yuan, J. and J. Chen, *N Terminus of CtIP Is Critical for Homologous Recombination-mediated Double-strand Break RepaiR..* Journal of Biological Chemistry, 2009. **284**(46): p. 31746-31752.
61. Wu, L., et al., *BLAP75/RMI1 promotes the BLM-dependent dissolution of homologous recombination intermediates.* Proceedings of the National Academy of Sciences, 2006. **103**(11): p. 4068-4073.
62. Sangild, P.T., et al., *Invited Review: The preterm pig as a model in pediatric gastroenterology.* Journal of Animal Science, 2013. **91**(10): p. 4713-4729.
63. Geyra, A., Z. Uni, and D. Sklan, *The effect of fasting at different ages on growth and tissue dynamics in the small intestine of the young chick.* British Journal of Nutrition, 2001. **86**(1): p. 53-61.
64. Faccin, J.E.G., et al., *Impact of increasing weaning age on pig performance and belly nosing prevalence in a commercial multisite production system.* Journal of Animal Science, 2020. **98**(4).
65. Novais, A.K., et al., *Weaning differentially affects mitochondrial function, oxidative stress, inflammation and apoptosis in normal and low birth weight piglets.* PLOS ONE, 2021. **16**(2): p. e0247188.

66. Le Bon, M., et al., *Gut transcriptome reveals differential gene expression and enriched pathways linked to immune activation in response to weaning in pigs.* BioRxiv, 2021: p. 2021.11.30.470420.
67. Schmeisser, H., et al., *Type I interferons induce autophagy in certain human cancer cell lines.* Autophagy, 2013. **9**(5): p. 683-696.
68. Powell, J.D., et al., *Regulation of immune responses by mTOR.* Annual Review of Immunology, 2012. **30**: p. 39-68.
69. Frier, B.C., R.L. Jacobs, and D.C. Wright, *Interactions between the consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2011. **300**(2): p. R212-R221.
70. Mana, M.D., et al., *High-fat diet-activated fatty acid oxidation mediates intestinal stemness and tumorigenicity.* Cell Reports, 2021. **35**(10): p. 109212.
71. Rath, E., A. Moschetta, and D. Haller, *Mitochondrial function — gatekeeper of intestinal epithelial cell homeostasis.* Nature Reviews Gastroenterology & Hepatology, 2018. **15**(8): p. 497-516.
72. Funk, M.C., J. Zhou, and M. Boutros, *Ageing, metabolism and the intestine.* EMBO reports, 2020. **21**(7): p. e50047-e50047.

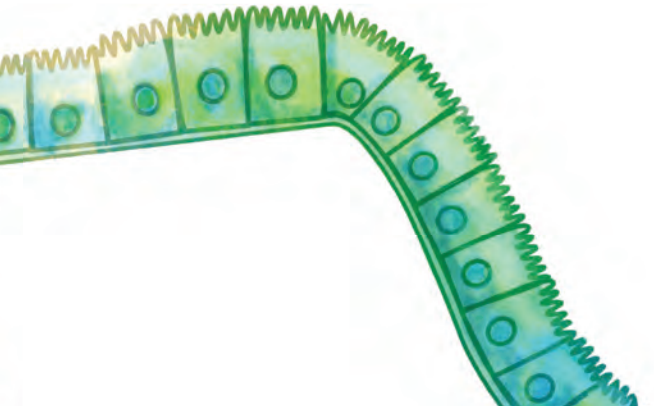
## Supplementary material




Supplementary Figure S7.1 **log2FC of the genes regulated in both suckling and feed habituated piglets in response to 48h fasting** for each of the following Reactome pathways: TCA cycle and respiratory electron transport (**A**), extracellular matrix organization (**B**), asparagine N-linked glycosylation (**C**), eukaryotic translation initiation (**D**), cell cycle checkpoints (**E**) and cytokine signalling (**F**).



# Chapter 8
General Discussion



## Main findings

The overall aim of my thesis was to increase our knowledge of how intestinal epithelial cell (IEC) metabolism adapts to changes in nutrient supply under various conditions. This was done by addressing two specific aims.

The **first aim** was to better understand how gut fermentation metabolites influence IEC metabolic function. In **chapter 2**, I reviewed the literature on the effects of protein-derived polyamines in the large intestine. This revealed that polyamines play a crucial role in the intestine and can influence the metabolism of IECs, but whether luminal polyamines affect IEC physiology *in vivo* remains unclear. This is a research gap to be investigated in future studies. In **chapter 3**, I experimentally investigated the effect of butyrate on metabolism of cultured colonocytes. I showed that butyrate itself was not oxidised in cultured cells, but that it instead increased the oxidation of pyruvate and increased lipid accumulation, presumably by being directly incorporated into lipid droplets. In **chapter 4**, I optimised a method to isolate viable primary pig IECs and measure their metabolic function *ex vivo* in the Seahorse XF analyser, allowing us to investigate the functional metabolic impact of *in vivo* applied challenges in isolated IECs. In **chapter 5**, I used this method to investigate the *in vivo* effects of low and high levels of fermentable fibres and well and poorly digestible protein on colonocyte metabolic function in pigs. I showed that the mitochondrial capacity of isolated colonocytes was only reduced by high fermentable fibre levels in an easily digestible whey-protein diet. In contrast, the glycolytic function of colonocytes was reduced by high fermentable fibre, irrespective of dietary protein source. Due to the complexity of the diets, it was difficult to link effects to specific colonic fermentation end products.

The **second aim** was to establish the functional metabolic and molecular consequences of fasting on IECs. In **chapter 6**, I studied the functional metabolic and molecular responses of IECs to fasting, in feed habituated piglets. I found that 48hrs of fasting simultaneously decreased functional mitochondrial and glycolytic metabolism in jejunal IECs of six-week-old feed habituated piglets. This also validated the global transcriptome results, where, upon fasting, mitochondrial pathways were among the top regulated Reactome pathways. Further gene expression analysis revealed that decreased metabolic function coincided with decreased cell proliferation and reduced expression of barrier function genes. In **chapter 7**, I investigated the differences in the response to fasting between jejunal IECs of four-week-old suckling piglets and six-week-old solid feed habituated piglets. I found that gene regulation upon fasting was strongest in suckling piglets. Furthermore, while some molecular pathways were similarly regulated in suckling and feed habituated piglets, indicative of a robust response to the fasting intervention, other vital processes were regulated in the opposite direction upon 48h fasting. This controlled fasting intervention proved to be a

valuable way to further unravel the relationship between metabolism and IEC function.

## The effects of specific fibre and protein-fermentation derived metabolites

Undigested carbohydrates and proteins, derived either directly from the diet or from endogenous losses (i.e. everything that originates from the intestine itself), end up in the colon [1-3]. Here, they are fermented by the microbial community, giving rise to a plethora of fermentation metabolites. These fermentation metabolites interact with the host's epithelium, and thereby potentially impact IEC function. To investigate how colonic IECs cope with such fermentation metabolites, we first studied specific fermentation-derived metabolites: butyrate as a major fibre-fermentation derived metabolite (**chapter 3**), and polyamines as a major class of protein-fermentation derived metabolites (**chapter 2**).

### *Fibre-fermentation derived butyrate*

Fibre-fermentation derived butyrate was the main focus of **chapter 3.** Fibres are dietary components that have a broad role in maintaining gastrointestinal health [4]. They help to maintain stool consistency by adding bulk to the stool and improving gut contractility [5]. In addition, they positively influence gut health through metabolites. Microbial fermentation of fibre results in the formation of metabolites that are in contact with the intestinal mucosa, and can thereby influence both mucosal and whole-body physiology. The major fibre fermentation metabolites acetate, propionate and butyrate were reported to decrease gut inflammation [6, 7], to protect against colorectal cancers [8], to increase whole-body fatty acid oxidation [9], and to increase insulin sensitivity [10]. From these fibre-fermentation derived metabolites, we investigated butyrate, as this is considered the primary energy source of colonocytes [11]. We explored how butyrate exposure impacted IEC metabolism in both cultured and primary colonocytes, giving special attention to the nutrient environment. This is relevant, because mechanistic studies into the effects of butyrate are often performed using *in vitro* cultures, where only a single specific nutritional condition and culturing environment is considered.

Our results **in chapter 3** highlighted that the nutritional environment and metabolic phenotype of cells are relevant considerations when interpreting metabolic responses to fermentation metabolites. Butyrate was found not to be oxidised in cultured cells, but instead, it rewired pyruvate oxidation in the presence of glucose and pyruvate. Although long-term exposure to butyrate did not affect the basal oxidative metabolism of cultured colonocytes, their glycolytic function was significantly decreased. We therefore concluded that the presence of glucose impacted butyrate metabolism, likely redirecting it towards incorporation in lipid droplets. Supporting this conclusion are

several reports that butyrate increased glucose oxidation in various cultured colon cell types [12-15]. However, it is important to note that cultured cells display the Warburg effect, meaning they primarily rely on aerobic glycolysis and are less dependent on mitochondrial OXPHOS than *in vivo* colonocytes [16, 17]. This could mean that our cultured cells are less able to oxidise butyrate [18, 19], which may have influenced the response of cultured cells to butyrate. However, we have found that isolated primary pig colonocytes, that do not necessarily rely on Warburg-type metabolism, also displayed decreased glycolytic function upon acute butyrate exposure. This indicates that this may be a conserved phenomenon that is not (solely) the result of a Warburg type of metabolism of cultured colonocytes.

### *Protein-fermentation derived polyamines*

Protein-fermentation derived polyamines were the main focus of **chapter 2.** Undigested protein is another dietary component that is fermented by the microbiome, but in contrast to fibre, it is mainly regarded as being harmful to gastrointestinal health. For instance, high protein diets have been associated with decreased fecal consistency and decreased fecal dry matter content in pigs [20, 21]. The negative effects of protein in the colon are principally attributed to the protein-fermentation derived metabolites that are formed as a result of protein fermentation [22, 23]. Prominent protein-fermentation metabolites are polyamines. To better understand their potential *in vivo* impact on IEC health we performed a literature review that summarised the current knowledge on the *in vivo* physiological effects of increased protein-fermentation derived polyamines in the colon. This resulted in a more nuanced picture. Polyamines can potentially impair IEC metabolic function, e.g. by depleting acyl-CoA levels or local production of toxic compounds [24-26]. But, polyamines were also found to be crucial for cellular physiology, because they are required for cell proliferation and protein translation, at least in part through post-translationally modifying the translation initiation factor EIF5a (as described in **chapter 2**, [24]).

An important research gap identified by the literature review performed in **chapter 2,** is whether *in vivo* luminal polyamine levels can change the intracellular polyamine concentration of IECs. To investigate this, I performed experiments in *in vitro* model systems using cultured HT29 colonocytes that were exposed extracellularly to polyamines. To assess whether extracellular polyamines could impact intracellular polyamine levels or function, I analysed protein post-translational modifications that can be altered upon a change in intracellular polyamine levels, and analysed metabolic profiles using Seahorse extracellular flux analysis. Exposing HT29 cells to polyamines did not result in post-translational modification of EIF5a, and neither did polyamine exposure alter the protein lysine-acetylation patterns or metabolic function of these cells. However, I used only one cell line thus far, and I exposed the cells to polyamines

for a relatively short duration (24h), which means that more experimental conditions should be included to draw definitive conclusions considering the aforementioned research gap. The *in vitro* experiments can be improved by using more cell lines or organoid cell-systems to better represent the *in vivo* situation. In addition, longer exposure times and more read-out measurements should be incorporated to better evaluate the outcome of extracellular polyamine exposure. Finally, a useful approach to study the impact of extracellular polyamine exposure could be to perform further analysis on the dietary intervention study performed in **chapter 5.** In the future, we will analyse the polyamine concentrations in the colonic digesta samples. Using western blot and immunohistochemistry, we can then associate luminal concentrations of polyamines with the expression of key enzymes involved in polyamine metabolism, or with the level of EIF5a that is post-translationally modified by polyamines in pig colonocytes. These analyses can give us insight into whether luminal polyamine levels impact intracellular polyamine metabolism. Such information is needed to understand whether and how high luminal concentrations of polyamines are potentially harmful to colonocytes.

## Challenging IECs with nutritional interventions

In **chapters 5, 6 and 7** I aimed to challenge in vivo IECs with nutritional interventions. Nutritional interventions can alter the luminal environment to which the intestinal epithelium is exposed. As the composition of the luminal environment can affect the function of IECs, nutritional interventions can be regarded as a challenge to the intestinal epithelium. A challenge is defined as a 'summons to take part in a contest or a trial of strength' by the Oxford dictionary, or as a 'stimulating task or problem' according to the Merriam-Webster dictionary. In physiology, the term *challenge* is often used to describe a method that aims to stimulate or trigger a response in an individual, organ or cell system [27]. The challenge allows the experimentalist to study how the system copes with the trigger: whether a new physiological state is reached or whether the system returns to the initial physiological state [27]. Characterizing this response on the cellular and molecular level generates relevant information about the (robustness of the) physiology of the system under study. Used in this sense, a challenge is something that triggers a response, but is not necessarily good or bad. In this thesis, I have challenged IECs with two nutritional interventions: a dietary intervention to impact the type and amount of fermentation metabolites in the colon, and a fasting intervention to eliminate luminal nutrient supply to small intestinal IECs. These nutritional interventions thus challenged IECs, since they needed to respond and adapt to the stimulus applied. Below, I will further discuss the two nutritional interventions applied in this thesis, elaborate on the main findings, and examine the way forward for studying the role of IEC metabolism in intestinal physiology.

