Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Mechanisms of methionine absorption in the porcine intestine

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Stella Romanet
Tierärztin aus Lyon, Frankreich

Berlin 2023
Journal-Nr.: 4402

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Table of contents

List of figures

List of tables

List of abbreviations

AA:	amino acids
ACE2:	angiotensin-converting enzyme 2
BCH:	2-amino-2-norbornanecarboxylic acid
D-HADH:	D-2-hydroxy acid dehydrogenase
D/L HMTBA:	D/L-2-hydroxy-4-methylthiobutyrate
GIT:	gastrointestinal tract
KMB:	2-keto-(4-methylthio) butyrate
L-HAOX:	L-2-hydroxy acid oxidase
MCT:	monocarboxylate transporter
MeAIB:	methylaminoisobutyric acid
Met:	methionine
MsrA:	methionine sulfoxide reductase A
NEM:	N-ethylmaleimide
NMDG:	N-methyl-D-glucamine
SAM:	S-adenosylmethionine
SAA:	sulfur-containing AA
SCFA:	short-chain fatty acids
SMCT:	sodium monocarboxylate transporter

1. Introduction

Production performance demanded of livestock is constantly increasing worldwide. To allow for such high growing performances, every single animal requires a specific, well-adjusted diet. Especially in pigs, a balanced supply of amino acids (AA) is essential to obtain good fattening performances. As an essential AA, methionine (Met) cannot be synthetized by the organism and needs to be obtained from the diet (Wang et al. 2009). In most diet formulations, Met concentration is limiting performance if not supplemented. A shortage in Met can result in deficiency of overall protein synthesis (Katsutoshi and Jun-Ichi 1987). Methionine has a crucial part in numerous metabolic pathways as well. It acts as a sulfur donor in the synthesis of the other two sulfur containing AA cysteine and cystine (Brosnan and Brosnan 2006). It is also used as a methyl donor in transmethylation pathways after conversion to S-adenosylmethionine (SAM). SAM is required in many metabolic pathways, e.g. DNA, creatine norepinephrine, dopamine or serotonin synthesis (Ouyang et al. 2020; Brosnan et al. 2011; Bauchart-Thevret et al. 2009a). Therefore, Met is not only significant on a financial level by ensuring good production and health, but is also of importance for the overall physical condition of the individual animal (Tian et al. 2016).

To ensure that livestock is appropriately provided with Met, the latter is often supplemented in its crystalline form to the diet of poultry, cows and pigs in the form of L-Met, its racemate D/L-Met or its synthetic analog D/L-2-hydroxy-4-methylthiobutyrate (D/L-HMTBA) (Zhang et al. 2015). The use of crystalline amino acids instead of “full-protein” diets was shown to enhance growth performance by Truong et al., probably, because diets with “rapidly available” protein in the form of supplemented crystalline amino acids had a positive effect on the utilization of amino acids. Additionally, an oversupply of protein can be avoided, thus decreasing the necessity of nitrogen detoxification and excretion (Truong et al. 2017). These findings underline the importance of understanding amino acid needs and their utilization in livestock.

The focus of the work presented in the current thesis was to assess whether the most used forms of dietary Met supplements (D/L-Met, L-Met and D/L-HMTBA) had an influence on the absorption of Met in the porcine intestine. Furthermore, this work aimed to describe the respective distributions of known and supposed Met and HMTBA transport systems along the gastrointestinal tract of pigs and whether these were regulated on a molecular level by aforementioned dietary supplements.

2. Literature review

2.1. Feed Intake and Digestion

As omnivorous animals, pigs are able to digest plant and animal protein with high efficiency. Animal protein, milk powder for example, is being processed with slightly higher efficiency than vegetable protein (from soybeans for example) (Mathai et al. 2017). However, studies showed the apparent ileal digestibility of amino acids (AA) from vegetable protein can be enhanced onto the same level as animal protein through fermentation, making it an excellent protein source for growing and fattening pigs (Jeong et al. 2016). Beside the digestibility of protein itself, AA patterns in the respective protein sources are essential to best cover the pig's requirements for every single AA. Also, feeding the appropriate AA profile allows to reduce overall protein supply, thereby, reducing feed costs and N excretion (Wang et al. 2018; Van Milgen and Dourmad 2015). Among the most used protein sources, soybean meal has appeared to be the best vegetable protein source for swine diets as it has very good ileal digestibility due to an AA profile rich in lysine, tryptophan and threonine (Gonzalez-Vega and Stein 2012). These AA are considered "essential" (Van Milgen and Dourmad 2015), which means they cannot be synthesized by the porcine organism and have to be obtained from the diet (National Research Council 2012). Other AA known to be essential for pigs are Met, phenylalanine, histidine, valine, isoleucine and leucine. In contrast, the AA which can be synthesized de novo by the body are considered non-essential. De-novo synthesis of these AA is not always sufficient to cover all dietary requirements of the animal (arginine in young pigs, for example); this means that a well-balanced diet should also contain sufficient amounts of non-essential AA (Van Milgen and Dourmad 2015). Crystalline AA are widely supplemented in the diets of livestock in order to fit the optimal AA pattern, assuring the best possible dietary supply with all essential (and non-essential) AA (National Research Council 2012).

The digestion of AA starts in the stomach and in the duodenum where the proteins are broken down into polypeptides by enzymes. Pepsin, trypsin and chymotrypsin play the most important role in this process. Pepsin is the principal acid protease of the stomach as it has a very broad specificity and is able to split proteins at numerous places (Berg et al. 2002). Trypsin and chymotrypsin are components of pancreatic secretions, both acting in the duodenum (Barrett et al. 2012). The polypeptides are later fragmented into AA and di- or tripeptides by various peptidases. These molecules can be transported into the intestinal epithelial cells by numerous different AA carriers and the intestinal peptide carrier PEPT1. The AA carriers are

mostly specific for the respective type of AA transported; some are specialized in neutral AA, others in anionic or cationic AA. Most of the known AA transporters are Na^+-coupled, but various Na^+-independent carriers also participate in the intestinal absorption of AA. Another set of carrier transports the intracellularly accumulated AA from the epithelial cell into the blood (Von Engelhardt et al. 2015).

2.2. Methionine supplementation in pigs

Methionine plays a very important role in the nutrition of pigs as an essential AA. Furthermore, it is also a limiting AA which means that it has to be supplemented with most diet formulations to improve growth rates, nitrogen efficiency and profitability in meat-producing livestock (Lemme et al. 2009). The recommended dietary Met intake is at approximatively 0.34% for growing pigs (Moehn et al. 2008) and between 0.32% and 0.38% for growing broilers (National Research Council 1994). Excessive supplementation of Met has to be avoided as this may have toxic effects. In humans, hypermethioninemia has been associated with acidosis, vascular endothelial dysfunction and impaired growth in infants (Garlick 2006). In rats and chickens, increasing the dietary Met concentration to 1.5-2.0% of the diet caused drastic decreases of growth and low feed intakes (Benevenga and Steele 1984). It follows that Met supplementation has to occur in a defined dosage range to fulfill its purpose.

Met deprivation or even restriction has been shown to lead to lower body weights and fat mass, but also to enhance overall energy expenses in mammals (Xiao and Guo 2021). Furthermore, a slight over supplementation of Met might have a positive effect on the development of pigs (Owen et al. 1995). It has been demonstrated that a rise in the Met content in the diet of freshly weaned piglets from 0.36% to 0.56% resulted in significantly higher weight gains. Others showed that supplementing the feed with 0.15% crystalline D/L-Met increased the daily weight gain by more than 13% (Ly et al. 2012). Besides improved growth, a higher amount of Met in the diets of growing pigs can raise muscular antioxidant capacity and improve meat quality after slaughter by increasing its pH and reducing drip loss (Li et al. 2017). Furthermore, as a sulfur containing AA (SAA), Met in its dietary form is a key ingredient for sustaining normal mucosal proliferation because dietary SAA deficiency resulted in intestinal mucosal atrophy (Bauchart-Thevret et al. 2009b). Despite the positive effects of slight over-supplementation of Met, it has been shown that a significantly higher Met intake than normal might have harmful consequences. In fact, Met has been described as the “most toxic” AA (Harper et al. 1970). These harmful impacts might include acidosis, coma and even death in some species (Scislowski and Pickard 1993). The source of the toxic effect is mainly attributed

to the fact that Met is a precursor of homocysteine which accumulates in the body when Met is supplemented in excess (Garlick 2006; Baker 2006), but also the rise in methanethiol and H_2S from transaminative degradation might play a role in the potential toxicity of this AA (Scislowski and Pickard 1993).

Several supplements are suited as Met source. It can be added in the form of L-Met, D/L-Met or as D/L HMTBA. Pure L-Met is the most expensive additive when compared to the two others, for this reason D/L-Met and D/L-HMTBA are most commonly used in animal nutrition. Whether the one or the other of the latter two is more cost efficient cannot be answered directly, as various factors have to be taken into account for a realistic economic model, especially overall feed price, the price of the used supplement and meat market price (Vedenov and Pesti 2010). The D/L-Met racemate is efficiently used by the pig's organism. The D-isomer is converted into the L-isomer in the liver and, in this form, it is fully utilized in metabolic processes (Zhang et al. 2015). Several studies used the hydroxyl analog D/L-HMTBA as an alternative to Met. They showed that D/L-HMTBA has a lower bioavailability than D/L-Met or L-Met (Zhang et al. 2015; Shoveller et al. 2010), however, it is still very efficient in promoting growth in pigs (Zhang et al. 2015). The reasons for the high growth rates reached by using D/L-HMTBA could be that the latter is metabolized to a considerable lesser degree in the intestinal epithelial cells than Met (European Food Safety Authority 2012). The Met requirement of epithelial cells is very high for the synthesis of glutathione and mucins, but also for transmethylation reactions (Fang et al. 2010a; Riedijk et al. 2007; Shoveller et al. 2003); furthermore, if the Met concentration in the epithelial cell increases due to high dietary supply, transsulfuration, transamination and transmethylation reactions are stimulated to cover the cells' needs for Met (Lemann and Relman 1959).

Nevertheless, even though the bioavailability of the different Met sources is well known, there are no studies directly comparing the absorption of Met and D/L-HMTBA in the intestine of pigs. It has also never been investigated whether these different Met sources had an effect on the expression of intestinal Met transport-systems in pigs, which could directly affect the absorption of this AA.

2.3. Methionine metabolism

Met has a very important role in protein biosynthesis as it is the AA used to initiate protein synthesis in the cytosol of all eukaryotes (Sherman et al. 1985). Its structure itself also makes Met a very interesting proteinogenic AA; the side chain of Met contains a sulfur atom and this

confers a certain flexibility to the proteins. This particularity can be used for stabilizing protein structures or facilitating protein-protein interactions, which makes Met a very important building block in a large variety of proteins. The Met metabolism itself mainly comprises transmethylation, remethylation and transsulfuration reactions (Figure 1) (Mastrototaro et al. 2016). Met can be converted to homocysteine through successive transamination and transmethylation via SAM. The latter is the main methyl donor in mammalian cells that is used for the methylation of nucleic acids, proteins and lipids (Bauchart-Thevret et al. 2009a). The produced homocysteine can then be methylated back into Met, or can be converted to cysteine and later taurine through transsulfuration; this last pathway being irreversible (Brosnan and Brosnan 2006). Another asset of Met is its ability to be oxidized to methionine sulfoxide which can be reduced back to Met by the same enzyme (methionine sulfoxide reductase A: MSR A). The oxidation of Met in proteins does not seem to have an effect on the their activity, but might be of interest in protecting the eukaryotic cell from oxidation (Aledo 2019). Evidence shows that Met even tends to show higher incorporation in proteins when cells are exposed to oxidative stress (Aledo 2019).

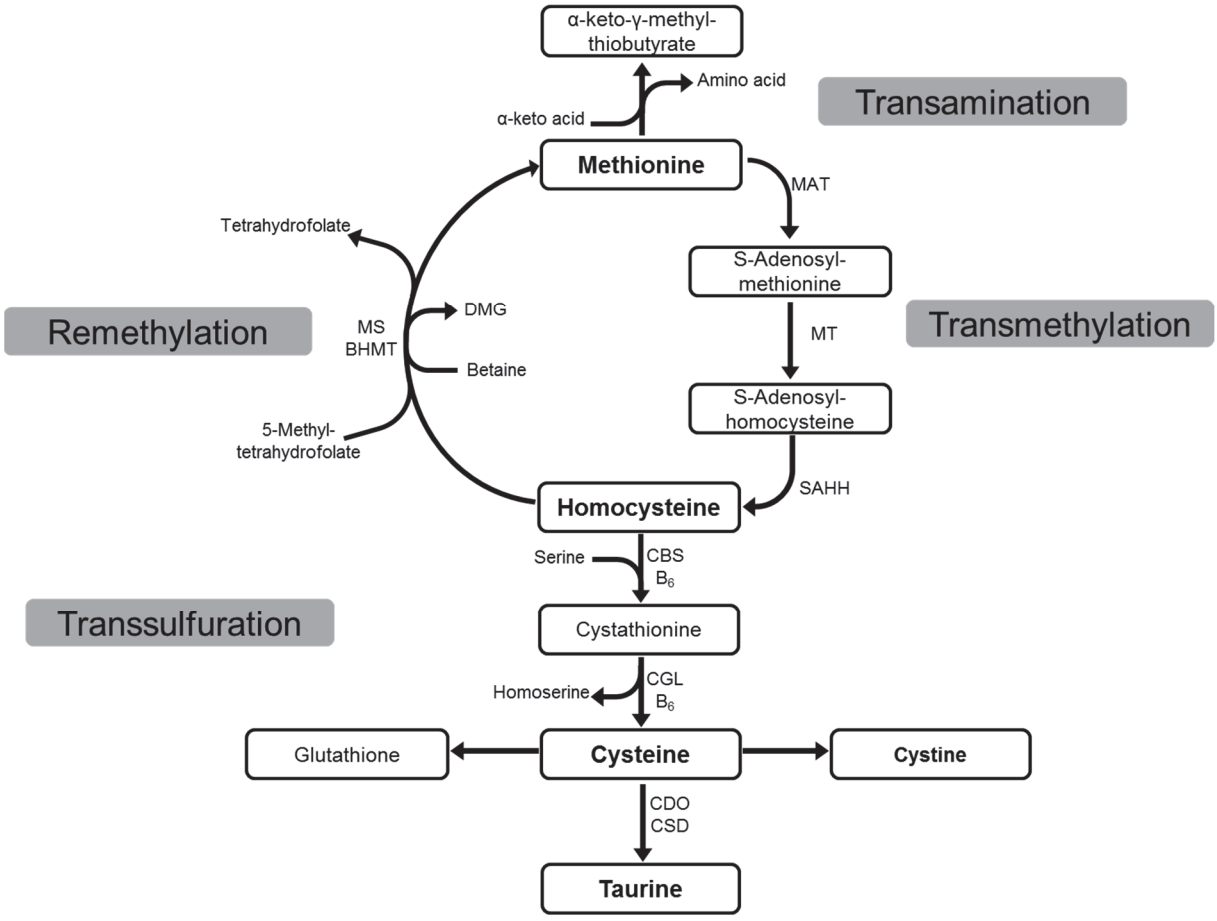

Figure 1: Methionine metabolism pathways according to Bauchart-Thevret et al. (Bauchart-Thevret et al. 2009a).

Methionine can be used in transamination reactions to produce other AA necessary for protein synthesis. Most Met absorbed in the intestinal epithelial cells is transmethylated into S-adenosyl-methionine (SAM). After demethylation into S-adenosyl-homocysteine (SAH), it is processed into homocysteine. Homocysteine can be methylated back into Met. In a transsulfuration reaction homocysteine can be metabolized into cysteine which can later be used in the synthesis of taurine, cystine or glutathione. MAT, methionine adenosyltranferase; MT, methyl transferase; SAHH, S-adenosylhomocysteine hydrolase; MS, methionine synthetase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; CDO, cysteine dioxygenase; CSD, cysteine sulfate decarboxylase.

Several studies showed that the dietary Met requirement can be lowered by supplementing cysteine. Indeed more than 40% of the SAA requirement can be met by cysteine (Bauchart-Thevret et al. 2009a). This “Met-sparing effect” seems to be a consequence of reduced homocysteine transsulfuration to cystathionine when cysteine is present in sufficient quantity (Finkelstein et al. 1988). Another product of Met metabolism is taurine. Taurine is used in the regulation of anti-oxidation reactions. As the intestine is constantly exposed to various endo- and exogenous oxidants, taurine is crucial to maintain gut health (Colovic et al. 2018). Next to that, taurine is involved in bile acid conjugation which makes it very important in intestinal lipid digestion (Bouckenooghe et al. 2006). Bauchart-Thevret et al. suggested that taurine can be synthetized in the intestine from Met and cysteine, as a sulfur AA-free diet leads to a decrease in ileal taurine concentration (Bauchart-Thevret et al. 2009a). In case Met is present in excess, it can also be converted into oxaloacetic acid to be used either in the glycolipid metabolism or in the synthesis of nonessential AA (Wan et al. 2017).

The intestinal epithelial cells have very high metabolism rates; indeed approximately 30% of dietary Met is directly metabolized in the gastrointestinal tract (GIT) (Shoveller et al. 2005). Evidence of the high intestinal metabolization is the low appearance of dietary Met in the portal blood, suggesting a high utilization of Met directly in the intestinal tissue (Stoll et al. 1998). As a matter of fact, the GIT of piglets shows relatively high transmethylation and transsulfuration rates for absorbed Met, a significant amount of the absorbed Met is directly used in the GIT for protein synthesis (Riedijk et al. 2007). Stoll et al. concluded that even though lysine is considered the first limiting AA, the limiting AA for extra-intestinal deposition can be considered being Met in pigs. This statement was made on the basis of the net portal AA balance, as only about 48% of the ingested Met appeared in the portal blood, presumably due to a very high “first-pass” Met utilization in the intestinal mucosa (Stoll et al. 1998).

2.4. HMTBA metabolism

2-Hydroxy-4-methylthiobutyrate is a synthetic hydroxy analog of Met. It is increasingly used as a Met substitute in the diet of livestock as it seems to be more cost efficient (Vedenov and Pesti 2010). While Met possesses the classic structure of an AA (an amino group, a carboxyl group, a hydrogen atom, and a side chain), the amino group is replaced by a hydroxyl group in HMTBA. This structural difference makes HMTBA similar to a monocarboxylate molecule, rather than an AA (To et al. 2021). This apparently small modification has a considerable influence on molecular properties and significantly alters metabolism and transport mechanisms of the substance. The bioefficacy of D/L-HMTBA has been the subject of various studies in poultry and swine (Shoveller et al. 2010; Lemme et al. 2002) and the use of this supplement is still controversial with regards to its effectiveness (Martin-Venegas et al. 2006). Commercially available D/L-HMTBA consists of 88% of a blend of mono-, di-, and oligomers. This product being a mixture of different molecule sizes might play a role in its lower dietary efficiency (Zimmermann et al. 2005). Several groups have investigated the effectiveness of D/L-HMTBA when compared to D/L-Met. Most studies used N balance as primary response criterion whereas others added animal performance in the form of weight gain as a criterion (Wang et al. 2020; Opapeju et al. 2012; Feng 2006; Kim et al. 2006; Zimmermann et al. 2005). The results ranged from 62 to 78% D/L-HMTBA efficacy when compared to D/L-Met on an equimolar base. Before it can be used by the organism, D/L-HMTBA has to be converted into L-Met. Studies have shown that this process occurs mainly in the liver (Lobley et al. 2006), but the GIT was found to be able to transform HMTBA into Met (Bauchart-Thevret et al. 2009a; Martin-Venegas et al. 2006). This could explain why one study identified higher fractional portal balance of Met (the amount of met in the portal blood after first-pass metabolism in the enterocytes) in animals that received D/L-HMTBA as a Met supplement compared to animals which were fed a D/L-Met-supplemented diet (Fang et al. 2010a). Nevertheless, the levels of protein synthesis are comparable in the tissue from animals fed D/L-HMTBA or D/L-Met (Knight et al. 1998). Another emerging concern about the substitution of Met with D/L-HMTBA might be that the Met analog is not able to replicate the antioxidative properties of the AA. Gasparino et al. tackled this question in 2018 and showed that enzymatic activity linked to antioxidant systems was similar in D/L-HMTBA-fed chicken when compared to the activity measured previously in birds which received D/L-Met as a dietary supplement (Gasparino et al. 2018). Other work groups also showed that D/L-Met and D/L-HMTBA similarly affected the antioxidant defense system under normal conditions (Zhang et al. 2018; Zeitz et al. 2018). In contrast, in heat stress situations, D/L-Met appeared to have a better antioxidant efficacy when compared to D/L-HMTBA (Liu et al. 2019). However, it is

important to notice that antioxidative capacity was not shown for D/L-HMTBA itself and could probably be linked to the capacity of D/L-HMTBA to be metabolized into Met. In another study it was hypothesized that a D/L-HMTBA-containing diet would lead to lower production of reduced glutathione, which is known for protecting cells from oxidative stress, eventually leading to the upregulation of alternative mechanisms against oxidative stress when compared to a D/L-Met or L-Met-supplemented diet. This group showed that D/L-HMTBA and D/L-Met supplementations led to similarly reduced glutathione levels, only pigs fed with L-Met showing higher levels. Nevertheless, the oxidative stress and defense status remained similar with all three diets (Rasch et al. 2020). Overall, these recent studies indicate that feeding D/L-HMTBA does not have an adverse influence on the antioxidative protective systems when compared to D/L-Met.

