



ESTONIAN UNIVERSITY OF LIFE SCIENCES
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**THE IMMUNOLOCALIZATION OF THE Na^+ / GLUCOSE
COTRANSPORTER 1 IN OSTRICHES' INTESTINAL
EPITHELIUM**
NAATRIUM/GLÜKOOSI KOTRANSPORTER 1
IMMUNOLOKALISEERIMINE JAANALINDUDE PEENSOOLE
EPITEELIS

Final Thesis

Curriculum in Veterinary Medicine

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Tartu 2024

Estonian University of Life Sciences Kreutzwaldi 1, 51014, Tartu Estonia		Abstract of Final Thesis	
Author: Victoria Ahlstedt		Curriculum: Veterinary Medicine	
Title: The immunolocalization of the Na ⁺ /glucose cotransporter 1 in ostriches' intestinal epithelium			
Pages: 46	Figures: 9	Tables: 1	Appendixes: 3
Chair: Chair of Clinical Veterinary Medicine Field of research and (CERC S) code: 3. Health, 3.2. Veterinary Medicine B750 Veterinary medicine, surgery, physiology, pathology, clinical studies Supervisors: Piret Hussar, Ilmārs Dūrītis Place and year: Tartu 2024			
<p>Glucose transporters are necessary for the absorption of monosaccharides from the small intestinal lumen into the body, thus knowledge about them is crucial for understanding the digestive capacity and nutritional requirements of different animals. Up to now, there has been a gap in the knowledge of the Na⁺/glucose cotransporter 1 (SGLT1) – a transporter essential for glucose uptake - in the ostriches' small intestine. The aim of this cross-sectional study was to immunolocalize SGLT1 in the duodenum and terminal zone of ileum in 1-, 14-, and 28-day-old ostriches to obtain new information about the small intestine's ability to transport glucose at a young age. Intestinal specimens were collected from 15 female ostriches (<i>Struthio camelus var. domesticus</i>) raised in a Latvian ostrich farm (Ozoloni AB), with five ostriches per age group. The samples were fixed with 10% formalin, embedded into paraffin, and sliced into 7 μm thick cuts according to standardized histological procedures. The samples were routinely stained with hematoxylin and eosin, and thereafter, stained immunohistochemically (IHC) using Rabbit anti-SGLT1 (Abcam, UK) as primary antibody and the corresponding immunohistochemistry kit (Abcam, UK) according to the manufacturer's guidelines. Photos of the samples were taken with a camera (AxioCam, HRc, Germany) and saved on the computer for analysing. The results showed that the expression of SGLT1 got progressively stronger with age, indicating that glucose transport, and thus absorption, is still developing in the small intestine during the first month of life. The findings provides valuable information about ostriches' nutritional requirements and is suggestive of ostriches not being able to properly utilize carbohydrates from feed at young age.</p>			
Keywords: Glucose transport, immunohistochemistry, SGLT1, ostrich intestinal epithelium			