### *First intervention: fermentable fibre and poorly digestible protein*

As dietary carbohydrates and proteins are important substrates for microbial fermentation, altering the diet can impact the type and amount of fermentation metabolites that are produced (e.g. [28-30]). Due to several recent societal developments that involve a shift in animal and human diet intakes, I was interested in better understanding the interactions between protein and fibre in the colon. For instance, pig nutrition is in transition to a more circular economy, involving increased use of high-fibre by-products that are also rich in moderately digestible proteins [31]. In human nutrition, plant-based diets are increasingly consumed, but such diets contain more fibres, while plant proteins have poorer protein digestibility, potentially increasing protein flow towards the colon [32].

To assess the *in vivo* effects of fermentation metabolites and the interaction between protein and fibre fermentation, we performed a dietary intervention in which we fed pigs diets in a 2x2 factorial arrangement, with protein source and fibre level as factors (**chapter 5**). Because we aimed to investigate how diets, through luminal metabolites in the distal colon, affected IEC metabolic function, we assessed metabolite concentration as colonic flux in mmol per hour. As expected, feeding poorly digestible collagen protein increased the flux of protein fermentation metabolites, and feeding high levels of fermentable fibre increased the flux of all the SCFAs measured in the colonic lumen. Based on the available literature, we expected that the presence of fibre in a poorly digestible collagen protein diet would decrease protein fermentation [28, 33]. Surprisingly, we observed that high fibre diets increased the colonic flux of protein fermentation metabolites, of ammonia, isovalerate, valerate, isobutyrate, and isocaproate (**chapter 5**). While this study was thus a relevant intervention to determine whether protein and fibre fermentation interact, it turned out to be less suitable to obtain a more mechanistic understanding of how the principal dietary constituents influenced functional IEC metabolism. The concomitant increase of fibre and protein metabolites upon feeding poorly digestible protein and fermentable fibres made it challenging to determine whether changes in the flux of specific fermentation metabolites could underlie altered IEC metabolism. In addition, the diets not only differed in metabolite flux, but also in mean retention time, stool consistency, ingredient composition (e.g. addition of synthetic amino acids in the collagen diets) and amino acid composition of the proteins used. All these differences could have interacted, which may have impacted their ultimate effect on colonic IEC function.

### *Second intervention: fasting*

Low feed intake, or periods of fasting, contribute to the onset of post-weaning diarrhoea in piglets [34-36]. Likewise, in humans malnutrition is recognised as one of the

underlying causes of potentiating diarrhoea development in children [37, 38]. As fasting and malnutrition are expected to alter the amount and type of nutrients and route IECs are exposed to, it is likely that IECs adapt their function and metabolism in response to these challenges, as is known to occur upon several nutritional interventions. For example, the small intestinal epithelium of piglets fed low protein feeds showed altered cellular metabolic substrate use compared to high protein fed pigs [39]. In addition, a study in rats found that arterial glutamine becomes the preferential substrate for enterocytes in the absence of luminal nutrients [40]. Also interesting is the finding that calorie restriction was shown to decrease the mass of most organs, while the alimentary tract is spared, possibly to ensure optimal uptake of available nutrients [41]. These findings thus show that the intestine can adapt to the unavailability or shortage of certain metabolic substrates. While luminal nutrients are recognised to be significant metabolic substrates for cells in both the small and the large intestine [42-44], the question remains whether the absence of said nutrients is indeed detrimental to IEC metabolism, or if it can adapt by switching to arterial nutrients.

In this thesis, I showed that fasting decreased the mitochondrial and glycolytic function of isolated IECs in feed habituated piglets, signifying that overall metabolic function was decreased (**chapter 6**). The RNA-sequencing results corroborated these functional metabolic measurements, showing that pathways related to mitochondrial energy production were decreased in both suckling and feed habituated piglets (**chapters 6 and 7**). Furthermore, I showed that extracellular matrix organisation gene expression was decreased in suckling and feed habituated piglets. This could imply that intestinal barrier function was decreased upon fasting [45]. Although weaning is well-known to induce decreased barrier function [46, 47], the role of low or no feed intake therein remained unclear. Although low or no feed intake at weaning is common, weaning is also associated with many other stressors that could contribute to intestinal barrier dysfunction at weaning, like maternal separation and a new environment [35, 48]. As the feed habituated piglets did not experience these additional weaning stressors, our findings strengthen the notion that low feed intake and mitochondrial dysfunction contribute to loss of barrier function. Together, our findings underscore the importance of luminal nutrient supply in maintaining IEC metabolic function.

### *Opportunities for marker development*

Comparing the fasting response between suckling and feed habituated piglets enabled us to identify a set of mitochondrial genes that was decreased in both suckling and feed habituated piglets (**chapter 7**). This gene set could be used as a starting point to develop robust markers for assessing mitochondrial function. Identifying robust marker genes is valuable, as they can potentially be applied and translated

to other tissues and species. In addition, these markers could be applied in future studies to assess (intestinal) mitochondrial function, or, if tissue is preserved in already performed studies, even retrospectively. The set of mitochondrial genes described by us in **chapter 7** in particular is valuable, since: 1) the decreased mitochondrial gene expression was validated by decreased mitochondrial flux analysis, and 2) the genes were regulated at two developmental time-points, and thus are more likely the direct result of energy deprivation. However, the gene set needs further validation e.g. in other species and other conditions, to ensure that the genes are indeed robustly regulated upon mitochondrial dysfunction. Further validation will need to incorporate functional metabolic analysis, to confirm that altered expression of the marker genes is indeed linked to altered metabolic function. As we have shown, functional IEC metabolism can be measured by isolating IECs and measuring their metabolic function, which I have optimised (**chapters 4, 5 and 6**). This tool has proven to be useful and sensitive to measure IEC metabolic function. Together, the set of overlapping mitochondrial genes as identified in **chapter 7**, and further validation of these genes using the functional assessment of IEC metabolism as described in **chapters 4, 5 and 6,** can help us to develop robust markers to assess the mitochondrial function of IECs in future studies.

### *Differentially regulated pathways indicate increased disease susceptibility of piglets upon fasting*

Further investigating the differentially regulated pathways upon fasting suckling and feed habituated piglets could help us to determine whether fasting itself primarily causes the differential expression of genes, or other concomitantly occurring stressors. For example, suckling piglets were effectively fasted at the time of weaning, meaning that they experienced additional stressors such as maternal separation, new pen mates, and a new physical environment [35, 48]. In addition, suckling piglets have a less mature intestine than solid feed habituated piglets, as solid feed habituated piglets were two weeks older at the time of the fasting intervention, and their intake of solid feed is expected to enhance intestinal maturation [46, 49, 50]. The differentially regulated pathways were already discussed in **chapter 7**, but a few will be highlighted in the remainder of this section and placed in a broader context.

Our findings potentially point to a predisposition to disease onset in piglets upon fasting. This is supported by the observed synchronous decreased expression of mitochondrial and barrier function genes upon fasting in both suckling and feed habituated piglets. Furthermore, we observed a striking divergent response in immune activation between both groups of piglets, which could further point toward increased disease susceptibility. Suckling piglets displayed a higher expression of immune pathways, which is indeed also observed in weaned piglets [51, 52]. This is most likely not primarily caused by low

feed intake around weaning, but by new pen mates and a new physical environment, considering that fasting downregulated immune pathways in feed habituated piglets. Decreased immune activation upon fasting was also described in the literature, as fasting lowered activation of mTORC1, a central regulator of metabolic signals, and this led to decreased immune function and interferon signalling [53, 54]. This implies that if piglets are fasted or have low feed intake at weaning, they could be less able to launch an immune response, while they experience more immune challenges, and a decreased barrier function. Together, these factors could translate to increased disease susceptibility of weaned piglets.

The findings in our study thus reveal primarily negative impacts of fasting in piglets, which is in contrast to the positive effects of calorie restriction and fasting in humans and mice, where this is recognised as a means to increase longevity and healthspan [55, 56]. I want to argue that such contradictions call for careful consideration of factors that might influence the outcome of fasting. Firstly, it is important to consider the different methods used to induce fasting or calorie restriction. Calorie restriction refers to a decrease in total calories consumed without causing malnutrition. In mice, calorie restriction is often enforced by limiting feed allowance and providing that feed only once per day, which results in the mice ingesting one meal, followed by a prolonged fast [57]. Calorie restriction thus not only limits the total amount of calories consumed, but also results in cycles of fasting and refeeding. These fasting/refeeding cycles are thought to prevent a build-up of damaged mitochondria [58], which aids in maintaining optimal mitochondrial dynamics throughout life [59, 60]. Although calorie restriction can thus resemble fasting in how it induces cycles of fasting and refeeding, a calorie restriction intervention is likely not as severe as 48hs of fasting, as was applied in this thesis. In part, the high severity of the 48h fasting intervention is due to the high growth rate of young piglets which at that time have few metabolic reserves to fall back on [61, 62], which means that they are more dependent on dietary nutrient supply. In addition, it is important to consider that our fasting intervention was only a single fasting cycle. Calorie restriction, on the other hand, is often performed much longer, potentially giving the tissue time to adapt to this nutrition intervention. Secondly, an important consideration with regard to the positive effects ascribed to calorie restriction is that these studies are primarily performed in animal facilities that are kept sterile; thus, these mice do not experience additional immune stressors. In our experiments, it appears that piglets at weaning experience a lot of immunological stressors, which they may be less able to cope with due to the fasting intervention as discussed above. This corresponds with findings that calorie-restricted mice are less able to launch an immune response against intact pathogens [63]. Thirdly, a factor that could influence whether fasting or calorie restriction is beneficial is the developmental stage of an individual, as will be further discussed later. Together, these considerations indicate

that applying fasting or calorie restriction requires careful consideration with regards to factors that may impact outcome of such an intervention.

## Regulation of IEC metabolic function beyond gene expression

An intriguing observation in this thesis is that we found an altered metabolic function of IECs, even though we analysed the metabolic flux of the isolated IECs *ex vivo* in a nutrient-rich environment (**chapters 5 and 6**). Especially for the decreased metabolic fluxes observed in **chapter 6**, we are confident that this reflects the *in vivo* situation. Here, decreased metabolic fluxes coincided with decreased mitochondrial gene expression, performed on mucosal scrapings snap-frozen immediately following slaughter. These results are exciting, as we know from the literature that metabolic function can rapidly respond to altered nutrient availability. For example, ingesting a meal following an overnight fast increased the expression of key glucose and amino acid metabolism proteins within two hours in human peripheral blood mononuclear cells [64]. As we can measure decreased mitochondrial and glycolytic function of isolated IECs even in a nutrient-rich environment, our results point towards longer-term regulation or imprinting of IEC metabolic phenotype by the nutritional interventions. Below, I will first highlight two regulatory mechanisms that are sensitive to nutritional interventions, which can imprint metabolic adaptations. Thereafter, I will explore how these mechanisms can program metabolic adaptations.

### *Post-translational modifications and their regulation of metabolic function*

A well-known mechanism by which metabolic fluxes can be modified is by reversible post-translational modification (PTM) of proteins. Proteins present in the nucleus (mostly histones), cytosol and mitochondria are known to be modified by PTMs, with diverse repercussions for their functionality. A form of PTM that was shown to specifically modify metabolic enzymes, and may thus regulate metabolic flux directly, is acylation. Acylation involves the modification of lysine residues through the covalent binding of an acyl group, often derived from a metabolic intermediate. Metabolic intermediates can react with proteins via acyltransferases or deacetylases, or directly modify proteins through non-enzymatic mechanisms [65]. In response to acylation, the function of acylated proteins changes for several reasons, including removal of the positive charge of the lysine residue or through steric hindrance. The most well-known protein acylation is acetylation, which derived from acetyl-CoA, but many others are known. Through acylations, the function of almost all metabolic proteins can quickly adjust to cellular needs [66]. For example, increased fatty acid concentrations in the cell culture medium induced the acetylation of a central fatty acid oxidation enzyme, leading to increased fatty acid oxidation [66]. Alterations in nutrient availability quickly

affect protein acylation and nutrient fluxes, because acylation is highly dependent on substrate concentrations [67].

There are some indications that changes in PTMs may have influenced our results. We observed an increase in colonic SCFA flux in response to the dietary intervention performed in **chapter 5**. The diets could thereby have changed PTMs in IECs, since microbial metabolites have been shown to alter the host's epigenome [68]. Especially the SCFAs acetate, propionate and butyrate have gained attention in this regard, since they were found to increase the host's histone acylation status, thereby increasing gene expression [69]. Butyrate especially was shown to inhibit histone deacetylase activity, thereby increasing histone and protein acylation patterns in enterocytes [18, 44]. Because we observed that butyrate altered pyruvate flux, we also performed an experiment in which we exposed HT29 cells to 5 mM of acetate, propionate and butyrate for 72h. We then analysed whether butyrate exposure led to post-translational modification of central glucose oxidation enzymes. Pyruvate kinase M2 (PKM2), the enzyme responsible for converting phosphoenolpyruvate to pyruvate, was previously reported to be a direct binding target of butyrate [19]. However, we did not observe acylation, butyrylation or crotonylation of PKM2 following 72h exposure of HT29 cells to 5 mM of the SCFAs. Nevertheless, exposure of the cell lysate to acetic anhydride did induce acetylation of PKM2, showing that acylation is possible but did not occur at detectable levels after exposure of cells to these SCFAs. We also assessed whether lactate dehydrogenase, the enzyme converting pyruvate to lactate, was butyrylated and acetylated, but this was also not the case. Even though we did not observe acylation of the analysed proteins after 72h exposure of HT29 cells to the SCFAs, we did observe that PKM2 can be acetylated, proving that acetylation of this protein is possible. In addition, other thus far unanalysed proteins could be acetylated. It would be therefore be interesting to dive further into this relatively new and underexplored field to identify novel protein targets that the SCFAs acylate.