The first step in L-Met synthesis from D/L-HMTBA is a stereospecific reaction involving either L-2-hydroxy acid oxidase (L-HAOX) or D-2-hydroxy acid dehydrogenase (D-HADH). This first reaction oxidizes D- or L-HMTBA into 2-keto-(4-methylthio) butyrate (KMB). KMB is then converted to L-Met in a transamination step catalyzed by the enzyme aminotransferase (Dibner and Knight 1984). These enzymes are not equally distributed in the organism. While aminotransferase is ubiquitously present in the tissue of animals, the activity of D-HADH and especially L-HAOX is much more restricted. As a matter of fact, L-HAOX has been found only in the liver and kidney of different animals. D-HADH is more common as it has additionally been found in other tissues, in intestine and skeletal muscle for example (Bauchart-Thevret et al. 2009a); it has even been stipulated that D-HADH is potentially present in every cell of the body (Vazquez-Anon et al. 2017). However, the activity of both L-HAOX and D-HADH has been described as highest in liver and kidney, especially when compared to tissue from the intestine (Fang et al. 2010b), indicating that these organs have their importance in the transformation of D/L-HMTBA.

2.5. Transport mechanisms for methionine

All AA possess an amino group ($-NH_2$) and a carboxyl group (-COOH). A third functional group may confer AA further structural characteristics; therefore, a large range of transport systems is necessary that all types of AA can be transported across epithelia in the organism. Met is a hydrophobic, neutral, non-aromatic and sulfur-containing AA (Berg et al. 2002); this determines which AA transporters can be involved in the intestinal absorption of Met. An overview of the hypothetical model of Met transport is shown in Figure 2. Met seems to have

a very high priority in the absorption of proteinogenic AA, as numerous transport systems appear to be involved in its absorption (Webb 1990). All of these systems have slightly different substrate spectra and characteristics. They can be specialized solely in neutral AA or also accept cationic AA. Most of them require sodium to carry their substrates; some also need chloride in addition. Others are AA exchangers and require the presence of an AA at the contralateral side of the membrane.

The **B^0AT1** (SLC6A19) system is probably the most important protein for Met transport. B^0AT1 is a strictly sodium-dependent and electrogenic system. This transporter is mainly located apically in epithelia of the small intestine and kidney. Its proper localization relies on the co-expression of a molecule called collectrin, or its analogue angiotensin-converting enzyme 2 (ACE2) in the intestine (Singer and Camargo 2011; Kowalczuk et al. 2008). B^0AT1 accepts all neutral AA as substrates but has a preference towards long-chained, branched and/or sulfur containing neutral AA like L-leucine, L-isoleucine and L-Met (Camargo et al. 2005). These are transported into to the cell with Na^+ in a 1:1 stoichiometry. The K_m described for L-Met is between 1,5 and 4,0 mM. Compared to other transport systems, the affinity of B^0AT1 for L-Met might appear relatively low (Bröer 2008; Bohmer et al. 2005; Jorgensen et al. 1990). Mutations in the *SLC6A19* gene are associated with a condition called Hartnup disorder, involving decreased absorption of neutral AA in the intestine and higher urinary loss. In patients with this disorder, the *SLC6A19* gene is mutated in such a way that the interaction with collectrin and ACE2 is reduced, thus hindering the proper localization of the transporter in the epithelial membrane (Jando et al. 2017; Kowalczuk et al. 2008).

ATB^{0+} (SLC6A14) is supposed to play a rather marginal role in the absorption of dietary Met (To et al. 2021). This system has a good affinity to Met and is mainly situated in intestinal segments with low Met availability (Chen et al. 1994). At neutral pH, it only accepts neutral AA, but the system accepts anionic AA at acidic pH. This system prefers large AA, especially glutamine and asparagine (Kekuda et al. 1997). Overall it is potentially the AA transport system with the broadest substrate spectrum, as it is able to transport 18 proteinogenic AA (Sikder et al. 2017). ATB^{0+} is a Na^+ and Cl^- dependent transport system and works with a 2:1:1 (Na^+: Cl^-: AA) stoichiometry. With a K_m around 14 µM, ATB^{0+} has a higher affinity to Met than B^0AT1 (Sloan and Mager 1999). The presence of ATB^{0+} in the porcine jejunum is subject to controversy. Munck et al. stipulated that ATB^{0+} might not be expressed in the intestine of pigs based on functional data (Munck et al. 2000). ATB^{0+} was shown to be present in the stomach and in the small intestine of pigs on the mRNA level (Yang et al. 2010) and the ATB^{0+} protein was already found in jejunum (Sun et al. 2015) although it is supposed to have a higher expression in more distal portions of the small intestine and in the large intestine (Hatanaka et

al. 2002). Hatanaka et al. showed that ATB^{0+} accepts several D-AA (D-Met was one of them among others). Because of the predominant location in the large intestine, Hatanaka's group stipulated that the primary function of ATB^{0+} was the absorption of bacterial AA (Hatanaka et al. 2002). ATB^{0+} also plays a role in various pathophysiological processes, for example in modulating the severity of cystic fibrosis, as well as being upregulated in various types of cancer (Ruffin et al. 2020; Sikder et al. 2017).

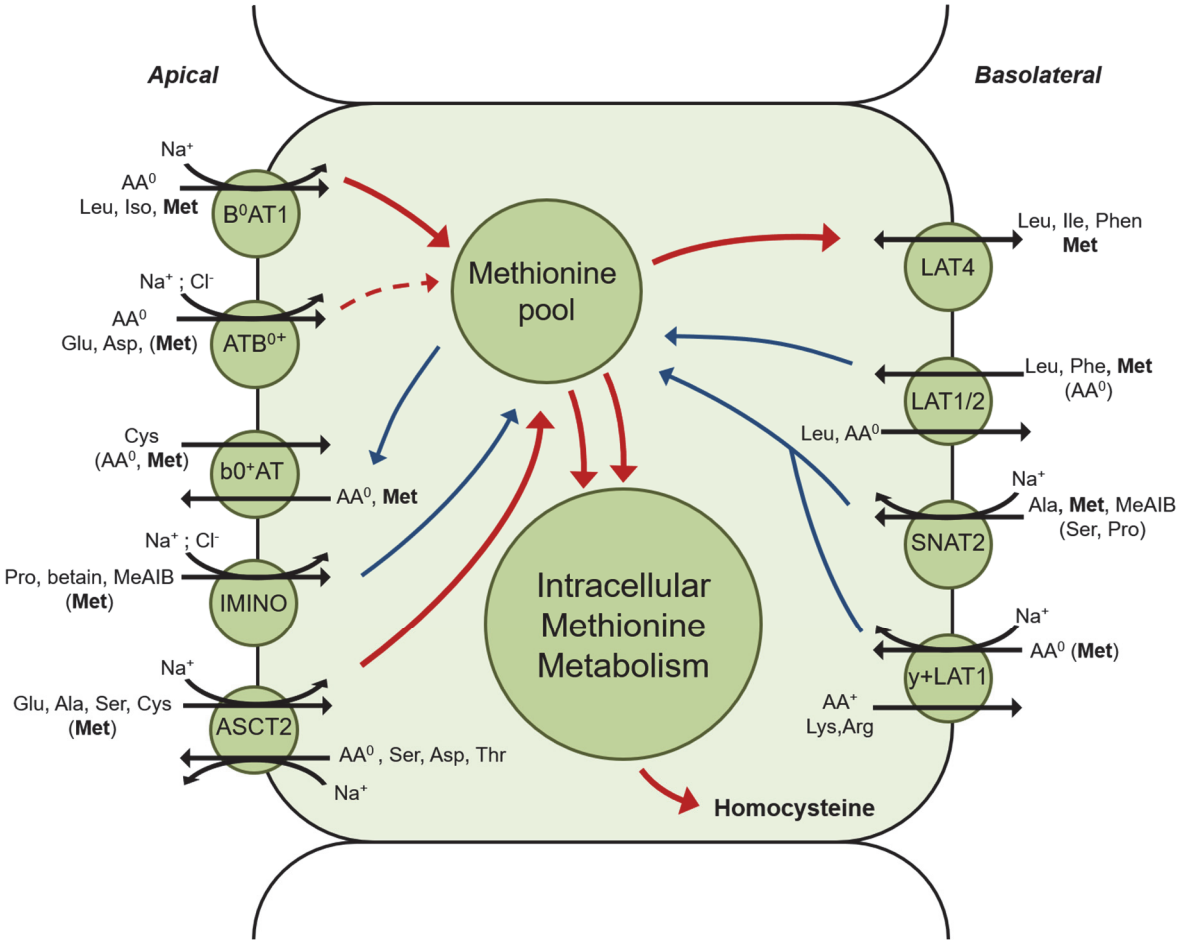

Figure 2: Supposed model of methionine transport in enterocytes of pigs according to Mastrototaro et al. (Mastrototaro et al. 2016).

The main ways of Met absorption in the intestine are through the Na^+-dependent transport systems B^0AT1 and ATB^{0+}. As the latter could not be found in the intestine of pigs, B^0AT1 is probably the main way of entrance for Met into the intestinal epithelial cell in pigs. The Na^+-independent $b^{0+}AT$ as well as the Na^+-dependent systems IMINO and ASCT2 may also play a role (although marginal) in the intestinal Met uptake. After import into the cell, the biggest fraction of the absorbed Met is rather quickly used up in the intracellular Met metabolism and metabolized to homocystein. However, a smaller proportion is released on the basolateral side through facilitated diffusion with the LAT4 transport-system. LAT4 is probably the only transport system responsible for Met efflux, as the other transport proteins on the basolateral side of the enterocytes (LAT1/2, SNAT2, y^+LAT1) may facilitate re-uptake of Met from the blood into the cell. The arrows show the Met fluxes in the intestinal epithelial cell, red full arrows represent the main flow in the porcine enterocytes, the red dotted arrow shows a significant fluxway, although probably inexistent in pigs. Blue arrows are likely of minor significance. AA^0: neutral amino acids ; AA^+: cationic amino acids ; MeAIB : methylaminoisobutyric acid.

Furthermore, a Na^+-independent system, **$b^{0,+}$AT** (SLC7A9) seems to participate in the intestinal Met absorption. This protein is part of a heteromeric AA transporter composed of a $b^{0,+}$AT and an rBAT (SLC3A1) subunit (Nagamori et al. 2016). Its principal function in the intestine is probably the absorption of cysteine from the lumen in the intestinal epithelial cell in exchange of neutral AA (for example Met) (Mastrototaro et al. 2016). The rBAt/$b^{0,+}$AT system has a relatively good affinity to Met (K_m = 130 µM in humans) (Nickel et al. 2009), however under physiological conditions, the Met absorption in the intestine by this transport system may be minor as it has a higher affinity to other AA, especially cysteine (K_m around 50µM has been described) (Bröer 2008). Mutations in the *SLC7A9* gene are associated with cystinuria and chronic kidney diseases (Corredor et al. 2020; Fazaeli et al. 2017).

The **IMINO** system is an electrogenic Na^+ and Cl^--dependent transport. For a long time, it had only been known functionally without being identified on a gene level. It was finally identified as the product of the *SLC6A20* gene (Takanaga et al. 2005). It mainly transports proline, betaine and methylaminoisobutyric acid (MeAIB) (Kowalczuk et al. 2005). The IMINO system also accepts Met as a substrate, but at a much lower affinity than proline and its other substrates; indeed, while the established K_m for proline and betaine are approximately 300 µM and 170 µM respectively, the K_m investigated for Met was around 7 mM (Nickel et al. 2009; Takanaga et al. 2005). Mutations in the *SLC6A20* gene might be involved in the pathogenesis of Hirschsprung disease (Lee et al. 2016). Recent findings associated *SLC6A20* together with other genes as a potential risk factor for severe responses to SARS-CoV-2 infections (Secolin et al. 2021; Ellinghaus et al. 2020).

ASCT2 (SLC1A5) is a high-affinity neutral AA transporter and is believed to be the main Met transporter in the intestine besides B^0AT1 (To et al. 2021). It is an obligatory Na^+-dependent, yet not electrogenic system with an antiport mode of transport (Scalise et al. 2018; Utsunomiya-Tate et al. 1996). It is not very clear whether Na^+ is co-transported with the AA or if it only binds to the transport system, thus stimulating the transport activity (Scalise et al. 2018). Its isoform, ASCT1 is found in the kidney and only accepts few AA as substrates (L-alanine, L-serine, L-cysteine) (Mastrototaro et al. 2016). ASCT2 has a slightly wider spectrum as it also accepts L-threonine and L-alanine with very good affinity (K_m ~ 20 µM) (Utsunomiya-Tate et al. 1996). L-Met is also accepted with significantly lower affinity (K_m ~300 µM) (Verma and Kansal 1993). Nevertheless, the main role of ASCT2 seems to be the uptake of L-glutamine, which it accepts with high affinity ($40 \leq K_m \leq 90$ µM) (Bröer et al. 2000). L-Glutamine is transported in a Na^+-dependent manner in exchange of other neutral AA such as L-serine, L-asparagine or L-threonine (Scalise et al. 2018). The transport of L-glutamine through ASCT2 seems to have major significance in fast growing cells, especially tumor cells (Mcgivan and

Bungard 2007). Through its high presence in tumorous cells, ASCT2 can be used as a predictor in cancer prognostics (Bernhardt et al. 2017; Sun et al. 2016), although its absence does not suffice to significantly slow down tumor growth. Indeed, the absence of this transport system in tumor cells has been shown to be compensated by the upregulation of other AA transporters, especially SNAT1 and SNAT2 (Bröer et al. 2019).

The Met efflux on the basolateral side of enterocytes is mainly regulated by a single protein, **LAT4** (SLC43A2) (Guetg et al. 2015). This transport system enables the outflow of a very small spectrum of AA. Indeed, only L-leucin, L-isoleucin, L-phenylalanine and L-Met are accepted as substrates. LAT4 is completely Na^+-, Cl^-- and pH-independent, and functions through facilitated diffusion, which means that not only the outflow of AA is regulated by this system; it is also able to allow the uptake of these same AA from the blood into the cell when needed (Bodoy et al. 2005). It has been demonstrated that LAT4 has a “two-step” kinetics for L-leucine and L-phenylalanine ($100 \leq K_m \leq 200$ µM und $K_m > 3$ mM). This feature makes LAT4 a very effective transport system at both low and high substrate concentrations (Bodoy et al. 2005). Tumor cells have been shown to highly express this transport system to ensure their own Met supply, to the point where especially immune cells in the surrounding area found themselves depleted of this AA (Bian et al. 2020).

The system-L transporters **LAT1** (SLC7A5) and **LAT2** (SLC7A8) are both located on the basolateral side of the intestinal epithelial cells and are electroneutral exchangers for neutral AA. Their significance for Met absorption is probably negligible as their first function seems to be equilibration of the relative AA concentrations across cell membranes (Verrey et al. 2004), especially the efflux of L-leucine and the re-uptake of L-Met from the blood into the cell in exchange of other AA (Mastrototaro et al. 2016; Yanagida et al. 2001; Chen et al. 1994). The functionality of LAT1 and LAT2 requires the association with the 4F2hc (SLC3A2) heavy-chained protein (Bröer et al. 2001).
LAT1 is a high-affinity transport system for L-leucine, L-isoleucine, L-phenylalanine and L-Met (K_m = 20 µM), though D-isomers can be accepted with similar affinity (Yanagida et al. 2001). LAT2 is known to accept the same substrates as LAT1, but with a slightly lower affinity (K_m = 204 µM for L-Met) and without accepting D-isomers (Segawa et al. 1999). Both systems can be inhibited by 2-amino-2-norbornanecarboxylic acid (BCH) (Christensen 1990). The significant functional difference between LAT1 and LAT2 is the pH dependence. While LAT2 is stimulated by acidic pH (Fraga et al. 2002), pH variations have no effect on the transport capacities of LAT1 (Prasad et al. 1999). LAT1 was shown to take part in the pathogenesis of cancer (Kanai 2021) but also that its presence is primordial for osteogenesis and maintaining bone density (Ozaki et al. 2019). The inactivation of LAT2 was associated with aminoaciduria,

but its absence seemed to be rather well compensated as growth and development were not significantly impacted (Braun et al. 2011).

Another transport system responsible for the re-entry of L-Met from the basolateral side into the enterocyte is **SNAT2** (SLC38A2). It is a secondary active transport system which transports its main substrates L-alanine and L-Met together with Na^+ from the blood into the intestinal epithelial cell. Other neutral AAs (especially L-serine and L-proline) are also accepted as substrates (Yao et al. 2000). In the mammary glands of mice, the K_m described for Met was around 400 µM (Verma and Kansal 1993). SNAT2 has a lower efficiency at lower pH and can be inhibited by MeAIB (Yao et al. 2000). Its isoform SNAT1 is also able to transport L-Met, but seems to have a very marginal role in the small intestine (Mackenzie et al. 2003). Even though SNAT2 is present in the intestine, the malfunction or absence of this transport system is not primarily linked to intestinal symptoms but impacts lung and placenta function (Vaughan et al. 2021; Weidenfeld et al. 2021).

4F2hc/**y^+LAT1** (SLC7A7) and 4F2hc/**y^+LAT2** (SLC7A6) are both located on the basolateral side of enterocytes and share a relatively high grade of sequence homology. Studies have shown that y^+LAT1 transports cationic AA in a Na^+-independent manner, whereas neutral AAs require the presence of Na^+ to be accepted as substrates (Pfeiffer et al. 1999). This particularity and the fact that the extracellular Na^+ concentration is physiologically high make these transport-systems efflux carrier of cationic AA (especially L-lysine and L-arginine) from the enterocytes in exchange of a large neutral AA (L-leucine, L-glutamine or L-Met for example) together with a Na^+ ion. This claim can be reinforced as it has been shown that neutral L-AA on the “trans” side stimulate the efflux of L-lysine from the cell (Rotoli et al. 2020; Deves et al. 1992). y^+LAT1 is believed to be expressed primarily in macrophages, intestine and in kidneys whereas y^+LAT2 is mainly expressed in erythrocytes and lymphocytes (Pfeiffer et al. 1999). Defects in the *SLC7A7* gene lead to an autosomal disease called lysinuric protein intolerance where the absorption and re-absorption of cationic AA in intestine and in kidney is disturbed. This leads to a collection of more or less severe symptoms ranging from hypotonia to pulmonary alveolar proteinosis, the last one being potentially lethal (Bodoy et al. 2019). Defects in y^+LAT1 are believed to be relatively well compensated by y^+LAT2, thus deletion of y^+LAT1 is normally associated with less severe symptoms (Rotoli et al. 2020). The loss of function of y^+LAT2 on the other hand, leads to other pathological patterns as it preponderantly disrupts the exchange of L-arginine and L-glutamine, upsetting the urea cycle in the liver and potentially leading to hepatic encephalopathy (Errasti-Murugarren and Palacín 2022).