Effects observed in the fasting study could also possibly be explained by alterations in PTMs. For example, fasting feed-habituated piglets induced expression of both ketogenic genes as well as the pyruvate dehydrogenase regulator, PDK (**chapter 6**). Interestingly, the ketone body ß-hydroxybutyrate was recently identified to be able to post-translationally modify histone lysine residues [65]. Histone ß-hydroxybutyrylation was also shown to induce *PDK4* expression in fasted mice's intestines [70]. Via ß-hydroxybutyrylation of histones, ketogenesis could thus contribute to the induction of lipolysis in the intestine upon fasting. In addition, lysine deacetylases appear to play a major role in the molecular fasting response. Fasting and calorie restriction are well known to increase the expression and activity of histone and protein deacetylases [71, 72]. For example, recruitment of class IIa histone deacetylases (HDACs) into

the nucleus was found to upregulate expression of gluconeogenic genes in the liver upon fasting [73]. We likewise observed upregulation of gluconeogenic genes upon fasting in pig intestines (**chapter 6**). At the same time, at least fasting of up to 24h seems to upregulate protein and histone acetylation in the liver and kidney of mice. This could be caused by increased mitochondrial oxidation, leading to increased availability of acylation substrates [74]. Although these findings seem contradictory (increased protein and histone acetylation with concomitant increased HDAC activity), this is likely needed to deacetylate specific proteins and gene promotor sites that have been acetylated [75]. For example, promotor sites needed for the transcription of fatty acid oxidation genes were shown to be deacetylated by SIRT3 upon fasting, enabling their transcription [75]. Upon fasting, we observed that genes belonging to the histone deacetylase superfamily were indeed primarily upregulated upon 48h fasting in both suckling and feed habituated piglets (fig. 8.1). However, the expression of individual deacetylases varies between suckling and feed habituated piglets, indicating the global protein acylation pattern may differ between these age groups. Thus, it would be interesting to further investigate how protein acylation is altered upon fasting in the suckling and feed habituated piglets, and how this contributes to the molecular regulation of gene expression.

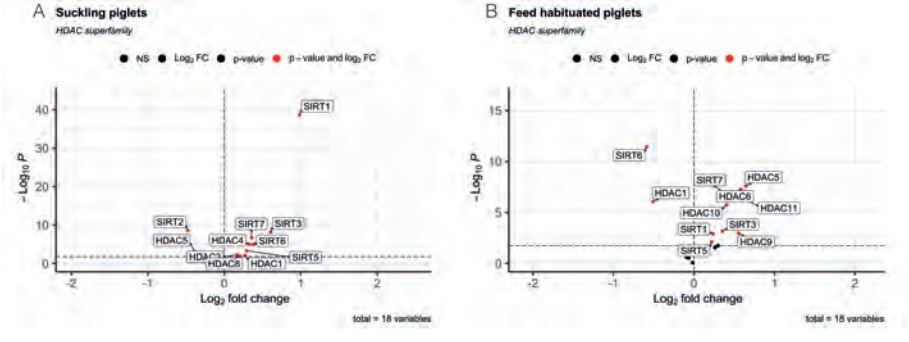



Figure 8.1 **Regulation of the histone deacetylase (HDAC) superfamily in the jejunum of suckling and feed habituated piglets upon 48h fasting.** Volcano plots of the genes belonging to the HDAC superfamily regulated in suckling (**A**), and feed habituated (**B**) piglets. Out of the eighteen genes in the gene set, eleven were significantly regulated in both suckling and feed habituated piglets. Significantly regulated genes are labelled with their gene symbol.

### *Mitochondrial remodelling as driver of mitochondrial efficiency*

Another mechanism that determines the efficiency of mitochondrial ATP production is mitochondrial morphology. Mitochondria are not single organelles, but operate in dynamic networks [76]. Fused mitochondria form tubular networks with high ATP synthesis capacity, but certain stressors can induce mitochondrial separation, called fission, to eliminate dysfunctional organelles [77]. A fused mitochondrial network improves metabolic efficiency by allowing the exchange of nutrients and cofactors.

In addition, it prevents auto-phagocytic clearance of mitochondria, also termed mitophagy [77, 78]. Interestingly, these processes of mitochondrial fission and fusion were found to be regulated through the previously discussed PTMs [79]. Currently, it is unknown whether and how quickly mitochondrial remodelling occurs in the intestine in response to the nutritional interventions applied in this thesis. However, we know from literature that the nutritional interventions applied potentially impact mitochondrial remodelling. For fasting, there are clear indications that mitochondrial morphology changes. Upon starvation, mitochondria were found to fuse into elongated tubes in cell culture systems [78], and in murine skeletal muscles [77]. Likewise, butyrate and acetate increased mitochondrial fusion in pancreatic islets and colon cancer cells [80, 81]. Whether butyrate, acetate and/or fasting also impact mitochondrial fusion in the colon *in vivo*, would be an interesting avenue for further research.

In addition to these morphological changes induced by fission and fusion, mitochondrial location within the cells is also important. Often, mitochondria reside in cellular locations with high energy demand. In the intestine, mitochondria were found close to the cell-cell junctions [82], suggesting that maintaining intestinal barrier function indeed is a highly energy-demanding process. Indeed, mitochondrial uncoupling decreases expression of tight junction proteins [83]. The processes of mitochondrial fission and fusion potentially influence mitochondrial localisation within the cell, leading to local energy shortages at critical cell locations, such as the cell-cell junctions. In future studies, it would be interesting to investigate the impact of challenges on intestinal mitochondrial morphology and localisation, to better understand the mechanisms underlying altered metabolic fluxes. This can be done for example via immunohistochemical staining of key mitochondrial proteins, such as COX4, to visualise the mitochondrial network and localisation within the intestinal epithelium. If alterations in mitochondrial network dynamics and localisation are observed, more detailed mechanistic studies are required to further investigate how the nutritional interventions altered mitochondrial remodelling and how this relates to the altered mitochondrial metabolic flux.

## Imprinting of cellular adaptations to nutritional interventions

As stated above, the processes of PTM and mitochondrial remodelling are dynamic and reversible, but they are known to be able to imprint metabolic adaptations. First, together with the altered nutrient environment following nutritional interventions, these processes impact metabolic fluxes. Thereby, the presence of mitochondrial and glycolytic metabolites is altered, and these metabolites communicate the metabolic status to the nucleus to align nuclear and mitochondrial activities. As we have also observed, gene expression can be altered, but the chromatin can also be remodelled with more permanent effects. For example, in humans, there is also evidence that *in*

*utero* exposure to malnutrition leads to a higher risk of developing non-communal diseases in later life [84]. Mechanistically, genetic imprinting can be induced by early life events through the mechanisms described above. For example, yellow-coated agouti dams that received a diet supplemented with methyl-donors (folic acid, vitamin B12, choline and betaine) during gestation were found to produce offspring with a coat colour closer to the brown pseudo agouti phenotype [85]. Furthermore, studies showed that intrauterine growth restriction (IUGR) results in an increased risk to develop insulin resistance [86]. In rats, it was found that this can at least be partially attributed to decreased acetylation and methylation of the promotor site of *Pdx-4*, a key transcription factor involved in pancreatic islet cell development [87].

Although especially *in utero* exposure was identified as a susceptible period for nutritional programming, the critical developmental window extends into the postnatal period [88]. In humans, the critical window that impacts life-long health is mainly described as being the first 1000 days of life, from conception to about two years of age. Indeed, multiple studies describe how post-natal exposure can program later life health. For example, undernutrition in childhood was found to worsen non-communal disease outcomes [89, 90]. In addition, protein malnutrition during the post-weaning period induced metabolic alterations in rats long after re-administration of a balanced diet [89]. Another study reported that in mice, consuming a diet rich in n-3 long-chain polyunsaturated fatty acids in the first four weeks of life decreased fat accumulation and improved plasma lipid profile [91]. Likewise, studies from our lab also found evidence for post-weaning programming by dietary monosaccharides [92]. Also, exposure to drugs in early life, such as pharmaceutical antibiotics treatment, was found to alter the composition of the microbiome in later life [93, 94], which also affects later life metabolism of IECs by altering nutrient availability [94]. Thus, there is clear evidence for post-natal programming by nutritional interventions, but the effects on intestinal programming have been poorly studied. Therefore, it would be of interest to study the impact of the dietary and fasting interventions on later life intestinal function, and the role of mitochondria therein.

### *Altered intestinal morphology as a result of transcriptional and post-translational regulations*

Apart from life-long programming effects, it is also interesting to investigate how long it takes for the intestine to recover after the dietary interventions. The fasting intervention reduced absolute small intestinal length (**chapter 6**), and this is possibly a first indicator of altered intestinal architecture. The previously discussed changes in gene expression, mitochondrial morphology and acylation patterns could impact intestinal architecture and physiology. The PPAR response genes, that are activated by e.g. lipid intake, were shown to increase intestinal length [95], which could impact

intestinal digestion and absorption abilities. In addition, a switch to secretory cell types, such as Paneth and goblet cells, requires a rewiring of mitochondrial metabolism and induction of a glycolytic phenotype [96, 97]. These metabolic changes are likely driven by mitochondria fission. Indeed, mitochondrial fission was found to be required for the differentiation of stem cells into Paneth and goblet cells [96], while defective fusion inhibited differentiation of enterocytes in *Drosophila* [98]. Furthermore, increased mitochondrial fusion induced by calorie restriction was shown to favour the stem-cell niche and decrease the differentiation into new absorptive enterocytes in mice [99]. Differentiation of enterocytes into either absorptive or secretory phenotypes was also shown to be steered through PTMs [100]. Thus, the intestinal architecture seems to adapt in response to metabolic cues and in response to mitochondrial dynamics, and these changes may last for some time, even when nutrients become available again

## Next steps to identify the role of IEC metabolism in intestinal function

An important factor remaining to be answered is to what extent, and how, the metabolic function of IECs contributes to intestinal functions. I have seen that the metabolic function of IECs is altered upon many challenges, in this thesis mainly in response to microbial fermentation products and fasting. However, thus far, I have not been able to establish whether the decreased metabolic function of IECs also causes intestinal dysfunction. To investigate this, it is first important to understand what a causal relationship is. According to classical logic, a causal relationship implies an asymmetrical relationship: 'A' causes something in 'B', while this does not happen vice-versa. Can we conclude that decreased metabolic function causes intestinal dysfunction, and not vice versa? Although I observed simultaneous decreased metabolic function and decreased expression of barrier function genes in the fasting intervention, this does not prove causality. Moreover, barrier function was only assessed using barrier function genes, and these results should be substantiated with a direct assessment of barrier function to solidify the conclusions. Together, I cannot conclude on a causal relationship based solely on the findings in this thesis. However, some findings do suggest there is at least a relationship between intestinal metabolism and barrier function. A study from our lab did show that disruption of mitochondrial energy production also decreased barrier function of trans-well cultured Caco-2 cells [83]. In addition, the concomitant decrease of both mitochondrial and intestinal barrier function genes upon fasting in both suckling and feed habituated piglets does suggest that metabolic dysfunction at least contributes to decreased barrier function (**chapters 6 and 7**). Three steps need to be taken to establish this causal relationship: 1) selective manipulation of IEC mitochondrial function, e.g. using mitochondrial inhibitors or genetic manipulations; 2) functional IEC metabolism and mitochondrial gene expression analysis, using e.g. the tools provided in this thesis as described previously; 3) measure the physiological impact of such manipulations.

### *Manipulation and variations in mitochondrial function in vitro and in vivo*

To establish a causal relationship, we need methods to manipulate or identify variations in mitochondrial function in physiologically relevant systems. *In vitro* cells are a relatively easy method to do this, because chemical mitochondrial inhibitors can easily be applied. Interesting new technological developments, such as organoids [101] and organ-on-chip [102], allow us to mimic the intestinal environment with increasing accuracy, because they allow for culturing in a 3D space, using various cell types. It is much more challenging to administer chemical mitochondrial inhibitors *in vivo,* since the route of administration towards the intestine is more difficult to control, meaning that the inhibitor could easily also impact other organs and cells than just the IECs. Target drug delivery, e.g. using coated particles with specific properties, such as pH-sensitive or starch coatings that are only degraded in the lower intestines [98], are being developed to help circumvent this difficulty. In this way, the mitochondrial function may be decreased *in vivo* to investigate how this affects intestinal physiology.

Another method to specifically target intestinal mitochondrial function is to create genetically modified mouse strains. Mice are well-established animal models, and many techniques have been developed to induce genetic modifications. For example, it is possible to perform IEC-specific knock-down of critical mitochondrial genes, or overexpression of mitochondrial uncoupling proteins to decrease mitochondrial efficiency. Gene expression can further be modified during specific time points or certain developmental periods using inducible promotor sequences that are activated using the antibiotics Tetracycline and Doxycycline [103]. The fasting intervention, as described in **chapters 6 and 7,** proved a valuable method to isolate the effects of luminal nutrient deprivation from other stressors, and could be employed in future studies, in combination with genetic manipulations, to further investigate the role of IEC metabolism in intestinal physiology.

### *Methods to assess physiological impact of altered IEC metabolism*

Apart from measuring and manipulating mitochondrial function, methods are needed to assess the impact of such manipulations on intestinal physiology. As maintaining a solid barrier is such a crucial function of the intestine, intestinal barrier function is often measured to assess intestinal function. A relatively simple and inexpensive technique is the ‘everted-sac’ method, first developed in 1954 by Wilson and Wiseman [104], and is most often applied to study drug kinetics [105]. But the method has potential for much broader application. Using the everted-sac method, it was for example reported that exposure to bacterial cell-wall components increases colonic permeability of a 40 Kilodalton (KDa) marker in rats [106].

I have applied the everted-sac method to two animal experiments. The first experiment was a prospective trial that aimed to investigate the mechanisms underlying diarrhoea development. To do this, faecal samples of piglets were collected pre-weaning, before the development of diarrhoea, and post-weaning, when some piglets had developed diarrhoea. Piglets with and without diarrhoea were dissected five days post-weaning. Using the everted-sac method, I assessed intestinal permeability in the large intestine by measuring the flux of undigestible fluorescently labelled dextrans of 4 and 40 KDa from the luminal to the apical side of the intestinal sac. For this, we optimized and applied a plate-reader based assay. Importantly, the 40KDa TRITC-labelled dextran was difficult to analyse, due to obtained signals that were around the detection limit, making these results technically less reliable. To accommodate this, we also measured glucose uptake as a marker for ATP-dependent transport. A negative control for glucose uptake was included by incubating intestinal sacs in 0.1% sodium azide, which blocks cytochrome c oxidase and thereby inhibits mitochondrial ATP production. The results showed no difference between control and diarrhoea piglets in permeability of the 4 and 40 KDa markers, nor was there any difference in glucose transport (fig. 8.2A-C). However, there was a significant decrease in glucose transport upon sodium azide exposure, confirming that glucose transport across the everted-sac is indeed ATP-dependent. However, inhibition of mitochondrial ATP production did not increase everted-sac permeability of the 4 and 40 KDa markers (fig. 8.2A-B). These results thus did not show increased permeability, nor decreased energy-dependent glucose uptake, in the colons of piglets with post-weaning diarrhoea. Neither did short-term energy deprivation, induced by sodium azide treatment, cause increased permeability. The second experiment involved applying everted-sac method in the dietary intervention study described in **chapter 5**. Here, we did not observe any effect of the dietary intervention on colonic permeability of the 4 and 40 KDa markers (fig. 8.2D-E). In conclusion, the everted-sac method did not reveal altered colonic transcellular permeability of piglets with diarrhoea, following dietary interventions, or upon mitochondrial energy deprivation induced by sodium azide.

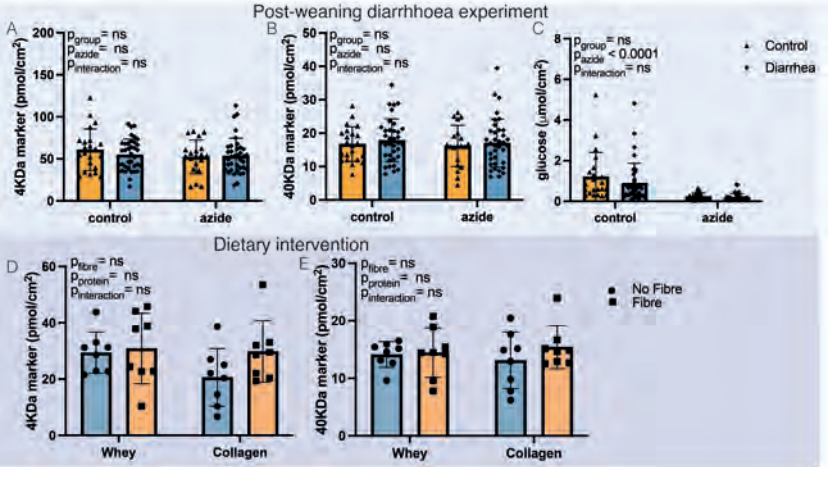


Figure 8.2 **Intestinal permeability measurement using the everted-sac.** Colonic permeability and ATP-dependent glucose transport of control piglets and piglets with diarrhoea were assessed in an on-farm experiment. Permeability to a 4KDa dextran (**A**) and a 40 KDa dextran (**B**) was measured to assess paracellular permeability. Glucose transport was measured as an ATP-dependent transport system (**C**). Sodium azide (0.1%) was a negative control for ATP-dependent transport. Colonic permeability of pigs fed diets differing in protein source and fibre level Permeability to a 4KDa dextran (**D**) and a 40 KDa dextran (**E**) was measured to assess paracellular permeability. KDa = Kilodalton.

There are several possible reasons why the everted-sac did not reveal differences in permeability following the interventions. As alluded to above, the technique may not be sensitive enough, e.g. because the markers are too large, and can only pass the intestinal barrier if there are relatively large paracellular gaps, or the signals are just too low to detect on our plate reader. In addition, the technique is subject to many *ex vivo* manipulations of the tissue (a good overview is given by [105]), which can impact permeability as measured using the everted-sac. The everted-sac method could be improved to more accurately measure intestinal permeability. For example, removing the muscle and serosa layers allows for more direct measurement of IEC paracellular permeability. Noorman *et al.* [107] recently showed that intact everted-sacs indeed result in an underestimation of jejunal permeability, and stripping away the serosa and outer muscle layer could increase the accuracy of the everted-sac method. Removal of the muscle and serosa layers is standard practice during permeability assessment using Ussing chambers, which measures both paracellular permeability and electrical resistance of the mucosal layer [108]. A significant advantage of everted-sac and Ussing chamber analyses is that they enable the measurement of intestinal permeability in specific intestinal locations. However, these are *ex vivo* analyses, and it would be good to assess permeability *in vivo*. A possible method to do this is by applying a multiple sugar test. This method allows for site-specific measurement of intestinal permeability with markers that can be analysed in both plasma and urine samples [109, 110]. Finally, combining manipulation of IEC metabolism with read-outs of intestinal permeability will aid in further unravelling the role of IEC metabolism in intestinal physiology.

### *Further recommendations for future research into IEC metabolic function*

In addition to the methods to manipulate and assess IEC metabolism and its impact on intestinal function as mentioned above, it would be valuable to study IEC metabolism further. In this thesis, I was able to analyse the impact of nutritional interventions on various levels of organismal organisation by combining multi-level analyses: the molecular (e.g. transcriptome analysis), the cellular (e.g. functional metabolic analysis) and the organismal (e.g. body weight). Although these multi-level analyses added complexity to the data interpretation, I believe this is the way forward to determine the true impact of nutritional interventions on IEC metabolism. Taking this further, this

calls for an extensive analysis at multi-levels, including functional parameters, of all animal experiments to better establish how interventions impact overall physiology. In addition, we can further improve our interpretation of how nutritional interventions impact IEC metabolism by simultaneously characterizing the luminal gut environment and the arterial nutrient supply to and drainage from the intestine. Organs constantly exchange nutrients through the bloodstream [111], and IECs can switch to these arterial metabolic substrates, as was discussed previously. In response to nutritional interventions, it is therefore likely that IECs adapt not only their mitochondrial and glycolytic phenotype, but also their metabolic substrate use. To investigate whether IECs alter their metabolic substrate use, we can adapt the Seahorse XF metabolic analysis of IECs. This can be done by, for example, adding or removing specific metabolic substrates, like SCFAs, long chain fatty acids or amino acids, or by blocking metabolic pathways to assess their contribution to cellular ATP production. Furthermore, the *in vivo* nutrient supply towards and away from the intestine can be analysed by sampling the afferent and efferent blood vessels and performing metabolomics. In this way, we can discern which nutrients are consumed or produced by the intestine during the nutritional interventions. Understanding what metabolic substrates are used by IECs during specific (nutrition) interventions, and how this affects intestinal metabolism, is helpful to design interventions that support IEC metabolism.

## Why understanding IEC metabolism is essential: a glance to current societal challenges

The results of this thesis show that IEC metabolism is responsive to nutritional interventions, but a better understanding of how this correlates with intestinal physiology, and how IEC metabolism is impacted by other relevant nutritional intervention is still needed. I would like to emphasise this need by highlighting some findings from our study and placing them into the perspective of current societal challenges.

First, our findings highlight that enteral nutrition is essential to maintain IEC metabolic function. We need further research to establish the link between IEC metabolism and intestinal function, to better support IEC function during times of limited or absent luminal nutrient supply. The importance of enteral nutrition to maintain proper intestinal barrier function and immune regulation is already recognised in human healthcare [112], where parental nutrition is only applied if tube feeding is not feasible. On a global scale, a significant challenge for the coming decennia will be to provide adequate nutrition and clean drinking water for the growing world population [113]. Understanding how to support the gastrointestinal tract, the ‘gateway to the body’, to maintaining a solid barrier, could reduce disease susceptibility in periods of nutrient and immunological stress caused by malnutrition or contaminated drinking water. Some suggestions for

how to support IEC metabolism will be given below.

Second, we found that diets high in poorly digestible protein and fermentable fibre altered IEC metabolic function. These results are relevant for human nutrition in light of the growing interest in switching to plant-based diets that have poorer protein digestibility and more fibres [32]. Although we do not yet understand the impact of the observed changes, it is clear that diets can impact IEC metabolism and possibly gut function. In addition, these results are relevant for pig nutrition. To reduce the competition of human edible food and animal feed sources for arable land, pigs will be fed increasingly with by-products or alternative feed sources that do not compete as much with human nutrition, such as grasslands or algae [114, 115]. As these sources are often high in fibre content, the question raised with regards to the results presented in this thesis is whether, and to what extent feeding such by-products will negatively impact animal health, both by decreasing protein digestibility and increasing fibre level.

For the described societal challenges, there is a need for more in-depth analysis of how they impact IEC metabolism and gut health in the long term. As IEC metabolism appears to be a critical determinant of IEC function, it would be useful to further understand the constraints and critical limits of IEC metabolic adaptations to tailor interventions and try to prevent the onset or worsening of disease. The tools presented in this thesis could be helpful to further elucidate the role of IEC metabolism in intestinal function, and to find ways to boost IEC metabolic function and gut health.

There are several attractive candidates to boost IEC metabolic function in the intestine. Specific co-factors that are essential for regulating and maintaining mitochondrial respiration, such as $NAD^+$, could be provided through the diet to support mitochondrial function [116]. Supplementation with a precursor of $NAD^+$, nicotinic acid, was found to increase stem cell proliferation [117], which could be beneficial to promote intestinal maturation in the post-weaning period or after times of intestinal injuries caused by infections. In addition, dietary supplementation of nutrients that are known to be important nutrient sources of IECs, such as glutamine for small intestinal IECs or targeted delivery of butyrate to colon IECs, could support IEC metabolic function, even if other substrates are limited [44, 118]. Finally, because the intestinal developmental stage seems an important factor in determining whether the gut can cope with challenges or not, increasing intestinal maturation could be a good strategy to improve intestinal resilience in infants and piglets. Polyamines were found to increase intestinal maturation, thereby improving intestinal resilience, especially around weaning and in pre-term infants with underdeveloped intestines [119-121].

## Conclusions

The tools provided in this thesis could aid in improving our understanding of IEC metabolism in health and disease. The isolation of primary pig IECs proved a valuable method to assess the impacts of dietary and fasting interventions. In addition, the set of mitochondrial genes that was decreased in both suckling and feed habituated piglets could assist in developing gene markers for assessing mitochondrial function in future experiments. Furthermore, the two nutritional interventions applied in this thesis, which focused on fermentation metabolites and fasting, clearly impacted IEC metabolic function. By altering IEC metabolic function, these interventions could impact the health of an individual, but the exact mechanisms, and implications for intestinal physiology need to be further investigated. Ultimately, understanding the role of IEC metabolism in intestinal physiology, and how it responds to the challenges of the present and future, can drive discoveries to beneficially manipulate IEC metabolism, to finally support the overall health of humans and animals.

## References


1. Grala, W., et al., *Flow of endogenous and exogenous nitrogen in different segments of the small intestine in pigs fed diets with soyabean concentrate, soyabean meal or rapeseed cake.* Journal of Animal and Feed Sciences, 1998. **7**(1): p. 1-20.
2. van der Wielen, N., P.J. Moughan, and M. Mensink, *Amino Acid Absorption in the Large Intestine of Humans and Porcine Models.* The Journal of Nutrition, 2017. **147**(8): p. 1493-1498.
3. Drochner, W., *Digestion of carbohydrates in the pig.* Archiv für Tierernaehrung, 1993. **43**(2): p. 95-116.
4. Reynolds, A., et al., *Carbohydrate quality and human health: a series of systematic reviews and meta-analyses.* The Lancet, 2019. **393**(10170): p. 434-445.
5. Gill, S.K., et al., *Dietary fibre in gastrointestinal health and disease.* Nature Reviews Gastroenterology & Hepatology, 2021. **18**(2): p. 101-116.
6. Segain, J.P., et al., *Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease.* Gut, 2000. **47**(3): p. 397.
7. Silva, J.P.B., et al., *Protective Mechanisms of Butyrate on Inflammatory Bowel Disease.* Current Pharmaceutical Design, 2018. **24**(35): p. 4154-4166.
8. Aune, D., et al., *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* The British Medical Journal, 2011. **343**: p. d6617.
9. Canfora, E.E., et al., *Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial.* Scientific Reports, 2017. **7**(1): p. 2360.
10. De Vadder, F., et al., *Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits.* Cell, 2014. **156**(1): p. 84-96.
11. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.* Gut, 1980. **21**(9): p. 793-798.
12. Tan, H.T., et al., *Quantitative and Temporal Proteome Analysis of Butyrate-treated Colorectal Cancer Cells.* Molecular & Cellular Proteomics, 2008. **7**(6): p. 1174-1185.
13. Ghorbaniaghdam, A., O. Henry, and M. Jolicoeur, *A kinetic-metabolic model based on cell energetic state: study of CHO cell behavior under Na-butyrate stimulation.* Bioprocess and Biosystems Engineering, 2013. **36**(4): p. 469-487.
14. Matthews, G.M., G.S. Howarth, and R.N. Butler, *Short-chain fatty acids induce apoptosis in colon cancer cells associated with changes to intracellular redox state and glucose metabolism.* Chemotherapy, 2012. **58**(2): p. 102-109.
15. Tabuchi, Y., et al., *Identification of genes responsive to sodium butyrate in colonic epithelial cells.* Biochemical and Biophysical Research Communications, 2002. **293**(4): p. 1287-1294.
16. Heiden, M.G.V., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation.* Science, 2009. **324**(5930): p. 1029-1033.
17. Warburg, O., *On the Origin of Cancer Cells.* Science, 1956. **123**(3191): p. 309.
18. Donohoe, Dallas R., et al., *The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation.* Molecular Cell, 2012. **48**(4): p. 612-626.
19. Li, Q., et al., *Butyrate Suppresses the Proliferation of Colorectal Cancer Cells via Targeting Pyruvate Kinase M2 and Metabolic Reprogramming.* Molecular & Cellular Proteomics 2018. **17**(8): p. 1531-1545.
20. Pieper, R., et al., *Fermentable Fiber Ameliorates Fermentable Protein-Induced Changes in Microbial Ecology, but Not the Mucosal Response, in the Colon of Piglets.* The Journal of Nutrition, 2012. **142**(4): p. 661-667.
21. Wellock, I.J., et al., *The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs.* Animal Science, 2006. **82**(3): p. 327-335.