The intestinal D/L-HMTBA uptake is quite distinct from the L-Met pathway; it has been shown that D/L-HMTBA is absorbed in the jejunum by a Na^+-independent carrier system associated with a diffusive component across the brush-border membrane (Brachet and Puigserver 1987). The exact apical D/L-HMTBA uptake mechanism has not been described yet (To et al. 2021). Evidence has been found that D/L-HMTBA is principally absorbed apically in an H^+-dependent way, and that this transport can be inhibited by L-lactate (Maenz and Engele-Schaan 1996). Due to these findings and to the morphologic resemblance between D/L-HMTBA and monocarboxylates, it has been suggested that D/L-HMTBA is transported into the intestinal cells by the **MCT1** transport system, probably with additional contribution of **MCT4** (To et al. 2021). MCT1 is a transport system which, in the intestine, is mostly localized on the apical cell membrane of enterocytes, whereas MCT4 is believed to be localized mainly on the basolateral side of enterocytes (Felmlee et al. 2020; Martin-Venegas et al. 2014). MCT1 and MCT4 have very similar substrate spectra with the main difference being that MCT4 demonstrates a lower affinity for most substrates than MCT1 (Halestrap 2013). Both MCT1 and MCT4 function as an H^+-dependent importer or an exporter in intestinal cells (Park et al. 2018; Morris and Felmlee 2008). The substrates accepted by MCT1 and MCT4 are mainly monocarboxylates, especially L-lactic acid (K_m = 3.5 - 6 mM for MCT1; K_m = 28 mM for MCT4), but it also accepts keto acids (e.g., pyruvate, K_m = 1.8 - 2.5 mM for MCT1; K_m = 153 mM for MCT4) (Felmlee et al. 2020; Halestrap and Wilson 2012; Martin-Venegas et al. 2007). High MCT expressions are associated with tumor proliferation in many cancer types, mainly for the efflux of L-lactic acid produced by the high glucose turnover in tumor cells. Discrepancies in the expression of MCT are mainly associated with metabolic diseases. Indeed, mutations of MCT1 have also been associated with exercise-induced hyperinsulinemia and ketoacidosis, and obese patients were shown to have increased expression of MCT4 in muscle (Felmlee et al. 2020).

3. Aims and objectives of this thesis

The literature review showed that the supplementation of AA, especially Met, is key to ensure good production rates and health in pigs. There has been a lot of research done on the effects of Met supplementation on the metabolism and health in various species, and we possess detailed information on individual transport systems. However, concerning the way these transport systems work together and react *in vivo* to changes in dietary supply, the information remains very sparse. To ensure that livestock is appropriately provided with Met, the latter is often supplemented in its pure form to the diet of poultry, cows and pigs in the form of L-Met, its racemate D/L-Met or its synthetic analog D/L-HMTBA. These forms of Met are immediately bioavailable; meaning they could have a direct impact on Met absorption and metabolism in the intestine. Thus, the hypothesis of this thesis is that Met absorption can be influenced by the form of Met supplemented through the diet.

To assess this hypothesis, the objective of this project was to analyze the mechanisms of Met absorption in the intestine of pigs. In the first part of this thesis, the main focus was to assess the effects of the different Met sources that are available in pig nutrition, on Met absorption in the intestine. For this purpose, a feeding study was designed, in which 3 groups of 9 pigs each received a different Met supplement: D/L-Met, L-Met or D/L-HMTBA. After a minimum of 10 days of feeding, the pigs were euthanized and sections from their duodenum, jejunum and ileum were used in Ussing-chamber experiments. In these experiments the flux of D-Met, L-Met and D/L-HMTBA were measured in the three small intestinal section of all 27 animals.

In the second part of this project, the aim was to describe the distribution of supposed Met and HMTBA transporters along the gastro-intestinal tract, as well as to evaluate if the different Met sources had an influence on the molecular expression of these transport systems. For this purpose, mRNA expression was examined by qPCR for the following genes: *B^0AT1, ATB^{0+}, rBAT, ASCT2, IMINO, LAT4, y^+LAT1, LAT2, SNAT2, MCT1 and MCT4.* Western blot experiments to measure protein expression were conducted for B^0AT1, ASCT2, LAT2 and LAT4.

The information gained through this thesis will deepen the understanding on the Met absorption pathways and their regulation. This knowledge would allow optimizing the Met supply in pig production, thereby, avoiding unnecessary over supplementation and reducing nitrogen emission while maintaining growth performance.

4. Results

4.1. Publication 1

Dietary supplementation of D/L-Methionine potently induces sodium-dependent L-methionine absorption in porcine jejunum ex vivo

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You have to read this part online.

*Authors' contributions are listed under Acknowledgements in Publication 1.

4.2. HMTBA-Flux in the small intestine

In the first part of this project we showed that feeding different Met sources can modify the absorptive flux of L-Met and D-Met (Romanet et al. 2020). The data presented in Publication 1 focusses on the flux of D-Met and L-Met in the intestine of pigs supplemented with three different Met sources (D/L-Met, L-Met and D/L-HMTBA). The flux of D/L-HMTBA was assessed in parallel in those experiments; the data will be presented in this chapter.

4.2.1. Materials and Methods

Diets, animal handling, methods and flux measurements, as well as statistical analysis are described in Publication 1 (Romanet et al. 2020). The only difference during flux measurement was that chambers received 37.0 kBq [^{14}C]-D/L-HMTBA together with unlabeled D/L-HMTBA to a final concentration of 50 µM on the mucosal side, which was increased for the second flux period to 5 mM. All other procedures of the Ussing chamber experiment remained the same.

4.2.2. Results

The means of 7-9 mucosal-to-serosal flux rates of radioactively labeled D/L-HMTBA in three different small intestinal regions at 50 µM and 5 mM D/L-HMTBA in the presence or absence of Na^+ are presented in Figure 3. For clarity, only *P*-values < 0.1 are shown. In Na^+-free condition, Na^+ was replaced with NMDG to maintain osmolarity.

At 50 µM D/L-HMTBA, significant effects on flux rates were neither observed for the factor diet nor for the factor Na^+ in any of the three investigated intestinal segments (Figure 3 A; C and E).

At 5 mM HMTBA, diet and the presence of Na^+ had also no significant effects on flux rates, except for a significant effect of Na^+ on D/L-HMTBA flux rates in the duodenum ($P < 0.05$). Peculiarly, the presence of Na^+ appeared to be associated with lower D/L-HMTBA flux rates (Figure 3 B).

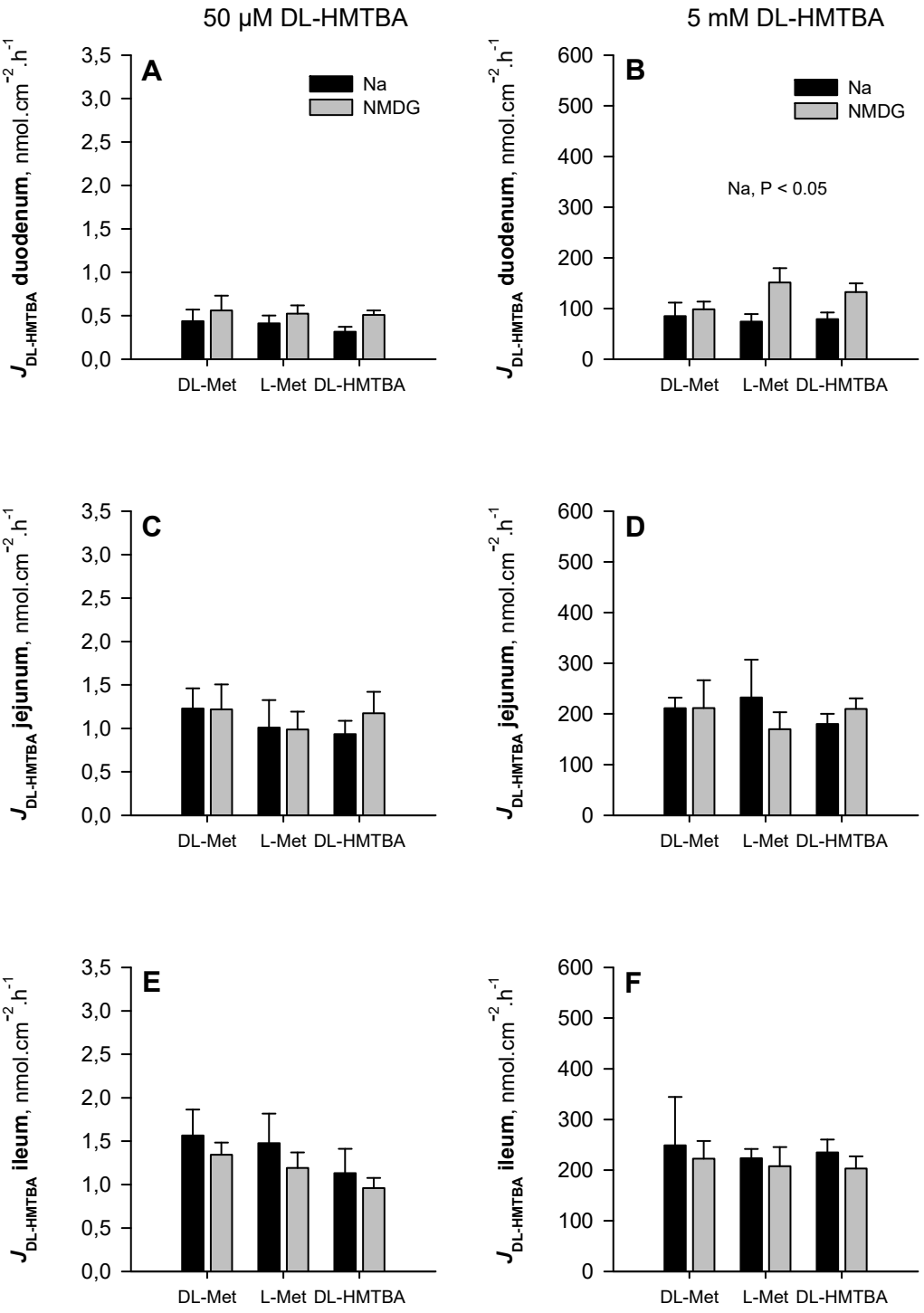

Figure 3: $J_{D/L\text{-}HMTBA}$ at a mucosal concentration of 50 µM D/L-HMTBA (A–C) or 5 mM D/L-HMTBA (D–F) in duodenum (A, D), jejunum (B, E), and ileum (C, F) in the mucosal presence of either Na^+ or $NMDG^+$.

Data represent means ± SEM of flux rates from n = 7 – 9 pigs fed diets supplemented with either D/L-Met, L-Met, or D/L-HMTBA. Data were compared using 2-factor ANOVA and a post-hoc Student–Newman–Keuls test. D/L-HMTBA, D/L-2-hydroxy-4-methylthiobutanoic acid; $J_{D/L\text{-}HMTBA}$, absorptive flux rate of D/L-HMTBA in the mucosal-to-serosal direction; Met, methionine; NMDG, N-methyl-D-glucamine.

In Publication 1, we compared the absorption capacity for D-Met and L-Met in the duodenum, jejunum and ileum. We showed that the absorption of D- and L-Met was lower in the duodenum at both low (50 µM) and high (5 mM) Met concentration. We performed the same analysis with the D/L-HMTBA flux rates (Table 1).

Table 1: Comparison of D/L-HMTBA absorption capacity in the duodenum, jejunum and ileum of pigs.

	Duodenum	***Jejunum***	***Ileum***	***P-Value***
Flux Rates				
50 µM D/L-HMTBA	0.46 ± 0.042[b]	1.09 ± 0.097[a]	1.26 ± 0.097[a]	< 0.001
5 mM D/L-HMTBA	102.87 ± 8.61[b]	202.23 ± 16.88[a]	222.26 ± 15.87[a]	< 0.001

Values represent mean flux rates ± SEM. Means were calculated irrespective of diet and irrespective of the presence of Na+. Data were compared using ANCOVA and a post-hoc Student-Newman-Keuls test. Different letters within one row indicate significant differences between intestinal sites at the same D/L-HMTBA concentration.

The ANCOVA showed that flux rates of D/L-HMTBA were lower in the duodenum when compared to the jejunum and ileum at both tested D/L-HMTBA concentrations. Jejunum and ileum showed similar D/L-HMTBA flux rates at low and high substrate concentrations.

4.2.3. Conclusion

The flux measurements of D/L-HMTBA showed that its absorption in the small intestine could not be influenced by different Met sources. The addition of sodium did not enhance D/L-HMTBA flux across the intestinal tissue in any tested segment; curiously, it seemed to lower the D/L-HMTBA flux in the duodenum. This effect reached statistical significance only at the higher D/L-HMTBA concentration. Interestingly, the duodenum showed overall significantly lower absorptive capacity for D/L-HMTBA when compared to the jejunum and to the ileum.

4.3. Publication 2

Expression of proposed methionine transporters along the gastrointestinal tract of pigs and their regulation by dietary methionine sources

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*Authors' contributions are listed in the Authors' contribution section of Publication 2.

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Genes & Nutrition

RESEARCH Open Access

Expression of proposed methionine transporters along the gastrointestinal tract of pigs and their regulation by dietary methionine sources

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Abstract

Background: Given the key role of methionine (Met) in biological processes like protein translation, methylation, and antioxidant defense, inadequate Met supply can limit performance. This study investigated the effect of different dietary Met sources on the expression profile of various Met transporters along the gastrointestinal tract (GIT) of pigs.

Methods: A total of 27 pigs received a diet supplemented with 0.21% DL-Met, 0.21% L-Met, or 0.31% DL-2-hydroxy-4-(methylthio)butanoic acid (DL-HMTBA). Changes in mRNA expression of B^0AT1, $ATB^{0,+}$, *rBAT*, *ASCT2*, *IMINO*, *LAT4*, y^+LAT1, *LAT2*, and *SNAT2* were evaluated in the oral mucosa, cardia, fundus, pylorus, duodenum, proximal jejunum, middle jejunum, ileum, cecum, proximal colon, and distal colon, complemented by protein expression analysis of B^0AT1, ASCT2, LAT2, and LAT4.

Results: Expression of all investigated transcripts differed significantly along the GIT. B^0AT1, *rBAT*, y^+LAT1, *LAT2*, and *LAT4* showed strongest mRNA expression in small intestinal segments. *ASCT2, IMINO*, and *SNAT2* were similarly expressed along the small and large intestines but expression differed in the oral mucosa and stomach. $ATB^{0,+}$ showed highest mRNA expression in large intestinal tissues, cardia, and pylorus. In pigs fed DL-Met, mRNA expression of *ASCT2* was higher than in pigs fed DL-HMTBA in small intestinal tissues and mRNA expression of *IMINO* was lower than in pigs fed L-Met in large intestinal tissues. Dietary DL-HMTBA induced a stronger mRNA expression of basolateral uptake systems either in the small (*LAT2*) or large (y^+LAT1) intestine. Protein expression of B^0AT1 was higher in the middle jejunum and ileum in pigs fed DL-Met when compared with the other Met supplements. LAT4 expression was higher in pigs fed DL-HMTBA when compared with DL-Met (small intestine) and L-Met (small intestine, oral mucosa, and stomach).

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Conclusion: A high expression of several Met transporters in small intestinal segments underlines the primary role of these segments in amino acid absorption; however, some Met transporters show high transcript and protein levels also in large intestine, oral mucosa, and stomach. A diet containing DL-Met has potential to increase apical Met transport in the small intestine, whereas a diet containing DL-HMTBA has potential to increase basolateral Met transport in the small intestine and, partly, other gastrointestinal tissues.

Keywords: Longitudinal heterogeneity of gene expression, Intestine, Methionine transport, qRT-PCR, Stomach, Western blot

Introduction

Methionine (Met) is an essential sulfur amino acid (AA), which must be provided by the diet because it cannot be synthesized *de novo* by the body [1]. Methionine plays several essential roles in cellular metabolism. It is a proteinogenic AA with special importance for the initiation of protein translation [2], a sulfur donor necessary to generate other sulfur-containing AA (cysteine and cystine), a main donor of methyl groups [3] and, finally, it influences the cellular redox state [4].

The optimum AA ratios in food or feed is a key element to ensure coverage of AA requirements with a minimum of protein intake [5, 6]. In animal production, reducing dietary protein levels is not only vital to reduce N excretion through urine and feces but also to lower greenhouse gas emissions [7, 8]. Additionally, the reduction of protein levels in the feed of livestock is a cost-reducing strategy [5, 9]. Therefore, providing adequate dietary methionine supply, as a main limiting essential AA in low crude protein diets, is crucial to ensure optimal growth and health with additional ecologic and economic benefits. Whereas humans rely primarily on naturally occurring L-Met from food materials, pig and poultry diets are often supplemented with L-Met, DL-Met, or a hydroxyl analogue DL-2-hydroxy-4-(methylthio)butanoic acid (DL-HMTBA) [10, 11]. Not only are the metabolism and utilization different for these Met sources; they also differ in their absorption mechanisms. Because HMTBA is a precursor without an amino group, it is not absorbed by AA transporters, but rather by sodium-dependent and sodium-independent monocarboxylate transporters, one prominent candidate of the latter being MCT1 [12, 13]. By sharp contrast, Met is mainly taken up via carrier-mediated systems that differ in their specificity for L- and D-isomers [11, 14]. The current knowledge on gastrointestinal Met transporters has been summarized by Mastrototaro et al. [14]. According to this proposed model, the apical transport systems are mainly Na^+-dependent (B^0AT1, $ATB^{0,+}$, ASCT2, IMINO) but are complemented by $b^{0,+}AT$ which is Na^+-independent. $b^{0,+}$ requires the heavy chain rBAT for membrane targeting; hence, expression of rBAT is considered critical for $b^{0,+}$/rBAT heterodimer function. Consequently, previous studies mostly analyzed the expression of subunit rBAT in preference over subunit $b^{0,+}$ [15–17]. On the basolateral side, the main transport system for Met efflux is represented by the Na^+-independent system L (LAT1, LAT2, and LAT4; where L stands for large neutral AA). Interestingly, the exact localization of LAT1 seems to be species-specific as it was located basolaterally in the chicken intestine and porcine kidney but apically in human intestine [12, 18]. System L is complemented by the Na^+-dependent transporters SNAT2 and y^+LAT1. SNAT2 takes up AA from blood in interdigestive phases, and y^+LAT1 mediates the Na^+-dependent influx of neutral AA (like Met) against an efflux of cationic AA [19–21].

The present study aimed to determine whether the supplementation of different dietary Met sources would modulate the distribution and the expression profile of presumed Met transporters along the porcine GIT. In a previous study on tissues from the same animals, we had identified increased transport of L- and partly D-Met in different small intestinal segments (duodenum, middle jejunum, and ileum) after feeding a DL-Met–containing diet [22].

Experimental procedures

Animals and diets

Diets and animal handling procedures have been described previously [22]. Briefly, 27 pigs (castrated male, Danbred x Piétrain) were used with an initial bodyweight of ~ 25 kg at ~ 10 weeks of age to assure the establishment of stable gastrointestinal health after weaning. Pigs were fed a basal diet deficient in standardized ileal digestible (SID) Met + Cys (0.46%) but adequate for all other AA. To meet Met + Cys requirements, the basal diet was supplemented with either 0.21% DL-Met (MetAMINO; Evonik Nutrition & Care GmbH, Essen, Germany; N = 9), 0.21% L-Met (Evonik Nutrition & Care GmbH, Essen, Germany; N = 9) or 0.31% DL-HMTBA (Novus International, Inc., Saint Charles, MO; N = 9). The higher dietary concentration of DL-HMTBA was used to account for its lower bioefficacy (approximately 70% compared with L-Met) [10]. Diets were provided for at least 10 days, after which pigs were euthanized for harvesting tissues. Dietary treatments

were blinded to the research investigators until gene expression data was summarized.

Tissue preparation

After euthanasia, tissue samples of all pigs were recovered from a total of 11 regions of the GIT for molecular analyses (quantitative real-time PCR and western blot) of Met transporter expression. Sections included four extraintestinal regions (oral mucosa as well as cardia, fundus, and pylorus of the stomach), four small intestinal regions (duodenum, proximal jejunum, middle jejunum, and ileum), and three large intestinal regions (cecum, proximal colon, and distal colon). The study targeted at selective quantification of epithelial transporters. Therefore, the tunica muscularis externa (longitudinal and circular muscle layers) was mechanically removed before collecting gastric and intestinal samples. Oral mucosa was harvested by perpendicular cutting of small tissue chips from the mucosal surface.

Tissues for transcript expression analysis were immersed in RNA*later*® (Sigma Aldrich, St Louis, MO, USA), stored at + 4 °C overnight and at − 20 °C thereafter until RNA isolation. Tissues for protein analysis were snap-frozen in liquid nitrogen and then stored at − 80 °C until analysis.

Gene expression analysis

Total RNA was extracted from the tissues using a commercial kit including a DNAse digestion step (Nucleospin RNA, Macherey & Nagel, Düren, Germany). Afterwards, all RNA samples were evaluated for quantity and purity using a lab-on-a-chip technique (RNA 6000 Nano Kit, Agilent, Waldbronn, Germany). Only samples with an RNA integrity number (RIN) > 6.5 were used for cDNA synthesis. Reverse transcription was performed with 1000 ng of RNA using iScript® cDNA synthesis kit (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions; reactions were then diluted to a final concentration of 5 ng/μL.

Changes in the relative expression of the Met transporters B^0AT1, $ATB^{0,+}$, *rBAT*, *ASCT2*, *IMINO*, *LAT2*, *LAT4*, y^+LAT1, and *SNAT2* were evaluated by qRT-PCR using intron-flanking or exon-spanning primers and double quenched probes synthetized by Eurofins MWG Operon (Ebersberg, Germany; for primer and probe sequences, see Table 1).

Table 1 Primer and probe sequences for the Met transporters and reference genes

Gene	Accession number	Primer	Sequence	Probe sequence
B^0AT1 (*SLC6A19*)	XM_003359855.4	Fwd	CTTCATCTTCACCCTGAACTC	CCCCTGCTCATCATCGCCTTCTTCGAGATGT
		Rev	GATGTCGCTGTTGAACCTG	
ATB^{0+} (*SLC6A14*)	NM_001348402.1	Fwd	CTGTGGCTTGGGGTGGTTTA	CCAACTCCCAGGTGGGCCAT
		Rev	AACCAAGCAGCAACCCAAAG	
rBAT (*SLC3A1*)	NM_001123042.1	Fwd	CAATGCAGTGGGACAACAG	TCCAAAAGACCCAGCCCAAATCAGCA
		Rev	GGCGTGAAGCAAACTTAATTC	
ASCT2 (*SLC1A5*)	XM_003127238.4	Fwd	CGATTCGTTCCTGGATCTTG	CTCCAACCTGGTGTCTGCAGCCTT
		Rev	TAGGACGTCGCGTATGAG	
IMINO (*SLC6A20*)	XM_003358406.4	Fwd	TCGTGTCCCTCATCAACAG	ACCTCCATCTTTGCCAGTGTCGTCACCTT
		Rev	AGGAAGCCATCTTCAAGGTC	
LAT4 (*SLC43A2*)	XM_003358191.3	Fwd	CAGATCCAGAAGATCACCAAC	TGACCTGCCTCATTCCCAACCTGC
		Rev	TGAAGGAGAGAATCTGTAGGG	
y^+LAT1 (*SLC7A7*)	NM_001110421.1	Fwd	CTCTGCTGTTCAATGGTCTC	GGCGCTGATCTACTTGTGCGTGGA
		Rev	ATAGAGCTGACCCACGATAG	
LAT2 (*SLC7A8*)	XM_003128550.5	Fwd	ACTACCTCTTCTATGGCATCAC	CGGACAGATAGTTCTTCGCTGGAAGAAGCCTAA
		Rev	GCAAGTAGATGATGGGGAACAG	
SNAT2 (*SLC38A2*)	XM_003126626.5	Fwd	TTCATTCTTCCATCTGCCTTC	GTTAAGTGGCATAGGGGTGATGACCGGA
		Rev	GGGCATTGTGTACCCAATC	
ACTB	XM_021086047.1	Fwd	GACATCAAGGAGAAGCTGTG	CTGGACTTCGAGCAGGAGATGGCC
		Rev	CGTTGCCGATGGTGATG	
GAPDH	XM_021091114.1	Fwd	CAAGAAGGTGGTGAAGCAG	TGAGGACCAGGTTGTGTCCTGTGACTTCAA
		Rev	GCATCAAAAGTGGAAGAGTG	
YWHAZ	XM_005662949.2	Fwd	AAGAGTCATACAAAGACAGCAC	ATCGGATACCCAAGGAGATGAAGCTGAA
		Rev	ATTTTCCCCTCCTTCTCCTG	

The qRT-PCR experiments used a 40-cycle two-step PCR protocol (20 s at 60 °C and 1 s at 95 °C) and were performed in a thermocycler (ViiA7, Applied Biosystems/Life Technologies, Foster City, CA, USA) with 4.5 μL of cDNA and three replicates per reaction. iTaq® Universal Probes Supermix (Bio-Rad Laboratories) in combination with the specific primers and probes was used as master mix in assay volumes of 10 μL. Thresholds were automatically calculated by the cycler software. Amplicons were validated by sequencing.

Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), and *β-actin* were tested for stable expression with geNorm, and all three were suitable and used as nonregulated reference genes. An inter-run calibrator (IRC), composed of a pool of 21 cDNA, was present on each plate and afterwards used as calibrator. The double delta C_t analysis was performed to analyze qRT-PCR data; so after normalization of Ct values with the reference genes, the normalized results were scaled to the calibrator to obtain the expression fold change of each sample relative to the IRC. Calibrated normalized relative quantities (CNRQ values) were used for statistical analysis.

Protein analysis

Proteins were isolated from ~ 200 mg of frozen tissue samples homogenized in 500 μL of RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, and 5 μL of Protease Inhibitor Mix G (Serva, Heidelberg, Germany). The samples were incubated on ice for 90 min and briefly shaken at 20,000 rpm for 1.5 min every 20 min by using a Mixer Mill (Retsch MM200, Hahn, Germany). Centrifugation (14,000 rpm, 4 °C, 30 min) was performed to a pellet-insolubilized material. The concentration of total extracted proteins was determined using the Pierce® 660-nm Protein Assay (ThermoFisher Scientific, Waltham, MA, USA). An aliquot of 15 μg total protein was resolved on an 8% SDS-polyacrylamide gel for LAT2, LAT4, and B^0AT1. The proteins for ASCT2 were loaded on a 10% TGX Stain-Free gel™ (Bio-Rad Laboratories). An IRC was loaded as reference sample on each gel.

Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc.), which was blocked with 5% dry milk in TBS + Tween (TBST, 50 mM Tris, 150 mM NaCl, 0.01% Tween-20, pH 7.6) for 2 h at room temperature. After blocking, immunoblotting was performed overnight at 4 °C with a primary rabbit antibody directed against B^0AT1 (SLC6A19, 1:1000; ABIN567031, Abnova Corp., Taipeh, Taiwan), mouse antibody against ASCT2 (SLC1A5, 1:1000; NBP1-89327 Novus biologicals, Littleton, CO), rabbit antibody against LAT2 (SLC7A8, 1:1000; ABIN2781629, Aviva Systems Biology, San Diego, CA, USA) or mouse antibody against LAT4 (SLC43A2, 1:1000; ABIN2781629 Abgent, San Diego, CA, USA). Primary mouse antibody specific for RPL19 (1:1500; Santa Cruz Biotechnology, Dallas, TX, USA) was used to quantify RPL19 as reference protein to control for loading efficiency for LAT2, LAT4, and B^0AT1. For ASCT2, total protein on the gel was quantified by the stain-free technology according to the manufacturer's instruction and used to correct for loading efficiency. After overnight incubation with the primary antibodies, the membranes were incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse, 1:1000; anti-rabbit, 1:2500; both from Cell Signaling Technology, Frankfurt, Germany). Proteins were visualized by use of the Clarity™ Western ECL Substrate (Bio-Rad Laboratories) and the Bio-Rad ChemiDoc™ MP Imaging System in combination with the software ImageLab 5.0 (Bio-Rad Laboratories), which allowed a densitometric analysis and a normalization of each band to the blackness of the respective lane (normalized intensities, NI). IRC was always assumed to have NI = 1; thus any other value represented the relative expression of the respective sample compared with IRC. Representative western blots are shown in Fig. 1.

Statistical analysis

The comparisons of mRNA or protein levels between all regions irrespective of the factor diet (Tables 2) were performed with IBM SPSS Statistics Version 26 (IBM, Armonk, NY, USA), using ANCOVA (Analysis of covariance) followed by post hoc Dunn's method (all pairwise multiple comparison), accounting for the covariate diet. The effect of diet on the expression of genes and proteins in comparable sets of regions (extraintestinal, small intestinal, and large intestinal regions) were subsequently compared by two-factor ANOVA with post hoc Student-Newman-Keuls' test (all pairwise multiple comparison) using the software SigmaPlot 11.0 (Systat Software, GmbH, Erkrath, Germany). Fixed factors were "diet" (DL-Met, L-Met, and DL-HMTBA) and "tissue". Results are given as means ± SEM. Differences of $P < 0.05$ were considered significant; trends are discussed if $0.05 \le P < 0.1$.

Graphs were plotted with SigmaPlot 11.0. *P* values for main factors and their interactions are listed in each graph. For clarity, however, only relevant *P* values < 0.1 are shown. If *P* values are not listed in a graph, they are ≥ 0.1.

Results

Effects of tissue and diet on mRNA expression

The regional distribution of Met transporters irrespective of the provided diet is shown in Table 2. Relative

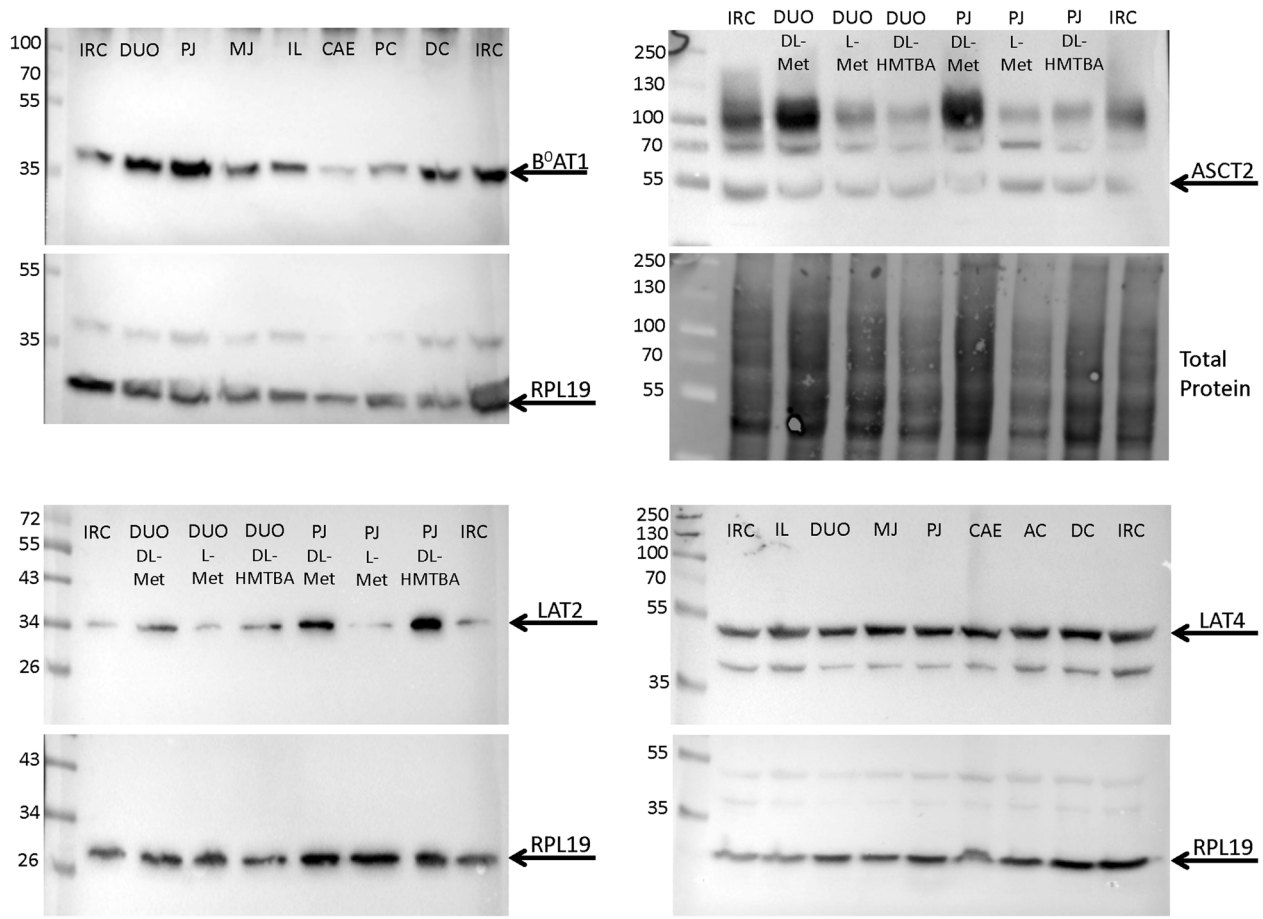

Fig. 1 Western blots of B^0AT1, ASCT2, LAT2, and LAT4 protein. Blots are representative of a total of 188 western blots evaluated for this study. Blots for B^0AT1, LAT2, and LAT4 were conventional western blots where specific signals were quantified relative to RPL19 as loading control. For ASCT2, stain-free technology was used and ASCT2 protein intensity was quantified relative to total protein. Tissues were grouped per animal on blots for B^0AT1 and LAT4, whereas tissues from the three feeding groups were compared on blots for LAT2 and ASCT2. The left lane in each blot shows the molecular weight marker. *IRC*, inter-run calibrator; *DUO*, duodenum; *PJ*, proximal jejunum; *MJ*, middle jejunum; *IL*, ileum; *CAE*, caecum; *PC*, proximal colon; *DC*, distal colon

expression data differed among the gastrointestinal sections for all investigated transcripts ($P < 0.001$). Of note, *B^0AT1*, *rBAT*, and *LAT4* had higher mRNA expression in the small intestinal segments compared with all other segments. *B^0AT1* had highest CNRQ values in the middle jejunum, *rBAT* in the duodenum and proximal jejunum, and *LAT4* in the proximal and middle jejuna. *y^+LAT1* showed highest CNRQ values in the middle jejunum and fundus, with intermediate CNRQ values in the duodenum, proximal jejunum, and ileum. *ASCT2*, *IMINO*, and *LAT2* had similar CNRQ values in most segments, except for a high CNRQ of *ASCT2* in the gastric fundus, gastric pylorus and distal colon, a comparatively low CNRQ of *IMINO* in the oral mucosa and gastric fundus, and a high CNRQ of *LAT2* in the gastric fundus and middle jejunum. *SNAT2* mRNA was highly expressed in the gastric fundus and pylorus. Expression of *$ATB^{0,+}$* appeared dominant in the large intestine with highest CNRQ values measured in the proximal and distal colons; intermediate values in the cecum, gastric cardia, gastric pylorus, and duodenum; and low expression in the jejunum, ileum, and gastric fundus. Oral mucosa was among the tissues with the weakest expression levels for all transporters tested ($P < 0.05$; Table 2).

As different gastrointestinal regions have different digestive functions, three functional subgroups with comparable functions were created to analyze the effect of diet on transporter expression: extraintestinal tissues (oral mucosa and the three gastric regions pylorus, cardia, and fundus), small intestinal tissues (duodenum, proximal jejunum, middle jejunum, and ileum), and large intestinal tissues (proximal colon, distal colon, and cecum).

Transport systems with supposedly apical and basolateral locations are shown in Figs. 2 and 3, respectively. The factor diet had no effect on any transporter in the

Table 2 mRNA expression of Met transporters among different intestinal and extraintestinal regions irrespective of the factor diet

Tissue	*B^{0}AT1*	*ATB^{0+}*	*rBAT*	*ASCT2*	*IMINO*	*y$^+$LAT1*	*LAT2*	*LAT4*	*SNAT2*
Oral mucosa	0.014 ± 0.006^{d}	0.48 ± 0.12^{cd}	0.019 ± 0.008^{d}	0.57 ± 0.15^{b}	0.065 ± 0.023^{b}	0.072 ± 0.016^{e}	0.14 ± 0.02^{d}	0.10 ± 0.02^{d}	0.69 ± 0.12^{c}
Cardia	0.023 ± 0.008^{d}	1.76 ± 0.25^{ac}	0.20 ± 0.03^{d}	0.74 ± 0.11^{b}	0.88 ± 0.09^{a}	0.37 ± 0.03^{ce}	0.49 ± 0.05^{bcd}	0.33 ± 0.07^{d}	0.92 ± 0.11^{bc}
Fundus	0.034 ± 0.011^{d}	0.040 ± 0.010^{d}	0.025 ± 0.005^{d}	1.74 ± 0.29^{a}	0.26 ± 0.04^{b}	1.51 ± 0.19^{ab}	2.23 ± 0.28^{a}	0.59 ± 0.07^{d}	1.59 ± 0.18^{a}
Pylorus	0.022 ± 0.005^{d}	1.09 ± 0.27^{bd}	0.025 ± 0.004^{d}	1.82 ± 0.30^{a}	0.75 ± 0.06^{a}	0.40 ± 0.04^{ce}	0.65 ± 0.08^{bcd}	0.23 ± 0.05^{d}	1.37 ± 0.13^{ab}
Duodenum	0.55 ± 0.08^{c}	1.04 ± 0.61^{bd}	2.34 ± 0.38^{ab}	0.80 ± 0.13^{b}	0.85 ± 0.06^{a}	1.06 ± 0.13^{bc}	0.64 ± 0.07^{b}	1.24 ± 0.19^{b}	0.70 ± 0.07^{c}
Proximal jejunum	0.91 ± 0.11^{bc}	0.43 ± 0.08^{cd}	3.13 ± 0.87^{a}	0.71 ±0.12^{b}	0.91 ± 0.11^{a}	1.12 ± 0.20^{b}	0.64 ± 0.09^{bcd}	1.92 ± 0.24^{a}	0.65 ± 0.06^{c}
Middle jejunum	1.73 ± 0.21^{a}	0.15 ± 0.06^{d}	1.31 ± 0.86^{c}	0.63 ± 0.11^{b}	0.96 ± 0.08^{a}	2.11 ± 0.36^{a}	2.02 ± 0.33^{a}	1.81 ± 0.32^{ab}	0.62 ± 0.06^{c}
Ileum	1.13 ± 0.12^{b}	0.21 ± 0.03^{d}	1.56 ± 0.25^{bc}	0.52 ± 0.08^{b}	1.04 ± 0.13^{a}	0.91 ± 0.16^{bcd}	0.63 ± 0.07^{bc}	1.32 ± 0.15^{ab}	0.85 ± 0.17^{bc}
Cecum	0.010 ± 0.002^{d}	1.60 ± 0.35^{ac}	0.17 ± 0.04^{d}	0.83 ± 0.13^{b}	0.79 ± 0.09^{a}	0.26 ± 0.02^{de}	0.28 ± 0.02^{cd}	0.28 ± 0.03^{d}	0.97 ± 0.11^{bc}
Proximal colon	0.026 ± 0.008^{d}	2.34 ± 0.30^{ab}	0.093 ± 0.020^{d}	0.81 ± 0.13^{b}	0.95 ± 0.16^{a}	0.28 ± 0.02^{de}	0.43 ± 0.05^{bcd}	0.27 ± 0.04^{d}	1.05 ± 0.14^{ac}
Distal colon	0.013 ± 0.003^{d}	2.82 ± 0.60^{a}	0.10 ± 0.02^{d}	1.17 ± 0.23^{ab}	1.04 ± 0.19^{a}	0.33 ± 0.03^{de}	0.55 ± 0.08^{bcd}	0.32 ± 0.04^{d}	0.84 ± 0.13^{bc}
***P* value**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

$^{a–e}$Gene expressions within one column are different at $P < 0.05$ if they do not share a common letter.

extraintestinal tissues. In selected intestinal tissues, the expression of *rBAT*, *ASCT2*, *IMINO*, *y$^+$LAT1*, and *LAT2* was affected by the factor diet. A diet containing DL-Met increased *ASCT2* gene expression across all small intestinal tissues compared with a diet containing DL-HMTBA ($P < 0.05$; Fig. 2). A diet containing L-Met induced a stronger expression of *IMINO* across large intestinal tissues compared with DL-Met ($P < 0.05$; Fig. 2). The diet containing DL-HMTBA increased the expression of the basolateral exchange systems *LAT2* in the small and *y$^+$LAT1* in the large intestines ($P < 0.05$; Fig. 3). It also caused an increase in the expression of *rBAT* selectively in the proximal jejunum ($P < 0.05$) as evidenced by a diet × tissue interaction ($P < 0.05$; Fig. 2).