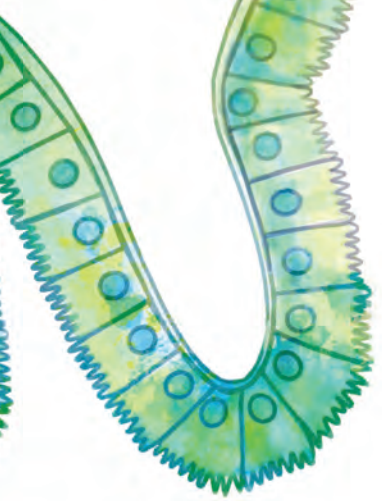
22. Gilbert, M.S., et al., *Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2018. **315**(2): p. G159-g170.
23. Pieper, R., et al., *Health relevance of intestinal protein fermentation in young pigs.* Animal Health Research Reviews, 2016. **17**(2): p. 137-147.
24. Bekebrede, A., et al., *The Molecular and Physiological Effects of Protein-Derived Polyamines in the Intestine.* Nutrients, 2020. **12**(1): p. 197.
25. Pegg, A.E., *Toxicity of Polyamines and Their Metabolic Products.* Chemical Research in Toxicology, 2013. **26**(12): p. 1782-1800.
26. Stevanato, R., et al., *Preliminary kinetic characterization of a copper amine oxidase from rat liver mitochondria matrix.* Amino Acids, 2011. **40**(2): p. 713-720.
27. van Ommen, B., et al., *Challenging homeostasis to define biomarkers for nutrition related health.* Mol Nutr Food Res, 2009. **53**(7): p. 795-804.
28. Jeaurond, E.A., et al., *Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs.* Canadian Journal of Animal Science, 2008. **88**(2): p. 271-281.
29. Pieper, R., et al., *Interaction between dietary protein content and the source of carbohydrates along the gastrointestinal tract of weaned piglets.* Archives of Animal Nutrition, 2014. **68**(4): p. 263-280.
30. Taciak, M., et al., *Interactive effects of protein and carbohydrates on production of microbial metabolites in the large intestine of growing pigs.* Archives of Animal Nutrition, 2017. **71**(3): p. 192-209.
31. Zijlstra, R.T. and E. Beltranena, *Chapter 11: Co-products in swine nutrition and feed formulation*, in *Poultry and pig nutrition: Challenges of the 21st century*, L. Babinszky, M.W.A. Verstegen, and W.H. Hendriks, Editors. 2019, Wageningen Academic Publishers: Wageningen, The Netherlands. p. 245-262.
32. Agnoli, C., et al., *Position paper on vegetarian diets from the working group of the Italian Society of Human Nutrition.* Nutrition, Metabolism and Cardiovascular Diseases, 2017. **27**(12): p. 1037-1052.
33. Kim, J.C., et al., *Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract.* British Journal of Nutrition, 2008. **99**(6): p. 1217-25.
34. Le Dividich, J. and B. Sève, *Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet.* Domestic Animal Endocrinology, 2000. **19**(2): p. 63-74.
35. Campbell, J.M., J.D. Crenshaw, and J. Polo, *The biological stress of early weaned piglets.* Journal of Animal Science and Biotechnology, 2013. **4**(1): p. 19-19.
36. Madec, F., et al., *Measurement of digestive disorders in the piglet at weaning and related risk factors.* Preventive Veterinary Medicine, 1998. **35**(1): p. 53-72.
37. Pelletier, D.L., et al., *The effects of malnutrition on child mortality in developing countries.* Bulletin of the World Health Organization, 1995. **73**(4): p. 443-448.
38. World Health Organization *Diarrhoeal disease.* 2017; Available from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease.
39. Van Der Schoor, S.R.D., et al., *The pattern of intestinal substrate oxidation is altered by protein restriction in pigs.* Gastroenterology, 2001. **121**(5): p. 1167-1175.
40. Windmueller, H.G. and A.E. Spaeth, *Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate.* Journal of Biological Chemistry, 1980. **255**(1): p. 107-112.
41. Mitchell, S.E., et al., *The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse.* Oncotarget, 2015. **6**(18): p. 15902-30.
42. Stoll, B., et al., *Substrate oxidation by the portal drained viscera of fed piglets.* American Journal of Physiology, 1999. **277**(1 Pt 1): p. E168-75.
43. Roediger, W.E., *Utilization of nutrients by isolated epithelial cells of the rat colon.* Gastroenterology, 1982. **83**(2): p. 424-429.

44. Donohoe, D.R., et al., *The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon.* Cell Metabolism, 2011. **13**(5): p. 517-526.
45. Vancamelbeke, M., et al., *Genetic and Transcriptomic Bases of Intestinal Epithelial Barrier Dysfunction in Inflammatory Bowel Disease.* Inflammatory bowel diseases, 2017. **23**(10): p. 1718-1729.
46. Boudry, G.I., et al., *Weaning Induces Both Transient and Long-Lasting Modifications of Absorptive, Secretory, and Barrier Properties of Piglet Intestine.* The Journal of Nutrition, 2004. **134**(9): p. 2256-2262.
47. Cao, S.T., et al., *Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets.* Journal of Animal Science, 2018. **96**(3): p. 1073-1083.
48. Moeser, A.J., C.S. Pohl, and M. Rajput, *Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs.* Animal Nutrition, 2017. **3**(4): p. 313-321.
49. Meng, Q., et al., *Weaning Alters Intestinal Gene Expression Involved in Nutrient Metabolism by Shaping Gut Microbiota in Pigs.* Frontiers in Microbiology, 2020. **11**: p. 694-694.
50. Pluske, J.R., D.J. Hampson, and I.H. Williams, *Factors influencing the structure and function of the small intestine in the weaned pig: a review.* Livestock Production Science, 1997. **51**(1): p. 215-236.
51. Novais, A.K., et al., *Weaning differentially affects mitochondrial function, oxidative stress, inflammation and apoptosis in normal and low birth weight piglets.* PLOS ONE, 2021. **16**(2): p. e0247188.
52. Le Bon, M., et al., *Gut transcriptome reveals differential gene expression and enriched pathways linked to immune activation in response to weaning in pigs.* BioRxiv, 2021: p. 2021.11.30.470420.
53. Schmeisser, H., et al., *Type I interferons induce autophagy in certain human cancer cell lines.* Autophagy, 2013. **9**(5): p. 683-696.
54. Powell, J.D., et al., *Regulation of immune responses by mTOR.* Annual Review of Immunology, 2012. **30**: p. 39-68.
55. Green, C.L., D.W. Lamming, and L. Fontana, *Molecular mechanisms of dietary restriction promoting health and longevity.* Nature Reviews Molecular Cell Biology, 2022. **23**(1): p. 56-73.
56. Colman, R.J., et al., *Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys.* Science, 2009. **325**(5937): p. 201-204.
57. Pak, H.H., et al., *Fasting drives the metabolic, molecular and geroprotective effects of a calorie-restricted diet in mice.* Nature Metabolism, 2021. **3**(10): p. 1327-1341.
58. Picard, M. and D.M. Turnbull, *Linking the metabolic state and mitochondrial DNA in chronic disease, health, and aging.* Diabetes, 2013. **62**(3): p. 672-8.
59. Weir, H.J., et al., *Dietary Restriction and AMPK Increase Lifespan via Mitochondrial Network and Peroxisome Remodeling.* Cell Metabolism, 2017. **26**(6): p. 884-896. e5.
60. Xiong, X., et al., *Activation of Drp1 promotes fatty acids-induced metabolic reprograming to potentiate Wnt signaling in colon cancer.* Cell Death & Differentiation, 2022.
61. Book, S.A. and L.K. Bustad, *The fetal and neonatal pig in biomedical research.* Journal of Animal Science, 1974. **38**(5): p. 997-1002.
62. Alpers, D.H., *Enteral feeding and gut atrophy.* Current Opinion in Clinical Nutrition and Metabolic Care, 2002. **5**(6): p. 679-683.
63. Kristan, D.M., *Calorie restriction and susceptibility to intact pathogens.* Age (Dordrecht, Netherlands), 2008. **30**(2-3): p. 147-156.
64. Zeng, Y., et al., *Peripheral Blood Mononuclear Cell Metabolism Acutely Adapted to Postprandial Transition and Mainly Reflected Metabolic Adipose Tissue Adaptations to a High-Fat Diet in Minipigs.* Nutrients, 2018. **10**(11): p. 1816.

65. Choudhary, C., et al., *The growing landscape of lysine acetylation links metabolism and cell signalling.* Nature Reviews Molecular Cell Biology, 2014. **15**(8): p. 536-550.
66. Zhao, S., et al., *Regulation of Cellular Metabolism by Protein Lysine Acetylation.* Science, 2010. **327**(5968): p. 1000-1004.
67. Reid, M.A., Z. Dai, and J.W. Locasale, *The impact of cellular metabolism on chromatin dynamics and epigenetics.* Nature Cell Biology, 2017. **19**(11): p. 1298-1306.
68. Lukovac, S., et al., *Differential modulation by Akkermansia muciniphila and Faecalibacterium prausnitzii of host peripheral lipid metabolism and histone acetylation in mouse gut organoids.* mBio, 2014. **5**(4): p. e01438-14.
69. Kebede, A.F., et al., *Histone propionylation is a mark of active chromatin.* Nature Structural & Molecular Biology, 2017. **24**(12): p. 1048–1056.
70. Terranova, C.J., et al., *Reprogramming of H3K9bhb at regulatory elements is a key feature of fasting in the small intestine.* Cell Reports, 2021. **37**(8): p. 110044.
71. Mihaylova, M.M. and R.J. Shaw, *Metabolic reprogramming by class I and II histone deacetylases.* Trends in Endocrinology & Metabolism, 2013. **24**(1): p. 48-57.
72. Houtkooper, R.H., E. Pirinen, and J. Auwerx, *Sirtuins as regulators of metabolism and healthspan.* Nature reviews Molecular Cell Biology, 2012. **13**(4): p. 225-238.
73. Mihaylova, Maria M., et al., *Class IIa Histone Deacetylases Are Hormone-Activated Regulators of FOXO and Mammalian Glucose Homeostasis.* Cell, 2011. **145**(4): p. 607-621.
74. Pougovkina, O., et al., *Mitochondrial protein acetylation is driven by acetyl-CoA from fatty acid oxidation.* Human Molecular Genetics, 2014. **23**(13): p. 3513-3522.
75. Hirschey, M.D., et al., *SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation.* Nature, 2010. **464**(7285): p. 121-125.
76. Westermann, B., *Bioenergetic role of mitochondrial fusion and fission.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2012. **1817**(10): p. 1833-1838.
77. Gomes, L.C., G.D. Benedetto, and L. Scorrano, *During autophagy mitochondria elongate, are spared from degradation and sustain cell viability.* Nature Cell Biology, 2011. **13**(5): p. 589-598.
78. Rambold , A.S., et al., *Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation.* Proceedings of the National Academy of Sciences, 2011. **108**(25): p. 10190-10195.
79. Giacomello, M., et al., *The cell biology of mitochondrial membrane dynamics.* Nature Reviews Molecular Cell Biology, 2020. **21**(4): p. 204-224.
80. Tailor, D., et al., *Sodium butyrate induces DRP1-mediated mitochondrial fusion and apoptosis in human colorectal cancer cells.* Mitochondrion, 2014. **16**: p. 55-64.
81. Hu, S., et al., *Acetate and Butyrate Improve Beta-cell Metabolism and Mitochondrial Respiration under Oxidative Stress.* International Journal of Molecular Sciences, 2020. **21**(4): p. 1542.
82. Crakes, K.R., et al., *PPAR-alpha-targeted mitochondrial bioenergetics mediate repair of intestinal barriers at the host-microbe intersection during SIV infection.* Proceedings of the National Academy of Sciences of the United States of America, 2019. **116**(49): p. 24819-24829.
83. JanssenDuijghuijsen, L.M., et al., *Mitochondrial ATP Depletion Disrupts Caco-2 Monolayer Integrity and Internalizes Claudin 7.* Frontiers in Physiology, 2017. **8**: p. 794.
84. Heijmans, B.T., et al., *Persistent epigenetic differences associated with prenatal exposure to famine in humans.* Proceedings of the National Academy of Sciences, 2008. **105**(44): p. 17046-17049.
85. Wolff, G.L., et al., *Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice.* The FASEB Journal, 1998. **12**(11): p. 949-957.
86. Sandoval, C., et al., *Maternal Nutrient Restriction and Skeletal Muscle Development: Consequences for Postnatal Health*, in *Amino Acids in Nutrition and Health: Amino acids in systems function and health*, G. Wu, Editor. 2020, Springer International Publishing: Cham. p. 153-165.