The two-factorial statistical evaluation of regions further supported the differences among tissues that had already been identified by ANCOVA (cf. Table 2). Additional findings were that cardia had a higher expression of *ATB$^{0,+}$* and *rBAT* than other extraintestinal regions; in the small intestine, the duodenum had highest expression of *ATB$^{0,+}$*, distal colon had higher expression of *LAT2* than caecum, and that expression patterns of *LAT2* and *LAT4* showed clear regional differences in extraintestinal tissues (Fig. 3).

Effects of tissue and diet on protein expression

Selected transporters with assumed high relevance for apical Met uptake (B^{0}AT1 and ASCT2) or Met basolateral efflux (LAT2 and LAT4) were further investigated on the protein level by western blot. As for mRNA, data from the three feeding groups were initially compared across all tissues by ANCOVA accounting for diet as covariate. Protein expression levels of all investigated proteins were significantly different along the GIT ($P < 0.001$; Table 3.

Protein expression of B^{0}AT1 was highest in the ileum and gastric cardia and lowest in the gastric fundus. Protein expression of ASCT2 was highest in parts of the large intestine, with lowest levels in all the three gastric regions and proximal jejunum. Protein expression of LAT2 was remarkably high in the oral mucosa and gastric cardia, whereas the pylorus, middle jejunum, and cecum had the lowest expression values. Protein expression of LAT4 was strikingly high in the oral mucosa with the lowest values in gastric fundus (Table 3).

The data were further divided into the three regional subgroups (similar to mRNA data) in order to investigate whether the diet has an effect on the protein expression in tissues of comparable physiological functions (Fig. 4). When comparing the expression of B^{0}AT1 within each regional subgroup, protein levels in the small intestine were significantly affected by diet ($P < 0.001$) and tissue ($P < 0.001$) with significant interaction of the two factors ($P < 0.05$). The basis for interaction were higher B^{0}AT1 protein levels in pigs fed DL-Met compared with L-Met or DL-HMTBA in the middle

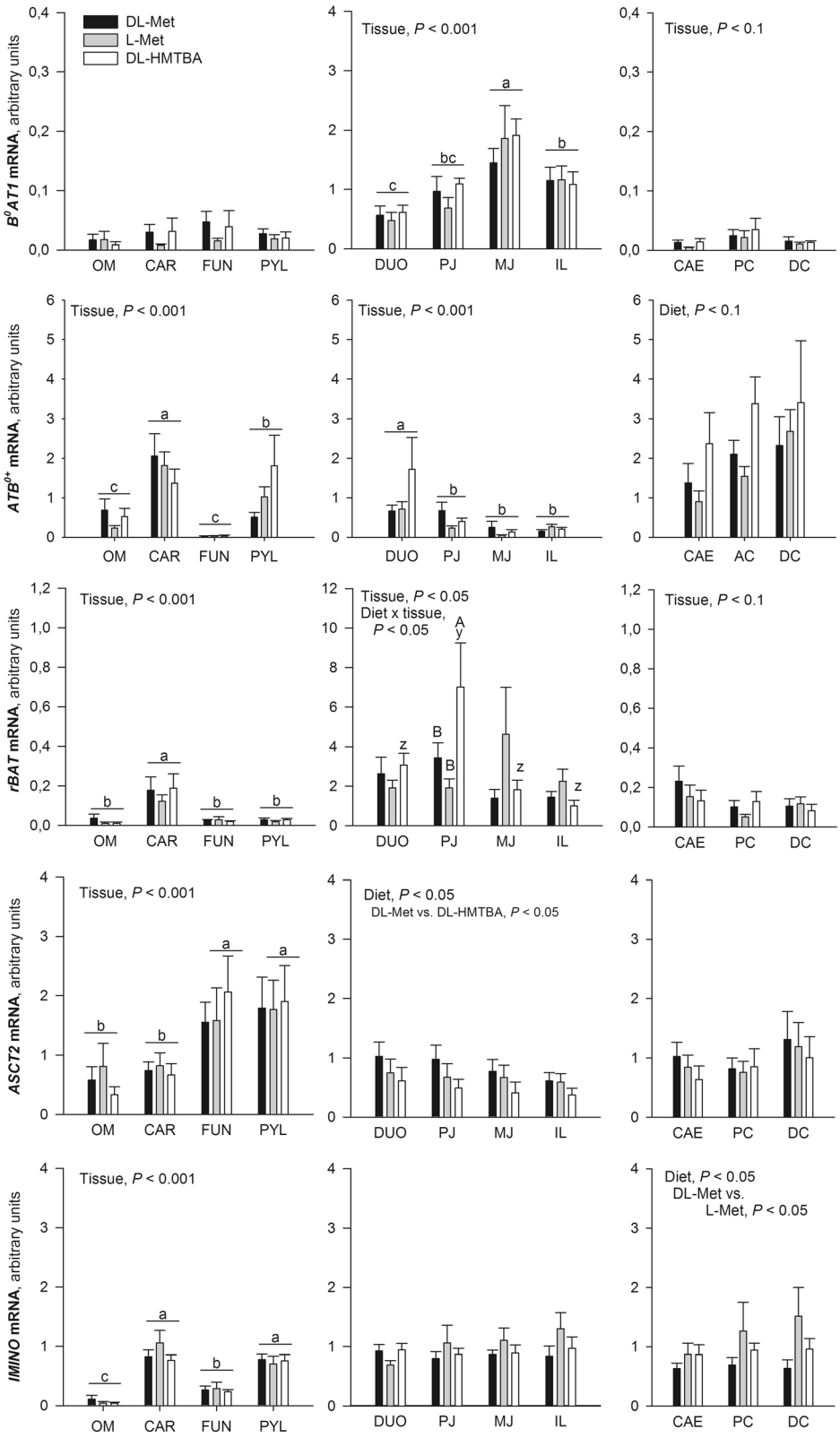

Fig. 2 (See legend on next page.)

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Fig. 2 Analysis of Met transporter expression with proposed apical localization along the gastrointestinal tract using qRT-PCR. Data was compared with 2-way-ANOVA for the factors "Tissue", "Diet", and their interaction "Tissue x Diet". Significant factor effects are mentioned in each graph. If column groups or columns do not share a common small letter within one graph, their expression values are either different irrespective of diet$^{(a-c)}$ or within a given diet$^{(y,z)}$ ($P < 0.05$). ^{A,B}Different capital letters indicate diet effects within a given tissue. *OM*, oral mucosa; *CAR*, cardia; *FUN*, fundus; *PYL*, pylorus; *DUO*, duodenum; *PJ*, proximal jejunum; *MJ*, middle jejunum; *IL*, ileum; *CAE*, cecum; *PC*, proximal colon; *DC*, distal colon

jejunum and ileum only ($P < 0.05$). In the extraintestinal regions, pigs fed DL-Met or L-Met tended to show higher B^0AT1 protein levels when compared with DL-HMTBA ($P < 0.1$; Fig. 4). Among the large intestinal regions, two-way ANOVA identified higher B^0AT1 expression in distal colon than the cecum—an effect that had not been significant with the all-tissue ANCOVA.

No diet effects were identified for ASCT2 protein (Fig. 4), which was unexpected because feeding a diet containing DL-Met had induced higher ASCT2 mRNA abundance in small intestinal segments (Fig. 2). Nonetheless, when only the proximal jejunum, middle jejunum, and ileum were tested in a two-way ANOVA, the effect of diet on ASCT2 protein was significant ($P < 0.01$) with higher expression in DL-Met–fed pigs compared with the other two groups.

No diet effects were observed for protein expression of LAT2 (Fig. 4). However, an effect of diet was observed for LAT4 in extraintestinal regions and small intestine ($P < 0.05$) and, as a trend also in the large intestine ($P < 0.1$). Pigs fed DL-HMTBA showed or tended to show highest LAT4 protein levels compared with pigs fed L-Met ($P < 0.05$ in extraintestinal and small intestinal tissues) and partly DL-Met ($P < 0.05$ in small intestinal tissues; Fig. 4).

Discussion

The present study intended to investigate the longitudinal heterogeneity of the expression of AA transporters along the GIT of pigs and to elucidate a possible impact of Met supplementation on the expression of these transporters. The effect of targeted Met supplementation on the expression of gastrointestinal Met transporters has not been investigated previously, except for a study in broiler chicken where a trend for higher mRNA expression of $ATB^{0,+}$ and B^0AT1 was observed in the ileum of L-Met– and DL-Met–supplemented versus non-supplemented control chickens [23]. Upon supplementation of a DL-Met–containing diet, we had observed an increased absorption of L-Met in the small intestinal segments (duodenum, middle jejunum, and ileum) and the induction of Na-dependent L-Met absorption in the middle jejunum for the same pigs used in the present study [14]. Therefore, the effects of the DL-Met–containing diet on transporter expression were of special interest.

Regarding the longitudinal distribution of Met transporters, it may appear somehow surprising that all investigated transporters were detectable in all investigated segments despite rather different physiological functions of these segments. The accepted textbook knowledge is

Table 3 Protein expression data of selected methionine transporters among different intestinal and extraintestinal regions irrespective of the factor diet

Tissue	B^0AT1	ASCT2	LAT2	LAT4
Oral mucosa	0.60 ± 0.11^{cd}	1.70 ± 0.26^{ac}	4.13 ± 0.44^{b}	7.50 ± 0.70^{a}
Cardia	1.41 ± 0.25^{ab}	0.78 ± 0.14^{c}	7.45 ± 1.70^{a}	0.98 ± 0.23^{bc}
Fundus	0.26 ± 0.05^{d}	0.62 ± 0.16^{c}	2.89 ± 0.64^{bc}	0.11 ± 0.03^{c}
Pylorus	0.67 ± 0.12^{cd}	0.77 ± 0.12^{c}	0.79 ± 0.14^{d}	1.00 ± 0.29^{bc}
Duodenum	0.79 ± 0.12^{bd}	0.99 ± 0.19^{ac}	2.09 ± 0.50^{cd}	1.02 ± 0.12^{bc}
Proximal jejunum	0.84 ± 0.12^{bd}	0.72 ± 0.10^{c}	1.60 ± 0.38^{cd}	0.93 ± 0.13^{bc}
Middle jejunum	0.67 ± 0.08^{cd}	0.87 ± 0.12^{bc}	0.73 ± 0.14^{d}	1.30 ± 0.22^{bc}
Ileum	1.92 ± 0.28^{a}	1.13 ± 0.20^{ac}	2.66 ± 0.64^{bd}	1.01 ± 0.17^{bc}
Caecum	0.48 ± 0.12^{cd}	1.99 ± 0.34^{ab}	0.91 ± 0.29^{d}	1.39 ± 0.20^{b}
Proximal colon	0.83 ± 0.12^{bd}	1.09 ± 0.16^{ac}	1.74 ± 0.35^{cd}	1.09 ± 0.18^{bc}
Distal colon	1.01 ± 0.14^{bc}	2.01 ± 0.35^{a}	2.07 ± 0.42^{cd}	0.87 ± 0.17^{bc}
***P* value**	<0.001	<0.001	<0.001	<0.001

$^{a-d}$Protein expressions within one column are different at $P < 0.05$ if they do not share a common letter.

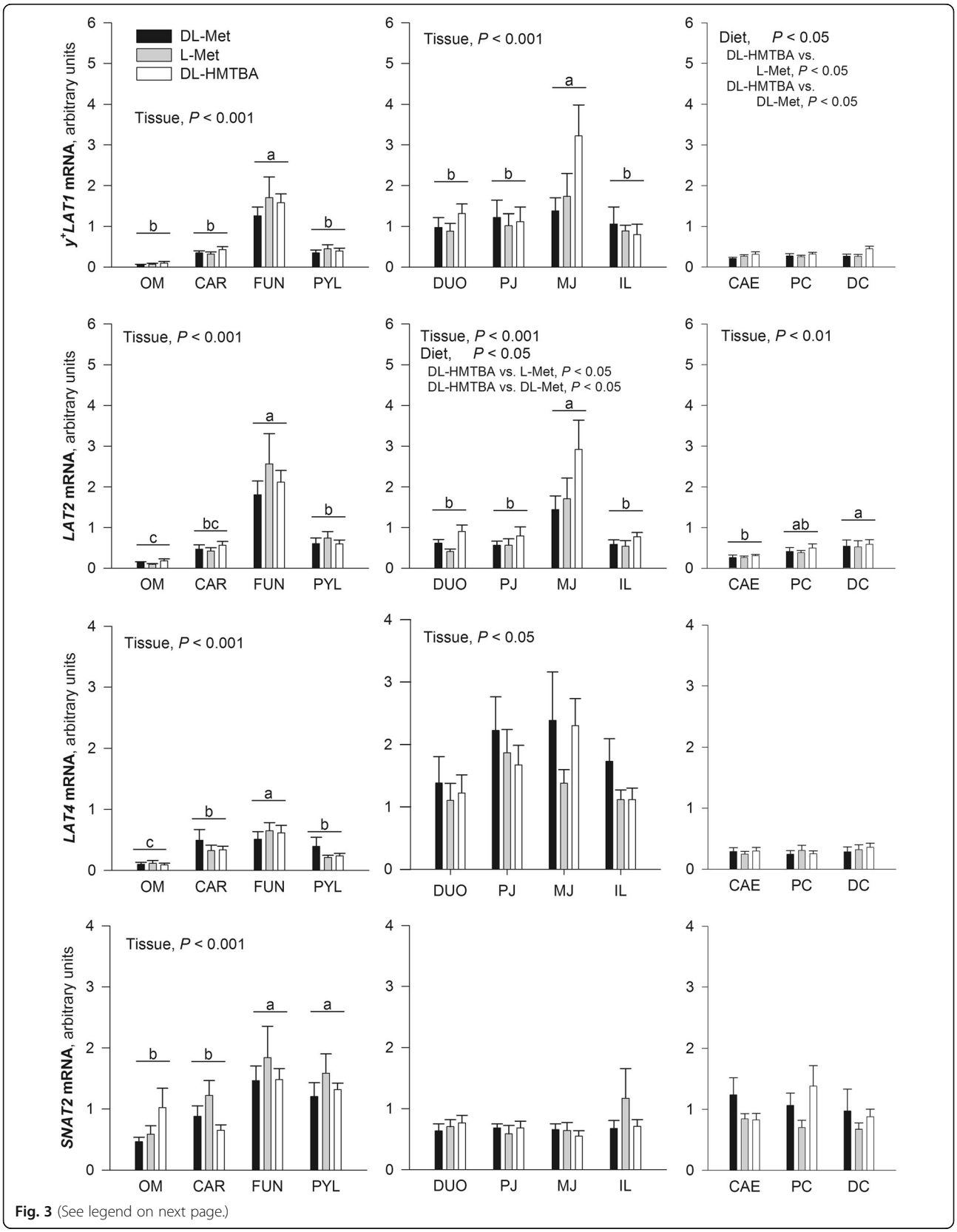

Fig. 3 (See legend on next page.)

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Fig. 3 Analysis of Met transporter expression with proposed basolateral localization along the gastrointestinal tract using qRT-PCR. Data was compared with 2-way-ANOVA for the factors "Tissue", "Diet", and their interaction "Tissue x Diet". Significant factor effects are mentioned in each graph. $^{a–c}$ Expression values among tissues within one graph (irrespective of diet) are different if they do not share a common letter ($P < 0.05$). For *LAT4*, multiple comparison could not identify differences in the small intestine despite of a significant effect of tissue. *OM*, oral mucosa; *CAR*, cardia; *FUN*, fundus; *PYL*, pylorus; *DUO*, duodenum; *PJ*, proximal jejunum; *MJ*, middle jejunum; *IL*, ileum; *CAE*, cecum; *PC*, proximal colon; *DC*, distal colon

that primarily the small intestine has relevance for AA absorption in mammals [24–27]. It has been shown specifically in pigs that their colon is also able to actively transport Met immediately after birth; however, this transport capability fades away in the first 10 days of life [28]. Nonetheless, several experimental findings provide indirect hints for a possible capacity to absorb AA also from the large intestine of adult mammals [29]. These hints include the appearance of microbial-derived AA nitrogen in the circulation and metabolism of pigs, humans and other non-ruminant mammals [30, 31], and the preferential presence of certain apical AA transporters like $ATB^{0,+}$ [32, 33] and ASCT2 [34] in the large intestine of mice. As these transporters accept D-AA as substrates [32, 35], it may be assumed that they have a specific role in the recovery of D-AA from bacterial metabolism [32]. Nonetheless, a final proof for quantitatively relevant absorption of AA from the large intestine is still missing in mammals, including pigs and humans. Similarly, a final proof for the relevance of apically located AA transporters in stomach and oral mucosa is missing, although the presence of all Na^+-dependent apical Met transporters investigated in the current study (B^0AT1, $ATB^{0,+}$, IMINO, and ASCT2) had been demonstrated in stomach, at least at the mRNA level, already in previous studies [14].

Coherent with a primary role of the small intestine in AA absorption, expression of *B^0AT1* and *rBAT* mRNA was rather low in most extraintestinal and large intestinal tissues in the present study. However, partly coherent with the just cited literature findings, *ASCT2* mRNA was highest in gastric fundus and pylorus among all investigated tissues, *IMINO* mRNA showed values in the cardia, pylorus, and large intestine that were comparable to those of the small intestinal segments, and *$ATB^{0,+}$* mRNA was highest in the large intestinal segments, gastric cardia, and pylorus. On the protein level, B^0AT1 was highest in the ileum; however, the cardia also showed a comparably high expression. For ASCT2 protein, highest values were observed in the oral mucosa, cecum, and distal colon.

The Na^+-dependent carrier B^0AT1 has previously been termed the Met-preferring system and is postulated to be a main carrier for Met absorption from the GIT of mammals [14]. Supporting its major role in AA absorption, we found transcripts predominantly in the small intestine with highest levels in the middle jejunum. Although *B^0AT1* mRNA levels were not influenced by the feeding regimen, B^0AT1 protein levels were regulated post-transcriptionally by the diet with a tissue × diet interaction in the small intestine. Pigs fed DL-Met showed a stronger B^0AT1 protein expression in the middle jejunum and ileum. This is in partial support of a functional induction of a Na^+-dependent transporter in the middle jejunum observed in our recent study on tissues of the same animals [22] and also partly confirms the previous results obtained in chickens receiving an L-Met– or DL-Met–supplemented diet [23]. It may thus be speculated that the increased expression of B^0AT1 protein upon feeding a DL-Met–containing diet may have functional significance.

$ATB^{0,+}$ has repeatedly been cited as a very important Met carrier in several species [36, 37]. Its expression in pigs is subject to controversy because a previous study was unable to detect $ATB^{0,+}$ on a functional level in the porcine jejunum [38, 39]. Nonetheless, another study observed *$ATB^{0,+}$* mRNA expression in the small intestine and stomach of White Duroc × Chinese Erhualian pigs [40]. In our study, the levels of *$ATB^{0,+}$* mRNA were comparatively low in the small intestine (except for the duodenum) when compared with areas in the stomach and especially to the large intestine. This partly confirms another study in mice where $ATB^{0,+}$ protein was predominantly expressed in the large intestine and, to a lower extent, also in the distal parts of the small intestine. The authors speculated that transport of Met and other AA into the intestinal epithelial cells might not be the primary function of $ATB^{0,+}$ as most AA coming from feed intake are not present in the digesta this far in the intestinal canal [32]. As stated earlier, however, other scientists considered a functional relevance of transporters like $ATB^{0,+}$ for large intestinal absorption of amino acid derived from microbial metabolism [29–31]. Dietary upregulation of *$ATB^{0,+}$* mRNA by a DL-Met or L-Met–containing diet, as previously observed in chicken [23], could not be identified in the present study. However, a trend for diet effect in the large intestine mRNA data indicates a possibility of dietary upregulation of $ATB^{0,+}$ by DL-HMTBA supplementation in the large intestinal segments. This finding appears concordant with the work of Malik et al. who showed that DL-HMTBA is available in the digesta further down the

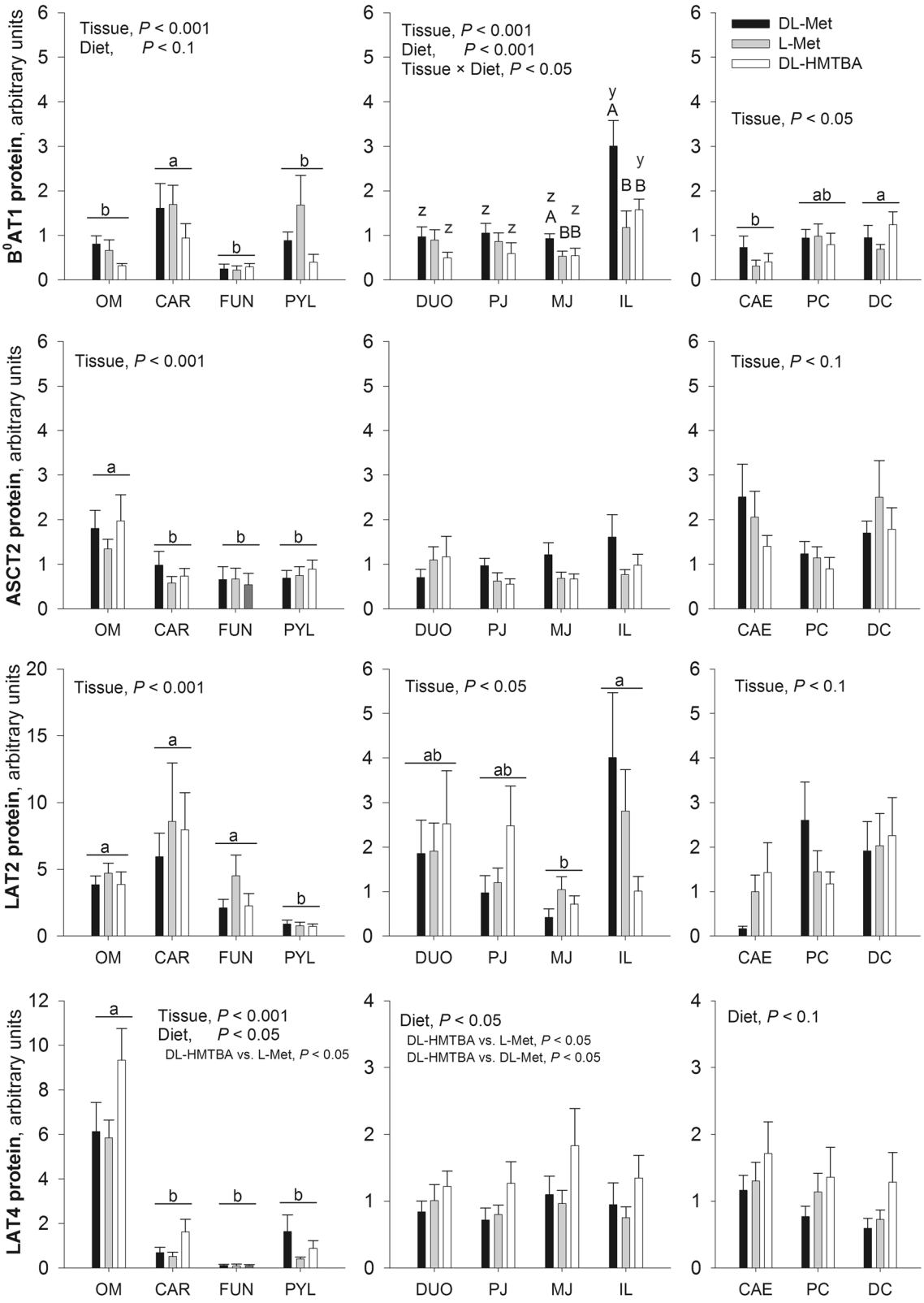

Fig. 4 (See legend on next page.)

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Fig. 4 Western blot analysis of B^0AT1, ASCT2, LAT2, and LAT4 in the intestinal and extraintestinal regions of pigs fed three different diets. Each value is the mean of 9 pigs and represents the relative expression to the IRC (IRC = 1). Data was compared with two-way ANOVA for the factors "Tissue", "Diet", and their interaction "Tissue × Diet". Significant factor effects are mentioned in each graph. If column groups or columns do not share a common small letter within one graph, their expression values are either different irrespective of diet$^{(a-c)}$ or within a given diet$^{(y,z)}$ ($P < 0.05$). ^{A,B}Different capital letters indicate diet effects within a given tissue. *OM*, oral mucosa; *CAR*, cardia; *FUN*, fundus; *PYL*, pylorus; *DUO*, duodenum; *PJ*, proximal jejunum; *MJ*, middle jejunum; *IL*, ileum; *CAE*, cecum; *PC*, proximal colon; *DC*, distal colon

lower intestine compared with Met [41]. Thus, DL-HMTBA is still present in the lumen of the large intestine and its possible microbial conversion to Met could potentially be associated with an upregulation of $ATB^{0,+}$ in this portion of the gastrointestinal tract.

The Na^+-independent transporter $b^{0,+}$/rBAT has been postulated as a main uptake system for L-Met in Caco2 cells [42]. As mentioned earlier, *rBAT* mRNA expression was analyzed representatively for the expression of the $b^{0,+}$/rBAT heterodimer in the present study. Transcripts of *rBAT* were also expressed highest in the small intestine, similar to B^0AT1, suggesting the possibility of functional relevance in these tissue segments. In the proximal jejunum, mRNA expression of *rBAT* was increased by a diet containing DL-HMTBA; however, no difference was observed in other segments of the small intestine. Furthermore, as we did not observe increased Na^+-independent methionine transport in the duodenum after supplementation of a DL-HMTBA–supplemented diet in our previous study [22], the functional significance of this finding remains to be determined.

The IMINO and ASCT systems have been characterized as uptake systems with low affinity for L-Met [34, 42]. They have been described as strongly expressed in the small intestine [14]. In the present study, the transcript levels of *IMINO* and *ASCT2* were rather similar in the small and large intestines. Additionally, ASCT2 protein levels showed only moderate variation across small and large intestinal segments with highest levels in the cecum and distal colon. Of note, a diet effect was observed for *ASCT2* mRNA in all small intestinal segments with highest values observed in pigs receiving the DL-Met–containing diet. Although the ASCT2 protein pattern almost mirrored its mRNA pattern in the proximal jejunum, middle jejunum and ileum, this effect did not penetrate towards statistical significance at the protein level when tested for all four small intestinal segments. When tested for only the proximal jejunum, middle jejunum and ileum, however, a DL-Met–containing diet significantly upregulated ASCT2 protein expression, which was coherent with the observed changes in mRNA expression. It was further coherent with the induction of a Na^+-dependent transporter by the DL-Met diet observed in the middle jejunum of these pigs in our companion study [22].

The basolateral exit of Met is mainly mediated by a single uniport system, LAT4. The other transport systems known to accept Met on the basolateral side of enterocytes either operate as exchange proteins (LAT1, LAT2, y^+LAT1) or mediate Na^+-dependent basolateral import of Met from blood (SNAT1, SNAT2) [14]. In the present study, we evaluated transcripts (y^+LAT1, *LAT2*, *LAT4*, and *SNAT2*) and proteins (LAT2 and LAT4) of basolateral transporters involved in Met shuttle. It needs to be acknowledged that y^+LAT1 and LAT2 have been suggested to have a function in methionine reentry from the blood into the intestinal epithelial cell [14, 43]. LAT1 and LAT2 transporters do not contribute to a net flux of AA since they only exchange abundant AA for less abundant AA [19]. Because of this characteristic, it is not surprising that LAT2 is also expressed in cells that have no absorptive function like Paneth's cells [44]. Transporter y^+LAT1 exchanges intracellular cationic AA against extracellular neutral AA like Met. Only LAT4 transports selected AA (L-Met, L-leucine, L-isoleucine, and L-phenylalanine) solely by concentration gradient, functioning as a symmetrical uniporter [45, 46].

The expression of mRNA or protein of basolateral Met transporters was not increased by dietary supplementation with either DL-Met or L-Met. However, DL-HMTBA supplementation enhanced gene expression of y^+LAT1 in large intestinal tissues and LAT2 in small intestinal tissues, as well as protein expression of LAT4 in small intestinal tissues, extraintestinal tissues, and, as a trend, in large intestinal tissues. A similar stimulating effect of DL-HMTBA on basolateral Met transport was suggested in a previous study in chickens [47]. Of note, the increased expression of basolateral transport systems was apparently not associated with increased transepithelial Met absorption as the latter was not stimulated by HMTBA in our previous study [22]. This argues against a rate-limiting role of basolateral Met transporters for transepithelial Met absorption as postulated earlier [14] based on studies in Caco2 cells [48]. Another study showed that even with feeding DL-HMTBA, the first pass utilization of Met remains at a constant proportion of about 30% [49, 50], indicating that basolateral transporters do probably not create an intracellular trap for Met in pigs as long as dietary Met concentrations are within requirement ranges.

Methionine metabolism in enterocytes plays a substantial role in the gastrointestinal absorption of Met. It is estimated that 20–30% of dietary Met is directly metabolized in intestinal epithelial cells [49, 50]. Dietary supplementation of DL-HMTBA is often seen as a way to avoid this first pass metabolism because HMTBA passes the intestinal epithelial cell largely unchanged and is only later converted into Met in the liver [51, 52]. However, such interpretation does not hold true. At least for pigs, HMTBA is absorbed from the intestinal lumen more slowly than L-Met and, thus, has a greater loss to intestinal bacterial degradation (as part of the first pass metabolism), associated with a lower amount of HMTBA absorption [41]. The latter may explain lower plasma Met concentrations by dietary HMTBA supplementation compared with Met supplementation [53]. Thus, it may be speculated that dietary DL-HMTBA supplementation induces a higher expression of basolateral Met "recovery systems" in order to compensate the lower dietary Met levels and to enhance the exchange of Met with other AA.

An interesting finding of the present study was that mRNA expression of two transporters, namely B^0AT1 and LAT2, was higher in the middle jejunum compared with the ileum, whereas their protein expression pattern was inverse. Bringing this data together with our previous flux study, it conformed functionally with higher absorptive capacity for L-Met in the ileum compared with the middle jejunum, at least at higher L-Met concentrations [22]. A quite similar finding was reported earlier for the glucose transporter GLUT2. Despite lower mRNA levels in the distal ileum compared with the middle jejunum, GLUT2 protein abundance was higher in the distal ileum than the middle jejunum [54]. Considering these findings together, it is tempting to suggest that a higher absorptive capacity for nutrients in the ileum of pigs compared with their middle jejunum originates partly from differences in the translation or turnover of certain nutrient transporters.

The present study was performed in pigs; however, Met supplements gain increasing popularity also in human nutrition [14]. As humans rely on L-Met supplements almost exclusively, it is desirable to explore in model animals whether other Met supplements may have nutritional benefits beyond those of L-Met. Pigs are an ideal model for humans [55]. Despite some minor differences, the anatomical and physiological similarities between the porcine and human intestines are striking and far superior when compared with rodent models or other non-rodent species [56]. Gut microbiota and nutrient digestibility of pigs show great resemblance to the human intestine [57]. The high correlation of ileal amino acid digestibility and the similarity in eating habits between pigs and humans make pigs a useful model for, especially, protein digestion in humans [58]. As such, the present results have a high potential to be transferable to man, suggesting that the type of Met supplementation may affect transporter expression and absorptive efficiency for Met and potentially other amino acids.

Conclusion

The present study showed that known Met transporters have a distinct longitudinal pattern of expression along the different sections of the GIT in pigs. A high expression of several Met transporters in small intestinal segments underlines the primary role of these segments in AA absorption. However, some transporters showed rather high expression in segments that do not have a proven role in AA absorption (extraintestinal and large intestinal tissues). Dietary Met source changed the expression of some transport systems. From these changes, it may be extrapolated that a diet containing DL-Met has potential to increase apical Met transport in the small intestine, which is congruent with recent functional findings of our group. On the other hand, a diet containing DL-HMTBA has potential to increase basolateral Met transporters in the small intestine and, partly, other gastrointestinal tissues. However, this was not previously found to improve functional methionine absorption and may be a result of a system compensation for lower free Met to be used for epithelial cell metabolism or as an exchange molecule for transport of other AA. Overall, the degree of regulation appeared small to moderate and, likely attributable to this fact, changes in mRNA expression did not clearly correlate with changes in protein expression. Importantly, the small changes in mRNA (ASCT2) and protein expression (B^0AT1) of apical Na^+-dependent transporters in the present study cannot explain the *de novo* induction of a Na^+-dependent uptake system in the mid jejunum identified in our previous study upon feeding a DL-Met–containing diet [22]. Therefore, further functional studies on intestinal Met absorption should complement the current findings, including investigations on post-translational mechanisms that possibly regulate AA absorption. A comprehensive knowledge on transcriptional, translational, and posttranslational regulation of AA absorption will greatly enhance our understanding of AA absorption in animals and eventually humans.

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Authors' contributions

The authors' contributions to the manuscript were as follows: LM, SR, and JRA designed the research; LM and SR conducted research; RP and JKH designed the feeding; RP and JZ took responsibility for all aspects around

animals and animal care; LM, SR, and JRA analyzed the data; SR, LM, and JRA wrote the paper. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval
Experiments were conducted following the approval of the local authorities responsible for animal care and use, the "Landesamt für Gesundheit und Soziales Berlin" (LaGeSo Berlin, Germany; Registration No. T 0264/15).

Consent for publication
Not applicable

Competing interests
JKH and RAW are employees of Evonik Operations GmbH. This fact had no influence on the acquisition and interpretation of data. All other authors have no conflict of interest to declare.

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4.4. Monocarboxylate transporters

The previous phase of this study showed that feeding D/L-Met, L-Met or D/L-HMTBA resulted in altered transcript levels of several AA transport systems in different sections of the porcine intestine. The data presented in Publication 2 (Romanet et al. 2021) concentrated on Met-associated transport systems. The main transport protein alleged to accept HMTBA as a substrate is MCT1, probably with the cooperation of MCT4 (To et al. 2021; Mastrototaro et al. 2016; Martin-Venegas et al. 2007). Therefore, their transcript expression in the porcine intestine depending on the fed Met supplement was of interest and was addressed with the following analyses.

4.4.1. Materials and Methods

Diet and animal handling are described in Publication 1 (Romanet et al. 2020). Methods for tissue preparation, gene expression measurement and statistical analysis are outlined in Publication 2 (Romanet et al. 2021).

Table 2 shows the primer and probe sequences used in the RT-qPCR experiments.

Table 2: Primers and probe sequences for MCT1 and MCT2.

		Primer and probe
MCT1	Fwd	CTGATGGACCTTGTTGGA
	Rev	TGTCATTGAGACGACCTAAA
	Probe	TCTCCAGTGCTGTGGGATTGGTGA
MCT4	Fwd	TGGACAGGTACCCTTGTATTA
	Rev	GGAGACAAACTGCTACCTTTAT
	Probe	TGGAGCTATCGCATTGCATTTGGTGC

4.4.2. Results

The mean expression values of MCT1 and MCT4 of 9 pigs for each gene in the different intestinal regions are summarized in the following figures. *P*-values obtained in the two-factor Anova for the factors “feeding” and “tissue” are shown next to the corresponding graph in Figure 4. For clarity, only *P*-values < 0.1 are shown.

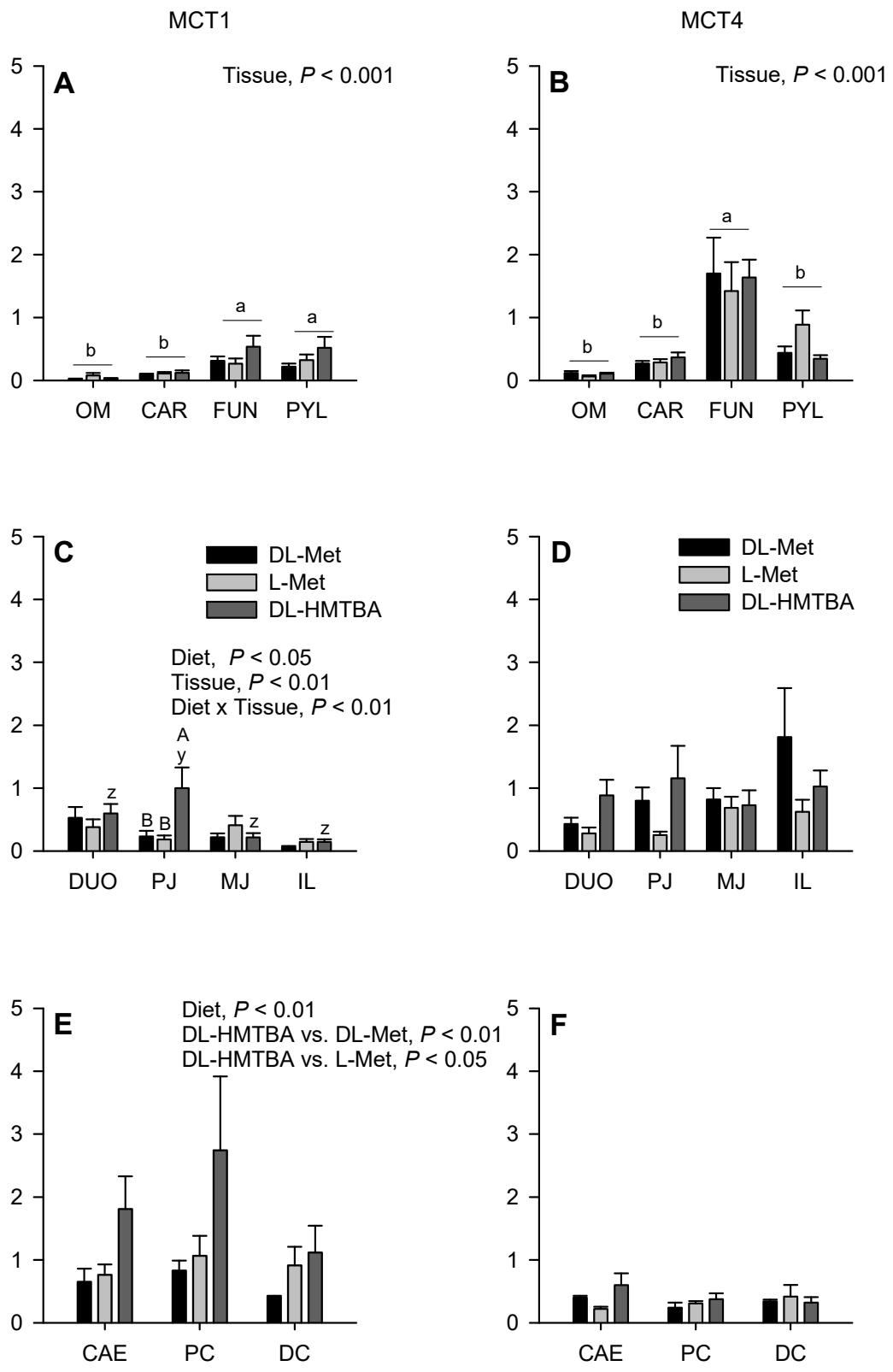

Figure 4: T-qPCR analysis of MCT1 (A, C, E) and MCT4 (B, D, F) in extra-intestinal regions (A, B), in the small intestine (C, D) and in the large intestine (E, F).