87. Park, J.H., et al., *Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1.* The Journal of Clinical Investigation, 2008. **118**(6): p. 2316-2324.
88. van der Beek, E.M. and A. Oosting, *Nutritional programming in early life: the role of dietary lipid quality for future health.* Oilseeds and Fats, Crops and Lipids, 2020. **27**(5): p. ocl190062.
89. del Carmen Miñana-Solis, M. and C. Escobar, *Post-weaning protein malnutrition in the rat produces short and long term metabolic impairment, in contrast to earlier and later periods.* International Journal of Biological Sciences, 2008. **4**(6): p. 422–432.
90. Carolan-Olah, M., M. Duarte-Gardea, and J. Lechuga, *A critical review: early life nutrition and prenatal programming for adult disease.* Journal of Clinical Nursing, 2015. **24**(23-24): p. 3716-3729.
91. Oosting, A., et al., *N-3 Long-Chain Polyunsaturated Fatty Acids Prevent Excessive Fat Deposition in Adulthood in a Mouse Model of Postnatal Nutritional Programming.* Pediatric Research, 2010. **68**(6): p. 494-499.
92. Bouwman, L.M.S., et al., *Replacing Part of Glucose with Galactose in the Post-weaning Diet Protects Female But Not Male Mice from High-Fat Diet-Induced Adiposity in Later Life.* The Journal of Nutrition, 2019. **149**(7): p. 1140-1148.
93. Ozkul, C., et al., *A single early-in-life antibiotic course increases susceptibility to DSS-induced colitis.* Genome Medicine, 2020. **12**(1): p. 65.
94. Mu, C. and W. Zhu, *Antibiotic effects on gut microbiota, metabolism, and beyond.* Applied Microbiology and Biotechnology, 2019. **103**(23): p. 9277-9285.
95. Stojanovi, O., et al., *Dietary excess regulates absorption and surface of gut epithelium through intestinal PPAR-alpha.* Nature Communications, 2021. **12**(1): p. 7031.
96. Ludikhuize, M.C., et al., *Mitochondria Define Intestinal Stem Cell Differentiation Downstream of a FOXO/Notch Axis.* Cell Metabolism, 2020. **32**(5): p. 889-900.e7.
97. Rodriguez-Colman, M.J., et al., *Interplay between metabolic identities in the intestinal crypt supports stem cell function.* Nature, 2017. **543**(7645): p. 424-427.
98. Deng, H., et al., *Mitochondrial dynamics regulates Drosophila intestinal stem cell differentiation.* Cell Death Discovery, 2018. **4**(1): p. 81.
99. Yilmaz, Ö.H., et al., *mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake.* Nature, 2012. **486**(7404): p. 490-495.
100. Lindeboom, R.G., et al., *Integrative multi-omics analysis of intestinal organoid differentiation.* Molecular Systems Biology, 2018. **14**(6): p. e8227.
101. Clevers, H., *Modeling Development and Disease with Organoids.* Cell, 2016. **165**(7): p. 1586-1597.
102. Bein, A., et al., *Microfluidic Organ-on-a-Chip Models of Human Intestine.* Cellular and Molecular Gastroenterology and Hepatology, 2018. **5**(4): p. 659-668.
103. Das, A.T., L. Tenenbaum, and B. Berkhout, *Tet-On Systems For Doxycycline-inducible Gene Expression.* Current Gene Therapy, 2016. **16**(3): p. 156-167.
104. Wilson, T.H. and G. Wiseman, *The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface.* The Journal of Physiology, 1954. **123**(1): p. 116-125.
105. Alam, M.A., F.I. Al-Jenoobi, and A.M. Al-mohizea, *Everted gut sac model as a tool in pharmaceutical research: limitations and applications.* Journal of Pharmacy and Pharmacology, 2012. **64**(3): p. 326-336.
106. Moyano-Porcile, V., et al., *Short-term effects of Poly(I:C) on gut permeability.* Pharmacological Research, 2015. **101**: p. 130-136.
107. Noorman, L., et al., *P112. Influence of removing serosal and outer muscle layers on estimates of intestinal permeability in everted sac segments.* Animal - science proceedings, 2022. **13**(2): p. 191.
108. Thomson, A., et al., *The Ussing chamber system for measuring intestinal permeability in health and disease.* BMC Gastroenterology, 2019. **19**(1): p. 98.
109. van Wijck, K., et al., *Novel analytical approach to a multi-sugar whole gut permeability assay.* Journal of Chromatography B: Analytical Technologies in the Bio-

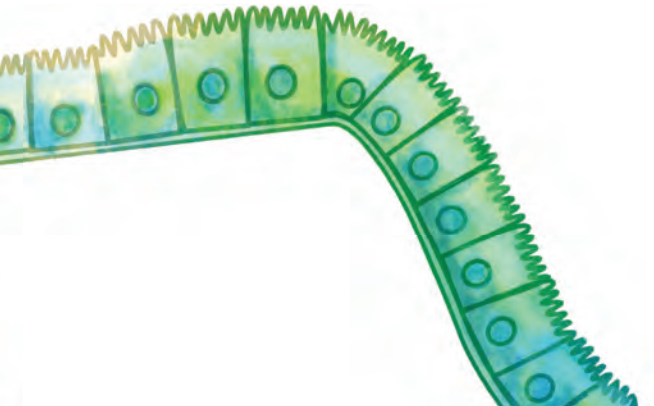
medical and Life Sciences, 2011. **879**(26): p. 2794-2801.
110. van Wijck, K., et al., *Novel multi-sugar assay for site-specific gastrointestinal permeability analysis: A randomized controlled crossover trial.* Clinical Nutrition, 2013. **32**(2): p. 245-251.
111. Jang, C., et al., *Metabolite Exchange between Mammalian Organs Quantified in Pigs.* Cell Metabolism, 2019. **30**(3): p. 594-606.e3.
112. Schörghuber, M. and S. Fruhwald, *Effects of enteral nutrition on gastrointestinal function in patients who are critically ill.* The Lancet Gastroenterology & Hepatology, 2018. **3**(4): p. 281-287.
113. FAO, I., UNICEF, WFP and WHO, *The State of Food Security and Nutrition in the World 2021: Transforming food systems for food security, improved nutrition and affordable healthy diets for all*. 2021, Food & Agriculture Organisation of the United Nations: Rome.
114. Schader, C., et al., *Impacts of feeding less food-competing feedstuffs to livestock on global food system sustainability.* Journal of The Royal Society Interface, 2015. **12**(113): p. 20150891.
115. Parisi, G., et al., *Protein hunger of the feed sector: the alternatives offered by the plant world.* Italian Journal of Animal Science, 2020. **19**(1): p. 1204-1225.
116. Cantó, C., et al., *The NAD+ Precursor Nicotinamide Riboside Enhances Oxidative Metabolism and Protects against High-Fat Diet-Induced Obesity.* Cell Metabolism, 2012. **15**(6): p. 838-847.
117. Kaiko, G.E., et al., *The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites.* Cell, 2016. **165**(7): p. 1708-1720.
118. Domeneghini, C., et al., *Gut-trophic feed additives and their effects upon the gut structure and intestinal metabolism. State of the art in the pig, and perspectives towards humans.* Histology and Histopathology, 2006. **21**(3): p. 273-283.
119. Plaza-Zamora, J., et al., *Polyamines in human breast milk for preterm and term infants.* British Journal of Nutrition, 2013. **110**(3): p. 524-528.
120. Sabater-Molina, M., et al., *Effects of dietary polyamines at physiologic doses in early-weaned piglets.* Nutrition, 2009. **25**(9): p. 940-946.
121. van Wettere, W.H., et al., *Effect of oral polyamine supplementation pre-weaning on piglet growth and intestinal characteristics.* Animal, 2016. **10**(10): p. 1655-1659.



# Appendices

Summary
Samenvatting

Acknowledgements
Curriculum Vitea
List of Publications
Education and Training Activities



## Summary

The intestinal tract is a complex organ that is vital for maintaining organismal health. It transports provides essential nutrients and minerals that are needed to sustain all bodily functions, while keeping out unwanted environmental agents and compounds. An optimally functioning intestine is thus indispensable, and suboptimal intestinal function can result in gastrointestinal diseases. Gastrointestinal diseases are prevalent, especially at a young age. Childhood diarrhea accounts for nearly 1.7 billion cases every year, leading to over half a million deaths in children under 5 years of age, primarily in low income countries. Likewise, also young animals like piglets frequently suffer from diarrhea, which globally accounts for large losses of lives and industrial profits. Pigs are thus an important target species in their own right. Moreover, they are a good model for humans, and pigs were therefore chosen as the main focus of the research presented in this thesis.

To perform all its tasks, the intestinal lining is made up of a layer of tightly connected, terminally differentiated cells called intestinal epithelial cells (IECs). The IECs, like all living cells, need to produce sufficient energy to support their cellular functions. Cellular energy metabolism is a highly dynamic process, and it can adapt and change in response to intrinsic and extrinsic stimuli. Currently, it is poorly understood how the metabolism of IECs responds when faced with altered nutritional environments. The **overall aim** of my thesis was therefore to increase our knowledge of how IEC metabolism adapts to changes in nutrient environment under various conditions. In my research, we have applied two nutritional interventions, constituting challenges to the intestine, that are relevant for both human and animal nutritional: diet and fasting.

My **first aim** was to study how IEC metabolic function is influenced by diet. Carbohydrates and proteins that are not digested in the small intestine end up in the colon, where they are fermented by the microbes present there, giving rise to a plethora of fermentation metabolites. These fermentation metabolites interact with the hosts' epithelium, and can thereby potentially impact IEC function. I investigated polyamines because they are a major class of protein-fermentation derived metabolites. and butyrate, because it is a major fibre-fermentation derived metabolite. In **chapter 2**, I reviewed the current knowledge of the biochemical, cellular and physiological functions polyamines in the intestine. This revealed that polyamines play a crucial role in the intestine, and can influence metabolism of IECs. However, it remains unclear whether luminal polyamines affect IEC physiology *in vivo*, and this is a research gap to be investigated in future studies. In **chapter 3**, I investigated the effect of butyrate on the metabolism of cultured colonocytes. Butyrate is considered the primary energy source of colonocytes, and it has been widely studied for its beneficial health effects. Because mechanistic studies on the effects of butyrate are principally performed using cultured cells, which differ from

*in vivo* cells in crucial aspects such as nutrient availability and metabolic phenotype, I investigated how exposure to butyrate affected the metabolism of cultured colonocytes. I showed that butyrate itself was not oxidized in cultured cells, but that it instead increased the oxidation of pyruvate and increased lipid accumulation, presumably by being directly incorporated into lipid droplets. These results highlight that the nutritional environment and metabolic phenotype of cells are important considerations when interpreting metabolic responses to fermentation metabolites.

In addition to assessing the effects of fermentation metabolites *in vitro* and using literature research, I assessed how the functional metabolism of IECs is impacted by relevant nutritional interventions *in vivo*. Therefore, I first optimised a method to isolate viable primary pig IECs and measure their metabolic function in the Seahorse XF analyzer in **chapter 4**. In **chapter 5**, I used this method to investigate the *in vivo* effects of fermentable fibres and poorly digestible protein on colonocyte metabolic function in pigs. I showed that the mitochondrial capacity of isolated colonocytes was only reduced by high fermentable fibre levels in an easily digestible whey-protein diet, while the glycolytic function of colonocytes was reduced by high fermentable fibre, irrespective of dietary protein source. Due to the complexity of the diets, it was difficult to link effects to specific colonic fermentation end products. However, this study did demonstrate that dietary interventions, and subsequent alterations in luminal fermentation metabolites, affect the metabolic function of IECs.

The **second aim** of my thesis was to establish the functional metabolic and molecular consequences of fasting on IECs. During periods of fasting, luminal nutrient supply is completely abolished for a certain amount of time, leading to altered supply of metabolic substrates to IECs for cellular ATP production. In **chapter 6**, I studied the functional metabolic and molecular responses of IECs of feed habituated piglets to fasting. I found that 48hs of fasting simultaneously decreased functional mitochondrial and glycolytic metabolism in jejunal IECs of six-week-old feed habituated piglets. This also validated the transcriptome results, where mitochondrial pathways were among the top regulated Reactome pathways upon fasting. Further analysis of the gene expression revealed that decreased metabolic function coincided with decreased cell proliferation and barrier function. In **chapter 7**, I investigated the differences in the response to fasting of jejunal IECs of four-week-old suckling and six-week-old solid feed habituated piglets. I found that gene regulation upon fasting was strongest in suckling piglets. Furthermore, while some molecular pathways were similarly regulated in suckling and feed habituated piglets, indicative of a robust response to the fasting intervention, other key processes were regulated in the opposite direction upon 48h fasting. Together, these findings underscore the importance of luminal nutrient supply to maintain IEC metabolic function, and reveal molecular pathways that may underlie

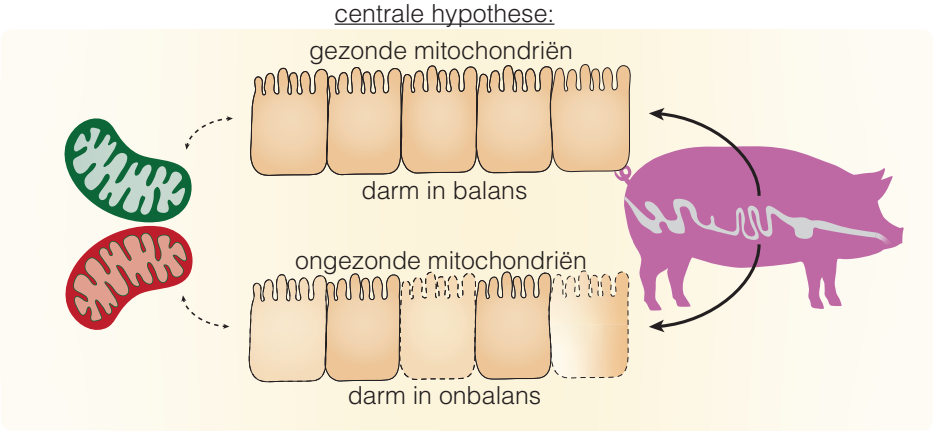
increased disease susceptibility during times of nutrient stress.