Data were compared using 2-factor ANOVA and a post hoc Student–Newman–Keuls test. Significant factor effects are mentioned in each graph. If column groups or columns do not share a common small letter within one graph, their expression values are either different irrespective of diet (a-c) or within a given diet (y,z) ($P < 0.05$). Different capital letters indicate diet effects within a given tissue. DUO: duodenum; PJ: proximal jejunum; MJ: middle jejunum; IL: ileum; Cae: caecum; PC: proximal colon; DC: distal colon; OM: oral mucosa; CAR: cardia; FUN: fundus; PYL: pylorus

In the extra-intestinal regions, no diet effect could be found for the mRNA expression of *MCT1* (Figure 4E) or *MCT4* (Figure 4F). In contrast, the factor "tissue" was statistically significant for both transport systems ($P < 0.001$). *MCT1* showed higher mRNA levels in fundus and in pylorus compared to oral mucosa and cardia ($P < 0.01$). *MCT4* had highest gene expression levels in fundus compared to the three remaining extra-intestinal regions ($P < 0.001$).

In the small intestine, *MCT1* mRNA expression (Figure 4A) was statistically different depending on which Met supplement the animals received (factor diet, $P < 0.05$) and depending on the segment tested (factor tissue, $P < 0.01$) with significant diet × tissue interaction ($P < 0.01$). The interaction effect was attributable to a higher *MCT1* mRNA expression in the proximal jejunum only, where the D/L-HMTBA group had significantly higher *MCT1* mRNA levels when compared to the D/L-Met and L-Met feeding groups. The *MCT4* mRNA expression in the small intestine (Figure 4B) did not differ regarding both factors tissue and diet.

In the large intestine, *MCT1* gene expression (Figure 4C) was significantly higher in pigs fed D/L-HMTBA when compared to pigs fed D/L-Met or L-Met ($P < 0.05$). This effect could not be found for *MCT4* (Figure 4D) ($P > 0.1$). Both *MCT1* and *MCT4* did not show any changes in the mRNA expression levels for the factor tissue.

In Table 3, the relative expression data of *MCT1* and *MCT4* was analyzed among all segments, irrespective of the factor diet using ANCOVA with diet as a covariate.

Table 3: Comparison of the relative mRNA expression data of *MCT1* and *MCT4* among different intestinal and extra-intestinal sections irrespective of the factor diet.

	MCT1	***MCT4***
OM	0.05 ± 0.01^{d}	0.1 ± 0.01^{c}
Pyl	0.36 ± 0.07^{bcd}	0.57 ± 0.1^{bc}
Car	0.11 ± 0.02^{cd}	0.31 ± 0.03^{c}
Fun	0.37 ± 0.07^{bcd}	1.59 ± 0.25^{a}
Duo	0.50 ± 0.08^{bcd}	0.54 ± 0.11^{bc}
PJ	0.49 ± 0.14^{bcd}	0.77 ± 0.21^{bc}
MJ	0.28 ± 0.06^{cd}	0.74 ± 0.11^{bc}
Ile	0.12 ± 0.02^{cd}	1.18 ± 0.30^{ab}
Cae	1.07 ± 0.21^{ab}	0.40 ± 0.08^{c}
PC	1.50 ± 0.40^{a}	0.31± 0.04^{c}
DC	0.82 ± 0.18^{abc}	0.36 ± 0.07^{c}
P-value	< 0.001	< 0.001

Different small letters within one column indicate significant differences of gene expression between intestinal and extra-intestinal sites at $P < 0.05$.

The gene expression distribution of *MCT1* and *MCT4* mRNA along the intestinal tract was rather different. While *MCT1* had a rather high expression in the large intestine, levels of *MCT4* were highest in the fundus of the stomach and in ileum (Table 3).

4.4.3. Conclusion

The gene expression of *MCT1* seemed to be partially influenced by the feeding of different Met supplements. While pigs fed D/L-Met and L-Met showed similar *MCT1* levels through the whole intestinal tract, pigs which received D/L-HMTBA showed higher *MCT1* levels in all large intestinal samples and ileum. *MCT4* levels were comparable for the factor diet in all tested samples.

Interestingly, *MCT1* and *MCT4* gene expression was not equivalently distributed along the intestinal tract. Whereas measured *MCT1* gene expression was highest in the large intestine and rather low in the small intestine, this pattern was inverted for *MCT4*. Apart from the oral mucosa and cardia, the samples of the large intestine showed the lowest *MCT4* levels; and small intestinal samples were among the samples with the highest *MCT4* gene expression.

5. Discussion

The importance of supplementing Met to the diet of livestock is elemental to ensure health and growth performance because it is a limiting and essential AA. As a precursor for SAM, Met is also primordial for cell metabolism. The importance of SAM for cell survival is directly linked to its metabolism. SAM is used, among other purposes, in the methylation of DNA and phospholipids, thus the availability of SAM has a direct influence on gene expression and membrane fluidity (Lu 2000). Another very important metabolic pathway of SAM is the trans-sulfuration to glutathione which is a major cellular anti-oxidant (Lu 2000). Methionine deprivation triggers various pathways to maintain the intracellular Met and SAM stock and a long-term absence of these metabolites leads to cell apoptosis (Shiraki et al. 2014). Maybe because of this fact, it is also the AA with the highest absorption rates in the intestine of mammals (Webb 1990) and a large portion of the absorbed Met is directly metabolized by the intestinal epithelial cell (Shoveller et al. 2005). This high Met demand of the intestine is not very surprising, as the intestinal mucosa forms a junction between the inner organism and the outer environment. Besides having to form an effective barrier, the intestinal mucosa also has to be permeable towards nutrients; this dual role makes the intestinal epithelium prone to oxidative damage by luminal components (Circu and Aw 2012). Damages to the enterocytes have a direct impact on nutrient utilization and, therefore, on production performance (Yang and Liao 2019). Methionine has been shown to have a beneficial effect on intestinal integrity and morphology, antioxidative status and immune response (Yang and Liao 2019). To provide sufficient Met supply for all metabolic functions and to ensure good production rates, Met has been supplemented to the diet of livestock (pigs and poultry especially) in its crystalline form. There is evidence of a beneficial effect on health and production rates when animals are offered a low crude protein diet supplemented with adequate levels of crystalline Met in poultry (Lemme et al. 2020) and pigs (Wang et al. 2018; Gloaguen et al. 2014). The most commonly used Met supplements in animal nutrition are D/L-Met and the synthetic precursor D/L-HMTBA. L-Methionine is also available but due to the high production cost of this supplement, it is mostly used in human nutrition and pharmacological applications. The adequate supplementation of D/L-Met or D/L-HMTBA is essential not only for commercial purposes, but also because over-supplementation of either supplement can cause reduced performance (Vazquez-Anon et al. 2017). While piglets fed L-Met and D/L-Met show very similar average daily weight gain during their growth (Shen et al. 2014), some studies suggested that piglets fed D/L-HMTBA had better growth rates than animals fed D/L-Met (Li et al. 2014) despite it being less bioavailable than D/L-Met (Shoveller et al. 2010). The relative effectiveness of D/L-HMTBA when compared to D/L-Met was described to be between 62% and 78% (Zimmermann

et al. 2005). D/L-HMTBA was not absorbed as effectively as D/L-Met in the small intestine (Drew et al. 2003). Interestingly, a study involving cecectomized chicken showed that D/L-HMTBA and D/L-Met could not be measured in the excreta of conventional chicken but that animal without ceca excreted significant amount of D/L-HMTBA indicating that the large intestine and especially the microbiome play an essential role in the utilization of D/L-HMTBA (Han et al. 1990). This also suggests that the lower bioavailability of D/L-HMTBA is not only caused by a limited absorption of the substance. This would mean that at least part of the differences in bioavailability are of metabolic nature. These variances in metabolism seem to affect overall feed intake, depending on supplementation levels of D/L-HMTBA and D/L-Met, making it difficult to compare the bioefficacy of both compounds. There is indication that the weight gain of animals fed D/L-HMTBA vs. D/L-Met develops differently depending on how low (or high) the compounds are supplemented to the feed. At lower dosages, broilers fed D/L-HMTBA seem to show lower growth rates when compared to the same dosages of D/L-Met. This effect seems to be existent in diets deficient for sulfur AA, whereas the effect seems to disappear or reverse at higher concentrations (Vazquez-Anon et al. 2017). Having extensive insight about the ways Met and its analogs are absorbed by the body, as well as their effect on said pathways is essential. However, this information is very sparse in literature. The objective of the present work was to obtain knowledge on the influence of the most used dietary Met sources on Met absorption in the gastrointestinal tract (GIT) of growing pigs.

Influence of methionine absorption depending on dietary supplementation

In its first phase, this work examined the trans-epithelial Met absorption along the small intestine, comparing L-Met absorption to the absorption of its stereoisomer D-Met. The duodenum had lower absorptive capacity than the more distal parts of the small intestine, jejunum and ileum. However, it had been described previously that Met is very quickly absorbed in the proximal parts of the small intestine (Malik et al. 2009). For this reason, the enhanced Met absorption in the distal parts of the small intestine might appear contradictory. However, the higher concentration of the transport systems in the more distal portions of the GIT may enable the organism to extract even smallest amounts of Met from the digesta, preventing the loss of this essential AA. Interestingly, this effect has been observed for both D- and L-Met.

L-Methionine is the biologically active isomer. D-Methionine has to be converted into L-Met in order to be utilized in cell metabolism. This transformation occurs in the liver and in the kidneys where the reaction is catalyzed by D-AA oxidases and followed by reamination into L-Met (Metzler-Zebeli et al. 2017). The bioavailability of D-AA is controversially described in literature. Certain authors describe a similar utilization based on the feed conversion ratio

(Kong et al. 2016; Chung and Baker 1992); others stipulate, with the same criterion as reference, that L-Met is more efficiently used (Shen et al. 2014; Kim and Bayley 1983). Based on this disparity, several groups preferred to measure N-retention and secretion as an indicator for D-AA bioavailability. Here again, the results were not consistent (Espinosa et al. 2021; Kong et al. 2016; Cho et al. 1980). An explanation for those disparities could be the different ages of animals used for the studies because it was stipulated that the age of animals could have an influence on the activity of D-AA oxidase and thus on the utilization of D-Met and other D-AA (D'aniello et al. 1993). At least in weanling pigs, as were used in the experiments of the present thesis, D-AA oxidase is present in high concentrations in the small intestine, potentially allowing for an immediate conversion of D-Met into L-Met directly after uptake, especially in proximal parts of the small intestine (Espinosa et al. 2021; Brachet and Puigserver 1992). In our experiments, the D-Met flux rates at physiological concentration were noteworthy and only the ileum showed significantly higher L-Met flux rates than D-Met flux rates, suggesting that a potentially lower bioavailability is not only caused by the limitation in absorptive capacity but also by metabolic factors. Overall, the uptake and utilization of D-Met in the intestinal tract is rather efficient when compared to some other D-AA (D-histidine or D-lysine, for example) (Friedman 1999; Jervis and Smyth 1959).

This work further showed that the choice of Met supplement had an influence on the absorption of L-Met in the intestine of pigs. In piglets fed with the D/L-Met racemate, D- and L-Met flux rates across the jejunal epithelium were significantly higher than in the tissue from animals which had received the L-Met or D/L-HMTBA supplement. Interestingly, the D/L-Met supplement caused a more evident increase in the L-Met flux rates than in the D-Met flux rates. Additionally, L-Met absorption was elevated only if the mucosal environment contained Na^+, suggesting that either the induced transport system accepts both Met enantiomers as substrates or the D/L-Met- containing diet induced two separate transport systems. It has been postulated that D-Met and L-Met are absorbed through distinct pathways in the intestine; L-Met being absorbed with higher affinity than D-Met, and each one inhibiting the uptake of the other (Brachet et al. 1987). In the present work, however, we were able to increase L-Met absorption with the dietary supplementation of D-Met, suggesting that both enantiomers take a rather similar path from the intestinal lumen into the enterocyte. Indeed, the main reason for the aforementioned suggested reciprocal inhibition might be that some transport proteins are known to accept both D-Met and L-Met though having a higher affinity for one of the enantiomers. For example ATB^{0+} was shown to allow flux of both L-Met and D-Met (Hatanaka et al. 2002). ASCT2 also accepts both isomers but seems to have a higher affinity towards D-Met when compared to L-Met (To et al. 2021). In the model for the intestinal Met transport proposed by Mastrototaro et al., nine proteins and transport systems are mainly responsible for the absorption of dietary Met in the GIT (Mastrototaro et al. 2016). Among the apically

located transport systems, four are Na^+-dependent: ATB^{0+}, IMINO, B^0AT1 and ASCT2. ATB^{0+} is described to be mainly expressed in the large intestine while being sparsely expressed in the small intestine (Hatanaka et al. 2002). The results obtained in this work support these allegations. To et al. even postulate that ATB^{0+} only plays a marginal role in dietary Met absorption (To et al. 2021). IMINO was shown to have a very low affinity for Met (Brachet and Puigserver 1987). Therefore, B^0AT1 and ASCT2 are more likely to have been induced by dietary D/L-Met. Both of the aforementioned transport-proteins accept other AA apart from Met and therefore it was no surprise to see that the Na^+-dependent Met flux that was stimulated with D/L-Met-containing diet was reduced by the mucosal presence of other AA. These results suggest that the induced transport system(s) is (are) selective not only for Met but also accept(s) other AA as substrates, potentially with higher affinity. Overall, a supplementation with D/L-Met might influence intestinal absorption of several AA in addition to Met. This dietary supplement might therefore raise the absorptive capacity of the small intestine and thereby improve dietary protein utilization.

Beside D- and L-Met flux rates, the flux rates of radiolabeled D/L-HMTBA were also investigated in the first phase of this project. Flux measurements of D/L-HMTBA showed that its absorption in the small intestine could not be influenced by different Met sources. The addition of Na^+ did not enhance D/L-HMTBA flux rates across the intestinal tissue in any tested segment at physiological concentration; peculiarly, it seemed to lower the D/L-HMTBA flux rates in duodenum. This effect reached statistical significance only at the higher D/L-HMTBA concentration of 5 mM, but was indicated numerically at 50 µM D/L-HMTBA. Interestingly, the duodenum showed overall significantly lower absorptive capacity for D/L-HMTBA when compared to the jejunum and to the ileum. The lack of diet effects on HMTBA flux rates is quite surprising as the transport system MCT1, which is supposedly the main protein responsible for the secondary active transport of HMTBA, is substrate-inducible (Cuff et al. 2002). Therefore, one could expect the HMTBA flux rates to be higher in HMTBA-fed animals. However, monocarboxylate transporters are proton-coupled and are dependent on a pH gradient in order to accept their substrates with high affinity (Felmlee et al. 2020). The lack of pH gradient in our experimental procedure between the apical and basolateral side of the intestinal tissue might have resulted in diffusion being the main flux pathway and explain why the flux rates were similar between dietary groups, as especially MCT1 might have had little activity, and the uptake of HMTBA in the intestine is suggested to have a diffusive component besides the active transport via MCT1 and MCT4 (Brachet and Puigserver 1987). In rainbow trout, the transport of D/L-HMBTA was shown to have a Na^+-dependent component, indicating the contribution of sodium monocarboxylate transporters (SMCTs) in some species (Pham Thi Ha To et al. 2020). The results obtained in the present study indicate that Na^+ does not enhance the flux of D/L-HMTBA through the porcine intestine whatsoever, indicating that

SMCTs had no major significance in the D/L-HMTBA transport in the small intestine of pigs. When considering the whole GIT, the intestinal absorption is not the only relevant factor when considering D/L-HMTBA as a Met replacement. The presence and activity of the enzyme D-HADH is primordial in the conversion of D/L-HMTBA into Met. This enzyme is regulated by the type of dietary Met supplement and its expression was remarkably high in the stomach, comparable to what was found in liver and kidney, suggesting that the stomach would be able to process dietary D/L-HMTBA (Martín-Venegas et al. 2011; Fang et al. 2010b). Therefore, a great part of dietary HMTBA might be absorbed in the stomach. Indeed, Malik et al. showed that around 50% of dietary HMTBA disappeared from the ingesta before the small intestine in pigs (Malik et al. 2009). In poultry, this effect was even stronger as almost the totality of the fed D/L-HMTBA seemed to be absorbed before the duodenum (Richards et al. 2005). As D/L-HMTBA has a rather low pKa (~ 3.86), diffusion could be quantitatively relevant at the acidic pH immanent to the upper GIT of pigs and poultry (To et al. 2021).

Overall, the functional experiments in the Ussing chamber showed that it is possible to influence intestinal Met absorption with dietary supplements. For a better understanding of the functional data, an analysis of the expression of possible met transport systems along the intestinal tract of pigs is helpful and is expected to give insight on the mechanisms that can be influenced by different Met supplements.

Expression of Met transporters along the gastrointestinal tract of pigs

In the feeding trial, the mucosal tissue was sampled in pigs fed D/L-Met, L-Met or D/L-HMTBA in different locations along the gastrointestinal tract. These samples were examined for gene and protein expression of known Met transporters in western blot and qPCR experiments. This research was complemented with the examination of the gene expression of transport systems that are suspected to accept HMTBA as substrate (MCT1 and MCT4). The goal of this part of the project was to identify the transport system(s) which were induced with a D/L-Met dietary supplement in the flux experiments of the first part of this project (Romanet et al. 2020). Six of the presented proteins (B^0AT1, ATB^{0+}, $b0^+AT$, IMINO, ASCT2 and MCT1) are principally located on the apical side of the intestinal epithelial cell and five (LAT4, LAT1/2, SNAT2, y^+LAT1, MCT4) are mostly located on the basolateral side. B^0AT1, ATB^{0+}, ASCT2, SNAT2 and y^+LAT1 require Na^+ for AA transport (Mastrototaro et al. 2016). Among these systems, few are known to accept both Met isoforms as substrate but especially ASCT2 and LAT1 seem to accept both isomers (Kobayashi et al. 2012; Yanagida et al. 2001).

ASCT2 is a rather interesting transport system. Firstly, ASCT2 expression and activity can be influenced by the composition of the diet, especially AA availability, in the intestine (Wu

et al. 2015) but also in skeletal muscle and mammary epithelial cells, potentially increasing AA availability (Dai et al. 2020; Hu et al. 2019). Secondly, ASCT2 is able to transport D-Met, possibly with higher affinity than L-Met (Kobayashi et al. 2012). This feature makes ASCT2 a promising target to enhance Met utilization, as in animal nutrition, Met is mostly supplied in its racemic form. In the present work, ASCT2 protein and mRNA was found all over the GIT with transcript levels being highest in the stomach and protein levels highest in sections of the large intestine. *ASCT2* mRNA levels seemed to be enhanced in the small intestine of animals which received a D/L-Met containing diet, especially when compared to D/L-HMTBA, indicating that this transport system might be a suitable target to improve dietary Met absorption. As an obligatory antiporter, ASCT2 seems essential in the AA homeostasis of the cell, endorsing a role as “harmonizer” of AA (Scalise et al. 2018). It has been stipulated that the activity of ASCT2 is closely related to the basolateral LAT1 transporter (Cormerais et al. 2018), which interestingly also accepts D-AA (Yanagida et al. 2001). LAT1 is, like ASCT2, also an AA exchanger and together these two proteins might take an essential part in balancing the levels of intracellular AA (Cormerais et al. 2018). Both transport systems seem to show similar expression patterns, they are either upregulated together or remain unchanged together when the expression of other AA transporters is being influenced, underlining their partnership (Mccracken and Edinger 2013; Amaral et al. 2008). This apparent interaction has been repeatedly described in association with tumor growth and cancer (Lopes et al. 2021; Zhang et al. 2020) but it is thinkable that it also takes part in physiological proliferative mechanisms that occur in the intestine. However, information on this topic outside of a pathological context remains very sparse.

The function of ATB^{0+} in the absorption of dietary Met is not very clear. While some assign ATB^{0+} an important role in L-Met absorption (Chen et al. 1994), others describe its role as marginal, mainly because of its supposedly low expression in the small intestine (To et al. 2021). Various working groups were able to identify the *SLC6A14* gene in the small intestine, whereas it has not been found in other studies based on its functional characteristics (Yu et al. 2020; Schweer et al. 2016; Sun et al. 2015; Yang et al. 2010; Munck et al. 1995). In the present work, we were able to detect ATB^{0+} gene expression throughout the whole intestinal tract of pigs. However, the large intestine showed significantly higher gene expression levels than the small intestine, reinforcing the postulate that ATB^{0+} does not have significance in the absorption of dietary Met or even dietary AA in general. Interestingly, ATB^{0+} gene expression trended to be higher in D/L-HMTBA fed animals in the large intestine only. Malik et al. showed that D/L-HMTBA is present much further down the GIT when compared to Met (Malik et al. 2009), indicating that D/L-HMTBA might be available for intestinal microorganisms in the large intestine, possibly leading to microbial conversion into Met. This theory is in line with another

group which hypothesized that ATB^{0+} is probably more involved in the absorption of AA coming from bacterial metabolism than in the absorption of dietary AA. (Hatanaka et al. 2002).

B^0AT1 was described by many authors as the main transport system for Met (To et al. 2021; Mastrototaro et al. 2016; Bröer 2008). In the present study, B^0AT1 was predominantly found in the small intestine, especially in the middle jejunum, thus suggesting it might be the most important transporter for dietary AA. As a matter of fact, mutations in the *SLC6A19* gene (as in Hartnup disease for example) result in excessive excretion of neutral AA (Hashmi and Gupta 2021), especially tryptophan, threonine, serin, glutamine, tyrosine and histidine. Methionine, isoleucine, alanine and phenylalanine have also been found to have a significantly higher renal clearance in patients with Hartnup disease (Bröer 2009). In another study, *SLC6A19* knockout mice suffered from protein malabsorption, including a delayed distribution of AA to tissues (Javed et al. 2018). These studies therefore suggest that lack or malfunction of *SLC6A19* result in severe changes in AA uptake, confirming its central role in the absorption of dietary neutral AA. In the present project, we also investigated the distribution of B^0AT1 protein and mRNA transcript levels along the digestive system in animals fed different Met sources (L-Met, D/L-Met and D/L-HMTBA). The type of diet did not have any influence on *B^0AT1* mRNA expression in this study, but mRNA transcripts were higher in middle jejunum and to a smaller extent in the ileum, when compared to the more proximal small intestinal portions. Protein levels of B^0AT1 were highest in the ileum. These results indicate a post-transcriptional upregulation of B^0AT1 and could support the data obtained in a functional study where D/L-Met induced a Na^+-dependent L-Met flux in the jejunum. In the jejunum and ileum, the flux rates of L-met were overall higher in pigs fed D/L-Met but a Na^+-dependency could not be shown on a statistical level, which is rather surprising as the upregulation of B^0AT1 protein by D/L-Met was numerically higher in the ileum than in jejunum. Thus, the sole presence of the B^0AT1 protein in the tissue does not seem to be a guarantee for B^0AT1 activity, post-translational pathways appear to be decisive in this case. The normal activity of B^0AT1 in the intestine was observed to depend on the partnership with ACE2, as the proper localization of this transport system in the membrane seems to rely on the latter's expression (Camargo et al. 2020; Singer and Camargo 2011; Kowalczuk et al. 2008), although the sole coexpression of these two proteins is seemingly not sufficient to guarantee B^0AT1 activity either (Yang et al. 2016). An explanation might be that B^0AT1 also associates with other proteins, such as syntaxin 1A. The pairing of B^0AT1 with syntaxin 1A has been shown to have the opposite effect to the partnership with ACE2, namely an inhibition of B^0AT1 activity (Fairweather et al. 2015). Overall B^0AT1 expression can be influenced on a molecular level by the diet as shown in the present study, but the post-translational processes that ensure its transport activity are rather complex and not yet fully understood.

On the basolateral side LAT4 seems to be the only relevant transporter for a net efflux of Met (To et al. 2021; Mastrototaro et al. 2016). The function of the other basolateral proteins seems to mainly be the equilibration of AA across the cell membrane rather than facilitating a net efflux (LAT1 and LAT2) (Bröer 2008; Verrey et al. 2004). Other basolateral amino acid transporters are mainly responsible for net uptake from the blood (SNAT2 and y^+LAT1) (Mastrototaro et al. 2016; Bröer 2008). This postulate has been confirmed in a recent trial which demonstrated that a lack of LAT4 resulted in the accumulation of Met in the enterocyte (Rajendran et al. 2020). Its indispensability has also been confirmed by Guetg et al., who reported that *SLC43A2* (LAT4) knockout mice showed severe postnatal growth deficiency and premature death (Guetg et al. 2015). The substrate spectrum of LAT4 has been shown to be rather narrow. As a matter of fact, it transports only a few essential AA, but interestingly other AA's plasma levels are also affected by the lack of LAT4, indicating that LAT4 is involved in the overall regulation of transepithelial AA flux (Oparija et al. 2019). A reason for this impact on AA homeostasis might be the tight cooperation of LAT4 with other basolateral AA transporter that have a broader substrate range (namely LAT2 and y^+LAT1) (Maric et al. 2021; Guetg et al. 2015). Together these systems control exit and entrance of AA at the basolateral side of the enterocyte. In the present study, LAT4 had highest mRNA expression in the small intestine, but transcripts were not influenced by the type of dietary Met supplement. In contrast, protein expression of LAT4 was significantly higher in the small intestine of pigs fed a D/L-HMTBA containing diet. D/L-HMTBA also influenced transcript levels of *LAT2* (in the small intestine) and *y^+LAT1* (in the large intestine), supporting the thesis that these proteins operate together to ensure AA balance across the basolateral enterocyte membrane. Enterocytes have a rather high need of Met, and feeding D/L-HMTBA is a popular method to lower first-pass metabolism of Met (Fang et al. 2010a). This may result in Met deficiency in the enterocytes, as HMTBA is mainly converted to L-Met in the liver (Lobley et al. 2006). By increasing the expression of LAT4 and other basolateral systems, the enterocyte might try to replace the missing dietary AA by others from the blood to guarantee sufficient supply for its own metabolism. This hypothesis is reinforced by the recent work of Fagundes et al. (Fagundes et al. 2020). They showed that *LAT4* mRNA transcripts were significantly higher in chicken fed a Met- deficient diet. Overall, besides being the major "exit door" for essential dietary AA from the intestinal cell, in particular for Met, there is some evidence suggesting that LAT4 might double as an "emergency backdoor" to guarantee the supply of those same essential AA in periods where nutritional supply is insufficient.

The present study showed that feeding HMTBA had an influence on the expression of some Met transporters. However, this upregulation of mainly basolateral transport systems might essentially be due to the lack of available serosal Met rather than to the action of HMTBA itself. Due to small (but essential) differences in their structure, HMTBA is not transported

through the same pathways as Met (Brachet and Puigserver 1987). MCT1 and MCT4 are supposedly the main transport systems for HMTBA; in consequence, their distribution along the GIT was also investigated. The data is presented in chapter 4.3 and shows that both investigated MCT genes are expressed along the porcine GIT. The results show that their distribution differs between extra-intestinal and intestinal regions. Whereas MCT1 shows higher gene expression in regions of the large intestine, MCT4 seems to have higher gene expression in the small intestine and in locations of the stomach. Furthermore, the different Met supplements affected the expression of MCT1 only. This data set is in accordance with literature that reports MCT1 as ubiquitously expressed but with highest levels in the colon where it is mainly responsible for the transport of short-chain fatty acids (SCFAs) produced by bacterial fermentation (Welter and Claus 2008; Ritzhaupt et al. 1998). The MCT1 mRNA levels seemed to be upregulated by a diet containing D/L-HMTBA in the large intestine and in the ileum. It has previously been shown that the MCT1 gene expression does not vary depending on the dietary supplements D/L-Met, L-Met and D/L-HMTBA in the jejunum of chicken (Zhang et al. 2017), which reflects the lack of regulation in this part of the porcine intestine in this project. It is reported in the existing literature that especially in the epithelium of the large intestine MCT1 is easily regulated by the presence of an appropriate substrate (Agyekum et al. 2015; Cuff et al. 2002). Therefore, the upregulation of MCT1 by D/L-HMTBA as oral supplement is coherent, because HMTBA transport across the apical membrane is known to be mediated by MCT1 (Zhang et al. 2015; Martin-Venegas et al. 2007). The results obtained in this project are also consistent with a study from Martin-Venegas et al. (Martin-Venegas et al. 2014) where they showed that HMTBA supplementation upregulates MCT1 mRNA expression and protein levels in Caco-2 cells.

In contrast to MCT1, which is ubiquitously distributed among tissues, MCT4 is mainly expressed in tissues with high glycolytic rates and probably located on the basolateral side responsible for lactate efflux in these tissues (Halestrap and Wilson 2012; Morris and Felmlee 2008; Bonen 2001). Investigations from other groups showed that MCT4 can be upregulated in skeletal muscle to allow the efflux of lactic acid under conditions of high energy demand, suggesting that MCT4 expression is regulated by the availability of its main substrate (Furugen et al. 2011). The quantification of MCT4 mRNA in the present study showed highest levels in the gastric fundus which is in accordance with the finding that a large portion of HMTBA is already resorbed in the stomach before reaching the intestine (Malik et al. 2009; Richards et al. 2005).

Feeding different Met sources did not induce any significant effect on MCT4 expression in any of the regions analyzed. This resembled the data of Martin-Venegas et al. (Martin-Venegas et al. 2014) who confirmed the expression of both MCT transporters in Caco-2 cells but reported that only MCT1 but not MCT4 expression was affected by HMTBA

supplementation. In the present work, the gene expression of only MCT1 was upregulated by the D/L-HMTBA-containing diet, indicating that MCT1 might represent the main active system involved in the absorption of HMTBA, as already suggested by Sepponen et al. (Sepponen et al. 2007). As MCT1 transcripts were among the lowest in the stomach, MCT1 is not a very likely explanation for the high disappearance rates of dietary HMTBA before the small intestine as observed in some previous studies (Malik et al. 2009; Richards et al. 2005). It could thus be speculated that metabolic conversion of HMTBA prior to reaching the small intestine or permeation by lipophilic diffusion facilitated by low pH (Vazquez-Anon et al. 2017; Pieper et al. 2016) may contribute to pre-intestinal disappearance of HMTBA.

Overall, the work showed that the absorption of Met is influenced by the diet, and that higher transport rates might be the consequence of the modulation of the expression of different AA transporters. Especially a D-Met-containing diet (in the form of a D/L-Met supplement) seemed to enhance L-Met absorption in the jejunum of pigs in the presence of Na^+. The two transport systems which are most likely causative for these higher transport rates are ASCT2 and B^0AT1. The molecular data showed that the mRNA expression of ASCT2 was slightly higher in pigs which received D/L-Met although the mRNA expression of B^0AT1 was not influenced by the diet. On protein level, ASCT2 was not influenced by the diet whereas B^0AT1 was higher in pigs fed D/L-Met. These findings do not allow to attribute the higher L-Met flux observed in pigs fed D/L-Met to only one transport system, especially because the full functionality of B^0AT1 does not only rely on its sole presence. To fully pinpoint the mechanisms involved in this process, more information, especially functional data is necessary.

As ASCT2 and B^0AT1 both accept a range of AA, especially neutral, as substrates, it is conceivable that overall AA absorption is influenced by the dietary supplementation of crystalline Met. This could have significance in supporting treatments against gastrointestinal malabsorption diseases also in humans. The porcine intestine has shown to be an excellent model of human digestive diseases because the human and porcine GIT are very similar in terms of anatomy and pathophysiology (Gonzalez et al. 2015). Especially diseases like short-bowel syndrome are well reproducible with the aid of a porcine intestinal model (Manithody et al. 2020). Moreover, understanding the exact mechanisms and learning how to influence AA absorption in pigs is essential to optimize pig production. This not only relates to enhancing yields and reducing N-excretion but it might be an important element in combating post-weaning diarrhea in piglets because adequate AA supply is a key component for intestinal health and metabolism (Mou et al. 2019).

6. Summary

Ensuring high growth performances is a central subject in the production of livestock. For this reason the diet of pigs is often supplemented with crystalline AA, especially Met as it is an essential and a limiting AA. The objective of this project was to gain insight into the mechanisms of Met absorption in the intestine of weaned pigs and to assess whether different dietary supplements might have different effects on the expression of Met transport systems in the intestinal tract.

In the first part of this project, a feeding study was run to give insight whether different Met sources had an effect on the absorption of Met in the small intestine. For this purpose, three groups of each 9 piglets received a pre-feeding which only differed by the Met source supplemented. The three sources were L-Met, D/L-Met and the hydroxy Met analog D/L-2-hydroxy-4-methylthiobutyrate) D/L-HMTBA. In a functional study using the Ussing chamber, we analyzed the mucosal-to-serosal flux rates of L-Met, D-Met and D/L-HMTBA through sections of duodenum, jejunum and ileum. The resulting data showed that a D/L-Met-containing diet increased the absorptive capacity for D-Met and L-Met. In the jejunum the flux rates of L-Met in the D/L-Met fed group were strongly Na^+-dependent, suggesting the induction of a Na^+-dependent transport system in the jejunum by a D/L-Met-containing diet. Na^+-dependent L-Met transport systems in the apical membrane include B^0AT1, ATB^{0+}, ASCT2 and system IMINO. The flux study of D/L-HMTBA in the small intestine showed that the different dietary sources had no influence on the intestinal flux of D/L-HMTBA itself, which was rather surprising as the systems which supposedly accept D/L-HMTBA as a substrate (MCT1 and MCT4) are known to be upregulated in the presence of appropriate substrates.

For a better understanding of the functional data obtained in the first part of this project, an analysis of the expression of possible Met transport systems was run along the whole gastrointestinal tract of pigs in the second part of this project. The samples came from the same pigs we used in the Ussing chamber experiments in the first part. Changes in mRNA expression of a total of nine apical or basolateral AA transporters, as well as MCT1 and MCT4, and changes in protein expression of four AA transporters were analyzed. In pigs fed D/L-Met, mRNA expression of *ASCT2* was higher when compared to D/L-HMTBA in the small intestine, but this induction was not statistically confirmed on the protein level. B^0AT1 protein expression was higher in the distal small intestine where it was upregulated by a D/L-Met containing diet. A D/L-HMTBA-containing diet seemed to upregulate certain basolateral transport systems on gene and protein levels, but also mRNA expression of *MCT1.* Overall, this part of the project showed that different Met supplements have an effect on the expression of several AA

transport systems, especially D/L-Met seemed to have an effect on apical transport systems and D/L-HMTBA more on basolateral transport systems.

Overall, this work showed that Met absorption in the intestine is influenced by the diet. Especially a D/L-Met-containing diet increased the L-Met flux in the jejunum of pig in the presence of Na^+. Following the functional experiments, the analysis of gene and protein expression showed that this effect might result from expression changes of ASCT2 and B^0AT1 which are both Na^+-dependent transport systems. They are both excellent candidates for being responsible for the induction of Na^+-dependent L-Met flux in the jejunum in the Ussing chamber experiment. To distinguish among those two contenders, more functional data is required to fully understand the mechanisms behind the changes observed in the feeding trial.

7. Zusammenfassung

Mechanismen der Methionineabsorption im Darm von Schweinen

Die Gewährleistung von hohen Wachstumsraten ist eine zentrale Zielsetzung in der Fleischproduktion. Aus diesem Grund wird die Ernährung von Schweinen oft mit Aminosäure-Supplementen ergänzt. Das Vorhaben dieses Projektes war es, einen Einblick in die Mechanismen der Methioninabsorption im Darm abgesetzter Ferkel zu gewinnen und zu erforschen, ob verschiedene Futterergänzungsmittel verschiedene Effekte auf die Expression von Methionintransportern im Gastrointestinaltrakt haben.

Im ersten Teil dieses Projektes, wurde eine Fütterungsstudie durchgeführt. In diesem Experiment sollte Verständnis gewonnen werden, ob die Absorption von Methionin von verschieden supplementierten Methioninquellen beeinflusst werden kann. Zu diesem Zweck wurden drei Gruppen aus je neun Absatzferkeln jeweils ein Futtermittel verfüttert. Jede Gruppe wurde mit je einer anderen Methioninquelle vorgefüttert. Die drei Quellen waren L-Methionin, D/L-Methionin und D/L-2-hydroxy-4-methylthiobutyrate (D/L-HMTBA), die restliche Zusammensetzung der drei Futtermittel war identisch. In einer funktionellen Studie wurden die mukoserosalen Fluxraten von L-Methionin, D-Methionin und D/L-HMTBA in verschiedenen Dünndarmabschnitten mit der Ussing-Kammer Technik analysiert. Die Daten zeigten, dass ein mit D/L-Methionin supplementiertes Futtermittel die Absorptionsfähigkeit für D- und L-Methionin erhöht. Im Jejunum der Tiere, die D/L-Methionin erhielten, waren die L-Methionin Fluxraten stark Na^+-abhängig. Dieses Ergebnis ist ein Hinweis, dass ein D/L-Methionin-haltiges Futtermittel ein Na^+-abhängiges Transportsystem im Jejunum von Absatzferkeln induziert. Als Na^+-abhängige Transportsysteme, die Methionin als Substrat akzeptieren, sind B^0AT1, ATB^{0+} ASCT2 und das IMINO System bekannt. Die D/L-HMTBA Fluxstudie im Dünndarm zeigte, dass die verschiedenen Methioninquellen keinen Einfluss auf die Fluxraten von D/L-HMTBA selbst hatten. Diese Erkenntnis war eher überraschend, da die Transportsysteme, die vermeintlich für den Transport von D/L-HMTBA im Dünndarm verantwortlich sind (MCT1 und MCT4), dafür bekannt sind, durch die Anwesenheit von eigenen Substraten hochreguliert zu werden.

Für ein besseres Verständnis der funktionellen Daten, die im ersten Teil dieser Arbeit erhalten wurden, wurde auch eine Analyse der Expression vermeintlicher Methionintransporter entlang des gesamten porzinen Gastrointestinaltraktes durchgeführt. Die Proben, die für diese Analysen verwendet wurden, stammten von denselben Tieren, die auch für die Ussing-

Kammer-Experimente des ersten Teils benutzt wurden. Veränderungen der mRNA-Expression von insgesamt neun apikalen oder basolateralen Transportsystemen, zuzüglich MCT1 und MCT4, sowie Veränderungen der Proteinexpression von vier Aminosäurentransportern wurden analysiert. Schweine, die mit D/L-Methionin gefüttert wurden, zeigten höhere *ASCT2* mRNA-Gehalte im Dünndarm verglichen mit den Tieren, die D/L-HMTBA erhielten; diese Induktion konnte auf Proteinebene statistisch nicht bestätigt werden. Die B^0AT1-Proteinexpression war höher im distalen Dünndarm, wo es durch die Zufütterung von D/L-Methionin hochreguliert wurde. Ein Futtermittel welches mit D/L-HMTBA supplementiert wurde schien einige basolaterale Transportsysteme auf der Gen- und Proteinebene hochzuregulieren. Die *MCT1*-mRNA-Expression wurde auch durch D/L-HMTBA erhöht. Insgesamt hat der molekularbiologische Teil dieser Arbeit gezeigt, dass verschiedene Methioninsupplemente einen Effekt auf die molekulare Expression verschiedener Aminosäurentransporter haben. Insbesondre D/L-Methionin scheint einen Einfluss auf apikale Transportsysteme zu haben, während D/L-HMTBA eher auf die basolateralen Systeme einwirkt.

Alles in allem hat diese Arbeit gezeigt, dass die Methioninabsorption im Darm über die Ernährung beeinflusst werden kann. Insbesondere eine Supplementierung der Diät mit D/L-Methionin hatte eine Zunahme des absorptiven L-Methioninfluxes in der Anwesenheit von Na^+ im Jejunum von Schweinen zur Folge. Anschließend an die funktionellen Experimente, hat die Analyse der Gen- und Proteinexpression eine Hochregulierung von zwei Na^+-abhängigen Transportsystemen, namentlich ACT2 und B^0AT1, gezeigt. Beide sind exzellenten Kandidaten für die Vermittlung eines Na^+-abhängigen L-Methioninfluxes in der Ussing-Kammer. Um die Mechanismen der veränderten Methioninabsorption aus der Fütterungsstudie voll zu verstehen, sind weitere funktionelle Daten nötig, insbesondere um unterscheiden zu können welches Transportsystem dafür verantwortlich war.

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

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