In **chapter 8**, the final chapter of my thesis, I discussed my main findings, future perspectives and recommendations for future research. There is a need to better understand how metabolic function of IECs contributes to intestinal functions intestinal physiology, and the tools provided in this thesis could aid in facilitating this research. Ultimately, understanding the role of IEC metabolism in intestinal physiology, and how it responds to the challenges of the present and future, can drive discoveries to manipulate IEC metabolism in a beneficial manner, to improve overall health of humans and animals.

## Samenvatting

Een fundamenteel criterium voor alle levende organismen en de cellen waaruit die zijn opgemaakt, is dat er voldoende energie beschikbaar moet zijn om de vitale processen in stand te houden. Dit geldt ook voor de darm, waar een dunne laag cellen voor de taak staat om het binnenkomende voedsel te verteren en de vrijgekomen nutriënten en mineralen op te nemen. Tegelijk moeten ongewenste organismen en stoffen die via de mond zijn binnengekomen, buiten worden gehouden. Deze taken vergen veel energie, die in onze cellen voornamelijk wordt geproduceerd in mitochondriën. Mitochondriën worden om die reden ook wel de energiefabriekjes van de cel genoemd. Uit deze informatie volgt direct de centrale hypothese van mijn proefschrift: goed functionerende mitochondriën zijn nodig om voldoende energie te produceren om alle cellulaire processen te laten werken. Daarmee dragen zij bij aan het in stand houden van een goede balans in de darm. Als mitochondriën om wat voor reden dan ook, niet goed functioneren, zorgt dit voor een cellulaire energiecrisis, waardoor essentiële processen niet in stand gehouden kunnen worden. Dat leidt uiteindelijk tot een onbalans in de darm (zie ook Fig. S1).

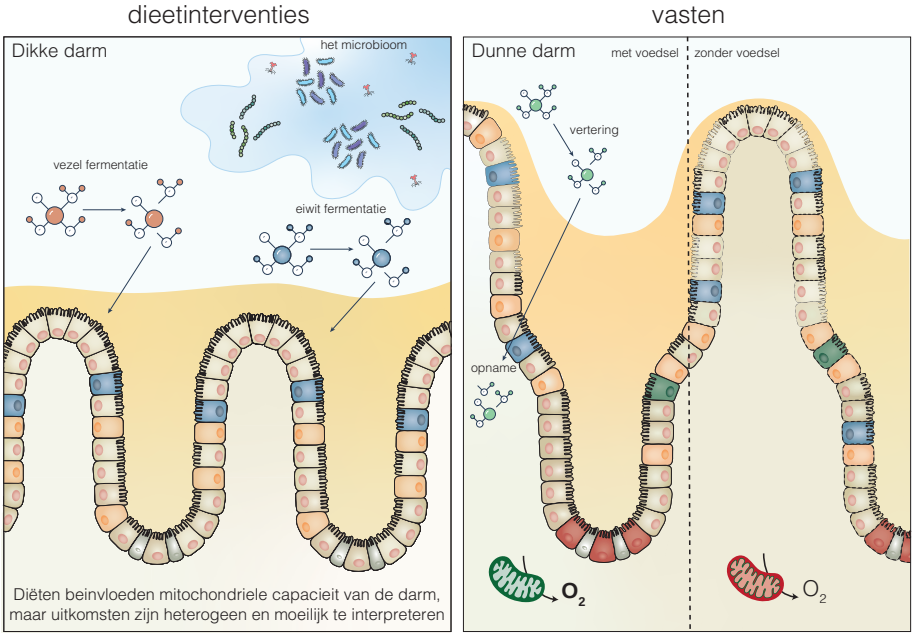
Om deze hypothese te testen hebben we onderzoek gedaan in varkens. In de eerste plaats omdat varkens een belangrijke doelpopulatie zijn wat betreft darmonderzoek. Vooral jonge biggen leiden namelijk veelvuldig aan darmziektes zoals diarree, wat resulteert in biggensterfte en grote economische schade. In de tweede plaats zijn varkens ook een model bij uitstek voor het menselijke maag-darmkanaal. Deze twee dingen samen maakte varkens ideaal voor dit onderzoek.




Figuur S1 **Centrale hypothese.** Om de darm in balans te houden, zijn goed functionerende mitochondriën nodig om voldoende energie te produceren waarmee alle cellulaire processen in stand worden gehouden. Als mitochondriën niet goed functioneren leidt dit tot een cellulaire energiecrisis, waardoor cellulaire processen niet in stand kunnen worden gehouden, wat uiteindelijk leidt tot onbalans in de darm.

Om te onderzoeken hoe de mitochondriële functie bijdraagt aan het optimaal functioneren van de darm, hebben we de darm uitgedaagd door blootstelling aan twee nutritionele interventies (zie Fig. S2). De eerste interventie had als doel om te onderzoeken hoe de mitochondriële functie van darmcellen wordt beïnvloed door het dieet. Eiwitten en vezels die niet zijn verteerd in de dunne darm, komen in de dikke darm terecht, waar ze worden afgebroken door de vele microben die hier leven. Tijdens de microbiële afbraak ontstaan er producten die in contact komen met de darmcellen, en daar een directe invloed op kunnen hebben. Eindproducten van vezelfermentatie worden over het algemeen gezien als positief voor de darmgezondheid, terwijl eiwitfermentatie-eindproducten juist negatief te boek staan. In onze studie gaven we varkens een dieet hoog aan vezel, hoog aan onverteerbaar eiwit of een combinatie. Hoewel we duidelijk zagen dat de diëten de mitochondriële capaciteit van darmcellen beïnvloedden, was het onduidelijk wat hieraan ten grondslag lag, en daardoor was het moeilijk om de resultaten te interpreteren. Wel zagen we dat de diëten de mitochondriële capaciteit van darmcellen beïnvloedden, en dat er een interactie was tussen vezel en eiwit. Onze tweede nutritionele interventie had als doel om te onderzoeken wat het effect is van vasten op de mitochondriële functie van darmcellen. Normaal gesproken halen darmcellen een belangrijk deel van hun voedingsstoffen direct uit het voedsel dat binnenkomt, wat wegvalt tijdens vasten. We vonden dat de mitochondriële functie van darmcellen sterk was verminderd na 48 uur vasten, wat we hebben bevestigd aan de hand van andere moleculaire analyses. Naast een vermindering van de mitochondriële functie, zagen we ook dat de celdeling, een belangrijk proces in de snel-delende darm, was afgenomen na vasten. Ook zagen we dat de expressie van genen die een rol spelen bij het in stand houden van de darmbarrière afgenomen was na vasten. Al deze resultaten wijzen erop dat vasten in jonge biggen kan bijdragen aan een onbalans in de darm, wat uiteindelijk kan leiden tot ziekte. Hoewel er zeker nog meer onderzoek nodig is, kunnen we uit mijn onderzoek concluderen dat de mitochondriële functie van darmcellen inderdaad wordt beïnvloed door nutritionele interventies, en het lijkt dat voldoende voeding inderdaad van belang is voor het in stand houden van darmgezondheid in jonge individuen. Met deze opgedane kennis hopen we bij te dragen aan ons begrip van darmgezondheid, en hoe we dit in de toekomst op een positieve manier kunnen beïnvloeden.

Nutritionele uitdagingen




Figuur S2 **Overzicht van de belangrijkste uitkomsten en interventies.** Twee nutritionele interventies zijn toegepast: dieetinterventies en vasten. Hoewel we zagen dat diëten de mitochondriële capaciteit van darmcellen beïnvloedde, waren de effecten heterogeen en daardoor moeilijk te interpreteren. We zagen dat vezel en eiwit elkaar beïnvloedden in de effecten op mitochondriële capaciteit van dikke darmcellen. Vasten zorgde voor een vermindering van mitochondriële functie van darmcellen.

## Acknowledgements

Finally, it is time for the best-read (but least cited) part of my thesis. Welcome to the acknowledgements! It took a lot of help, support, coffee-break puzzles, chocolate and the occasional beer to complete the work described in this booklet, and I would like to take the time to thank everyone for their input and support.

First and foremost, I would like to thank my supervising-team: **Jaap**, **Vincent** and **Walter**. Jaap, I greatly appreciate all your enthusiastic scientific input during our project-meetings. You always inspired me to research a little bit more. Your supervising strategy was always more focussed on training, and less on output, which allowed me to learn a lot as researcher and gave me at least the illusion of autonomy. Thank you for your continued support by giving me the opportunity to develop my career further at HAP. Vincent, I still remember the first time we met, where we quickly discovered our shared love for Seahorse Analysis. Thank you for always pushing for the best in me; your critical eye made every presentation, poster and manuscript so much better. I hope to one day reach your level of scientific independence and writing fluency. Walter, the piglet-work of my thesis would not have been possible without your input. My extremely limited knowledge on pigs has been greatly improved, but I still have much more to learn. Thank you for your interest in my thesis, and for always checking up on me and my work.

This project was supported by a TTW-DSM partnership program, which gave me the opportunity to interact with people from DSM and other universities and chair groups. **Pim** and **Arie**, you pushed for translatable research, which challenged me in new ways. **Anna-Maria**, you always gave interesting scientific input that sparked nice discussions. Thank you for the support! The yearly meeting with **DSM clients** was a nice experience that allowed me to visit new places and discover current issues in the field of animal nutrition. These meetings also challenged me to think about the relevance of my research for industry and the general population. A special thanks to **Jan**, **Koen** and **Bart** from Voergroep Zuid for helping me find a farmer willing to accommodate a young researcher on their farm for several weeks. Daarnaast wil ik ook graag de **familie Waijers** bedanken voor hun gastvrijheid en goede zorgen tijdens de dierproef. Het was een druk heen-en-weer van studenten en medewerkers, maar jullie hebben het zonder morren doorstaan. Het resultaat van al dat gedoe is er dan eindelijk! A big thanks also to the other PhDs and Postdocs within the project: **Bart**, **Joanna**, **Judith**, **Kim**, **Miranda**, **Myrthe**, **Rianne** and **Stefania**. **Lonneke**, you deserve an additional thank you here. It was great to perform our first animal experiment together, and to continue our collaboration with joint publication(s). I look forward to your defence in the future, and hope to continue our (working) relationship.

During my PhD, I was lucky to be embedded in chair group with so many nice colleagues that provided practical aid and mental support. The coffee breaks, and crossword puzzles were the highlight of many of my days! **Corine** and **Irene**, thanks for your support in all things practical. But also your shoulders to cry on, and your sweets to keep me going. **Annelies**, thank you for the laughter and the singing! I hope we will have many more borrels in the future. **Marcel**, your support during my last animal study was very much appreciated. You introduced me to my favourite Youtube cooking show, and I anticipate a good summer BBQ to test all the great recipe idea's we share. **Inge**, you are always a positive presence in and outside of the lab. I really admire your kindness and perseverance (you even started a PhD, wow!). **Melissa**, you are always will to lend a hand, with a smile on your face. Your patience is legendary! **Jur**, I anticipate a lot more singing and bike-ride adventures in the future! **Mel**, you add a bright and new perspective to HAP, thank you for the nice coffee-break chats! **Merel**, I'm sure you will contribute in a positive way to the HAP-vibes. To my new roommates: **Arie**, **Evert** and **Sander**, thanks for accepting me in the fold; I look forward to learning from your expertise. The plant is here to stay, I'm sorry. **Claudia**, your Italian passion shines through, adding laugher and interesting discussions to our chair group. I admire your helpful and supportive attitude. **Katja**, I learned a lot from your experience with teaching, writing and even grant-reviewing. Thank you for always providing us with the crossword puzzles, aka 'best relaxation agent'. **Silvie**, thank you for your critical input during meetings, your listening ear and support. **Sandra**, **Deli**, **Marlou**, **Werner** and **Zhuohui**, thanks for the input and attention in TuMoMeetings and coffee breaks.

A special paragraph is dedicated to my lovely PhD colleagues: **Alexia**, **Andrea**, **Bart**, **Chris**, **Cresci-Anne**, **Evelien**, **Eveline**, **Ferran**, **Jelle**, **Jeske**, **Jingmeng**, **Jingyi**, **Joëlle**, **José**, **Liangyu**, **Lianne**, **Maria**, **Marianne**, **Melissa**, **Miranda**, **Natasja**, **Peixin**, **Qi**, **Sarmad**, **Taolin**, **Wei Wei**, **Wenbiao**, **Xi** and the honorary PhD students **Gerwin** and **Gül**. Thanks for all the lovely time we send together. We've done it all: eating, drinking, amusement, but also support and a listening ear in when times were particularly stressful. A special 'good luck and I-wish-you-all-the-best' to all the participants of the post-HAP baby boom (you know who you are). Team Mito, I have reserved a special spot for you all on this page and in my heart. **Bart**, thank you for the fun times and training in the lab. Every time I may take a short-cut in the lab, I hear your voice telling me to take the extra step and save myself time in the future. Great advice! I hope we can work together sometime again in the future, and otherwise at least continue having drinks together. **Taolin**, you taught me so many things in the lab, but we also had a lot of fun outside of the lab. I miss your lively discussions, but luckily, we can still talk on the phone. **Joëlle**, your support was indispensable for finishing this PhD. Thank you for the many, many walks, calls, sparring sessions and dinners. I look forward to being an honorary aunt to your little one! All three of you allowed me the honour of being your

paranymph; I hope it has prepared me well for my own defence.

Throughout my PhD, I was entrusted with supervising both MSc and BSc students. They have contributed on paper in terms of data, but have also allowed me to learn how to transfer skills, knowledge and perhaps even a little bit of scientific enthusiasm. **Susanne**, you were my first student, and together we tackled the on-farm everted sac experiments. Thanks for sticking with me and pulling through. **Thirza**, you really taught me a lot, and the work that you started even resulted in a very nice publication, thank you! **Karthikeyan**, you ended up staying for an additional internship after your thesis, which was very nice! You were dedicated to the project, and were always willing to lend an extra hand when needed. **Monique**, you really made the project your own, and managed to balance your thesis, while even helping me out during the fist animal trial, thank you! **Daniela**, you started the project with so much enthusiasm, but unfortunately the pandemic cut your lab-work short, which was such a pity! I still have hope we can pick it up sometime. Who knows, maybe after you finish your PhD in the US? **Bo**, the pandemic also hampered your lab-work, but you were still able to write a very nice literature overview. It was a new challenge for both of us. **Ruiyu**, **Jorden** en **Carla**, you were of crucial importance during the last animal experiments. Thank you all for the help, your laughs, your willingness to always stop at the MacDonalds. I look back on a great time together, and am sure Lil' bacon has a permanent spot all of our hearts. **Ana**, I was so happy with your work, to get the first glimpses of the hard-earned data from the final animal study. A final big thank you to you all, and I hope our paths will cross again in the future!

Also a many thanks to all the people from Carus that helped with the animal studies. **Arjan**, **Adri**, **Bert**, **Jacolien** en **Ries**, speciale dank aan jullie voor de inzet tijdens al mijn proeven! Zonder jullie biggen-kennis was het zeker nooit wat geworden. Ik ben ook erg dankbaar voor de hulp van **Sebastiaan**, **Pieter** en **Ralph** (WBVR) voor hun kunde tijdens de dissecties. Bedankt voor de spoedcursussen sectie en pathologie tijdens de dissecties!

Gelukkig heb ik gedurende de vier (oké vooruit, bijna vijf) jaar van mijn promotieonderzoek ook altijd nog genoeg tijd gehad om te ontspannen. Daarvoor kon ik altijd terecht bij veel lieve vrienden, waarvoor mijn grote dank! Een paar mensen verdienen toch wel even een extra vernoeming. Alle leden en ere-leden van **Huize op de Fles – Marjolein, Hester, John, Rein, Nelline en Arne -** ben ik van harte dankbaar voor de vele gezellige avondjes uit eten, weekendjes weg en bank hangen. Het zegt denk ik heel wat dat ik zélfs heb genoten van ons weekendje Friesland terwijl ik daar nog mijn discussie aan het typen was. Zonder jullie had ik ook zeker nooit de geweldige wielersport ontdekt. **Marjolein**, **Hester** en **John**, ik ben erg blij dat jullie voor nu in elk geval nog lekker dichtbij wonen, hou dat nog maar even zo! Een speciale dank voor al jullie tijd, steun

en vriendschap. Op nog vele jaren! **Anne-Dieke**, ook met jou heb ik heel wat avonturen meegemaakt. Bedankt voor je luisterende oor en gezellige gesprekken. **Lara**, we begonnen bijna tegelijk met onze thesis bij HAP, en eindigen allebei met een PhD. Gelukkig hebben we genoeg momenten samen bier kunnen drinken om wat stoom af te blazen tussen de stress door. Ik zie uit naar nog vele thee, bier en wielrentochtjes samen! Tot slot wil ik ook graag alle leden van **GewooneenGroep** bedanken voor de voortdurende vriendschap en gezelligheid. Al zien we elkaar niet dagelijks, de basis blijkt steeds weer erg solide te zijn.

Tot slot kan uiteraard mijn familie niet ontbreken in dit dankwoord. Ik ben gezegend met de liefste opa's, oma's, (schoon)ouders en een hele trits aan broertjes en zusjes. Zonder jullie allemaal was het leven een stuk saaier geweest! **Papa** en **mama**, jullie hebben me altijd op een goede manier gestimuleerd om mezelf te blijven ontwikkelen. De vrijheid die jullie me daarin boden heb ik altijd erg gewaardeerd. Hoewel mama altijd gekscherend zegt dat we dat studeren niet van haar hebben, weet ik zeker dat de dorst naar kennis in elk geval wel met de paplepel is ingegoten. Pap, ik heb jouw goede voorbeeld gevolgd door in Wageningen te komen studeren. Bij deze zijn mijn studiemogelijkheden in Wageningen toch wel zo'n beetje uitgespeeld, tijd voor een nieuwe keer uit eten om dat te vieren. Bedankt voor al jullie steun en liefde! **Lydia**, jij bent altijd mijn goede voorbeeld geweest. Ik blijf altijd trots je mijn grote zus te mogen noemen. Gelukkig ben ik altijd welkom bij jou en **Hans** thuis, en kan ik tegenwoordig extra genieten van tante spelen voor **Joas**. Bedankt voor je gezelligheid en steun, en een speciale dank dat je tijdens mijn promotie naast me wilt staan op het podium! **Melissa**, hoeveel we qua uiterlijk op elkaar lijken, zo erg verschillen we qua karakter. Misschien daarom dat we elkaar toch zo goed kunnen vinden. Ik ben trots op al de vaardigheden die je jezelf steeds meer eigen maakt. Ook bij jou en **Samuël** staat de deur altijd open, en kan ik lekker komen knuffelen met baby **Bram**. **Janine**, wat gezellig dat je in Wageningen bent komen studeren! Je weet me goed te vinden voor de koffie-momentjes. Dat er nog vele mogen volgen. Ook mijn schoonfamilie zorgt altijd voor voldoende mogelijkheid tot ontspanning en gezelligheid. Dank allereerst aan mijn schoonouders, **Leonard** en **Maddie**. Jullie hebben me vanaf het begin met open armen ontvangen, en hebben me altijd volledig onderdeel laten voelen van de familie, waar ik erg dankbaar voor ben. En wat voor familie. **Mark Jan**, **Myrthe** en **Darryl**, **Ronald** en **Lisa**, **Julia** en **Geerten**, **Jan-Ruben** en **Nathan**: mijn grote dank aan jullie allemaal voor de afgelopen jaren. Ik geniet altijd ontzettend van de tijd die we samen hebben. Nooit een saai moment, en altijd liefdevol. Ik zie uit naar nog vele jaren samen! **Myrthe**, jij bent al lang meer een vriendin dan een schoonzus. Bedankt dat je me op het podium komt steunen tijdens mijn promotie! **Benjamin**, jij bent altijd mijn steun en toeverlaat geweest. Bedankt voor al de jaren dat je het al met me hebt volgehouden, en al de liefde die je me gegeven hebt. Ik hou van je, en kijk uit naar onze toekomst samen.

A

## Curriculum Vitae

Anna Bekebrede was born on the 29th of July, in Amersfoort. Six weeks later, her parents took her and her older sister with them to Mozambique, and later Angola, where they worked for ZOA (a Christian NGO providing refugee care). At the age of almost eight, the whole family, now enriched with two more daughters, moved back to the Netherlands. Anna's interest in human biology and food led her to enrol in the BSc program Human Nutrition and Health at Wageningen University in 2012. She successfully applied for the Biomedical research master Infection and Immunity at the Erasmus University in Rotterdam. During her master, she wrote her thesis on the role of microRNA's 34 and 199a in macrophage metabolism. She was supervised by Pieter Leenen at the department of Immunology (Erasmus MC) and Jan van den Bossche at the department of Medical Biochemistry (AMC). This project also facilitated her first encounter with the Seahorse XF Analyzer, which sparked her interest in metabolism as a driver of cellular physiology. For her second internship, Anna returned to Wageningen University, where she continued working on macrophage metabolism and the Seahorse XF Analyzer, under the supervision of Vincent de Boer at the chair group of Human and Animal Physiology. After graduating in 2017, she was appointed as a PhD candidate at the same chair group. During her PhD project, she investigated how nutritional interventions impacted the metabolic function of intestinal epithelial cells, both *in vitro* using cultured cells and *in vivo* in pigs. Apart from doing her research, Anna was actively involved in the teaching activities of the chair group and supervision of BSc and MSc students. She also completed the educational program of the WIAS graduate school and presented her work at national and international meetings. Currently, Anna is appointed as a postdoctoral researcher at the chair group of Human and Animal Physiology, where she continues working on understanding intestinal epithelial cell metabolism, which she combines with data analysis and teaching.

## List of publications

### *Peer-reviewed publications*

**Anna F. Bekebrede,** Jaap Keijer, Walter J.J. Gerrits and Vincent C.J. de Boer, *The Molecular and Physiological Effects of Protein-Derived Polyamines in the Intestine.* Nutrients, 2020. 12(1): p. 197.

Bart Lagerwaard, Olga Pougovkina, **Anna F. Bekebrede**, Heleen te Brinke, Ronald J.A. Wanders, Arie G. Nieuwenhuizen, Jaap Keijer and Vincent C.J. de Boer, *Increased Protein Propionylation Contributes to Mitochondrial Dysfunction in Liver Cells and Fibroblasts, but not in Myotubes*. Journal of Inherited Metabolic Disease, 2021. 44(2): p. 438-449.

**Anna F. Bekebrede**, Jaap Keijer, Walter J.J. Gerrits and Vincent C.J. de Boer, *Mitochondrial and Glycolytic Extracellular Flux Analysis Optimization for Isolated Pig Intestinal Epithelial Cells*. Scientific Reports, 2021. 11(1): p. 19961.

**Anna F. Bekebrede**, Thirza van Deuren, Walter J.J. Gerrits, Jaap Keijer, and Vincent C.J. de Boer, *Butyrate Alters Pyruvate Flux and Induces Lipid Accumulation in Cultured Colonocytes*. International Journal of Molecular Sciences, 2021. 22(20): p. 10937.

**Anna F. Bekebrede,** Lonneke Noorman, Jaap Keijer, Vincent C.J. de Boer, Walter J.J. Gerrits, *Functional Metabolic Capacity of Pig Colonocytes is Differentially Modulated by Fermentable Fibre and Poorly Digestible Protein*. Animal, 2022. 16(11): p. 100625.

### *Expected publications*

**Anna F. Bekebrede,** Vincent C.J. de Boer, Walter J.J. Gerrits, Jaap Keijer, *Functional and Molecular Profiling of Fasted Piglets Reveals Decreased Energy Metabolic Function and Cell Proliferation in the Small Intestine*. Submitted.

**Anna F. Bekebrede,** Vincent C.J. de Boer, Walter J.J. Gerrits, Jaap Keijer, *Small Intestinal Fasting Responses of Suckling and Feed Habituated Piglets*. In preparation.

## Education and Training Activities

### *The Basic Package – 3.0 ECTS*

| | |
|---|---|
| WIAS Introduction Day | Wageningen, NL |
| Course on philosophy of science and/or ethics | Wageningen, NL |
| Course on essential skills | Wageningen, NL |

### *Disciplinary Competences – 9.7 ECTS*

| | |
|---|---|
| Writing of a literature review | Wageningen, NL |
| Agilent Seahorse XF Users Group Meeting: Measure What is Important to Your Cell | Freiburg, DE |
| Advanced statistics: design of experiments | Wageningen, NL |
| Pig-specific course laboratory animal science | Utrecht, NL |
| Mechanisms of Metabolic Signaling | New York, USA (virtual) |

### *Professional Competences – 6.3 ECTS*

| | |
|---|---|
| Teaching and supervising thesis students | Wageningen, NL |
| Start to teach | Wageningen, NL |
| Efficient writing strategies | Wageningen, NL (virtual) |
| Carreer Perspectives | Wageningen, NL (virtual) |
| Popular Scientific Presenting | Groningen, NL |
| Popular Scientific Writing | Wageningen, NL |
| Finnishing Touch | Wageningen, NL |

### *Societal Relevance – 5.0 ECTS*

Yearly meeting and presentation for and with DSM clients
Communication with DSM clients via two-pagers

### *Presentation Skills – 4.0 ECTS*

| | |
|---|---|
| WIAS Science Day 2018, poster presentation | Wageningen, NL |
| WIAS Science Day 2019, poster presentation | Lunteren, NL |
| WIAS Science Day 2020, poster presentation | Lunteren, NL |
| WIAS Science Day 2021, oral presentation | Lunteren, NL |
| (Mechanims of Metabolic Signalling 2021, poster presentation) | New York, USA (virtual) |

### *Teaching Competences – 6.0 ECTS*

Supervision various practicals and give lectures in courses given by Human and Animal Physiology chair group
Supervision 1 BSc thesis student
Supervision 9 MSc thesis students

## Colophon

This research was funded by the research partnership program TTW-DSM with Project Number 14942, which is partly financed by the Dutch Research Council (NWO) and partly financed by DSM Nutritional Products. Financial support NWO and Human and Animal Physiology for printing this thesis is gratefully acknowledged.

**Layout** by Anna Bekebrede

**Cover design** by Sieben Medical Art

**Chapter title illustrations** by Sieben Medical Art

**Printed** by Ipskamp Printing