Eesti Maaülikool Kreutzwaldi 1, 51014, Tartu Eesti		Lõputöö lühikokkuvõte	
Autor: Victoria Ahlstedt		Õppekava: Veterinaarmeditsiin	
Pealkiri: Naatrium/glükoosi kotransporter 1 immunolokaliseerimine jaanalindude peensole epiteelis			
Lehekülgi: 46	Jooniseid: 9	Tabeleid: 1	Lisasid: 3
Õppetool: kliinilise veterinaarmeditsiini õppetool ETIS-e teadusvaldkond ja CERC S-i kood: 3. Terviseuuringud, 3.2. Veterinaarmeditsiin B750 Veterinaarmeditsiin, kirurgia, füsioloogia, patoloogia, kliinised uuringud Juhendajad: Piret Hussar, Ilmārs Dūrītis Katsimiskoht ja -aasta: Tartu 2024			
<p>Peamiseks energiaallikaks kõigis elusorganismides on monosahhariid glükoos. Glükoosi omastamist loomsetes kudedes teostavad transmembraansed valgud- glükoosi transporterid, mis transpordivad glükoosi ja sellele sarnaseid aineid läbi rakumembraanide. Suhkrute imendumisel soolestikus on väga oluline roll glükoosi transporterite GLUT2 ja GLU5 kõrval ka naatrium/glükoosi kotransporter 1 (SGLT1). Kuigi teadmised membraanivalkude kohta on väga olulised mõistmaks loomade ja lindude toitumisvajadusi ja seudevõimet, on seniajani teadmised SGLT1 lokaliseerimise kohta kõige suuremate ja väga oluliste põllumajanduslindude – jaanalindude – seedesüsteemis puudulikud. Samal ajal kui jaanalinnu farmides ligikaudu pooled vastkoorunud jaanalindudest esimese elukuu jooksul hukuvad, mille üks põhjustest võiks olla alimantaarne, on teaduslikud uurimustööd jaanalindude seedesüsteemi kohta sellel aja perioodil veel puudulikud. Käesoleva uuringu eesmärgiks oli immunolokaliseerida SGLT1 kaksteistsõrmiksoole ja niudesoole terminaalset tsooni limaskestast epiteelis 1-, 14- ja 28-päevastel jaanalindudel. Uuringumaterjal – koelõigud peensoole erinevatest osadest - koguti Läti jaanalinnufarmis kasvanud 15 emaselt jaanalinnult (<i>Struthio camelus var. domesticus</i>). Standardiseeritud histoloogiliste protseduuride kohaselt koelõigud fikseeriti 10% formaliinis, sisestati parafiini ja lõigati 7 µm paksusteks koelõikudeks, millele järgnes rutiinhistoloogiline värving hematoksüliin-eosiin meetodil ja immunohistokeemia (IHC). Immunohistokeemia puhul kasutati primaarse antikehana Rabbit anti-SGLT1 (Abcam, UK) ja sobivat sekundaarset antikeha sisaldavat immunohistokeemia komplekti (Abcam, UK) vastavalt tootja juhistele. Koelõikudest valmistatud preparaate vaadeldi AxioCam, HRc kaamera (Saksamaa) ja arvutiga ühendatud mikroskoobi Zeiss Axioplan-2 Imaging (Saksamaa) abil. Uuring näitas, et naatriumist sõltuv glükoositransporter-1 immunolokaliseerub soole epiteelirakkudes – karikrakkudes ja enterotsüütide mikrohattudes ning visuaalsel kontrollil täheldati SGLT1 värvumise intensiivsuse kasvu jaanalinnu tibude vanuse tõusul. Uuringu tulemused viitavad sellele, et esimestel elunädalatel pärast koorumist ei ole jaanalindude peensoole morfoloogia veel lõplikult välja kujunenud, mistõttu ei suuda jaanalinnu tibud söödast täielikult suhkruid omastada. Teostatud eksperimentaalne uuring SGLT1 immunolokalisatsioonist peensoole epiteelirakkudes eri vanuses jaanalinnu tibudel annab väga olulist teavet jaanalinnu tibude glükoosi transpordi ja ühtlasi toitumisvajaduste kohta.</p>			
Märksõnad: Glükoosi transport, immunohistokeemia, SGLT1, jaanalinnu soole epiteel			

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS.....	5
INTRODUCTION.....	6
1. LITERATURE REVIEW.....	8
1.1. Overview of glucose transporters.....	8
1.1.1. Facilitated-diffusion glucose transporters (GLUTs).....	9
1.1.2. Na ⁺ /glucose cotransporters (SGLTs).....	11
1.1.2.1. SGLT1 localization in mammals.....	13
1.2. Ostriches as agricultural animals.....	15
1.3. Morphology of ostriches' small intestine.....	17
2. AIM OF THE THESIS.....	19
3. MATERIAL AND METHODS.....	19
3.1. Animals and sampling.....	19
3.2. Laboratory methods.....	20
3.2.1. Tissue sample preparation.....	20
3.2.1.1. Fixation.....	20
3.2.1.2. Embedding.....	20
3.2.1.3. Microtome cutting.....	21
3.2.1.4. Deparaffinization and rehydration.....	21
3.2.2. Tissue sample staining.....	21
3.2.2.1. Hematoxylin and eosin staining.....	21
3.2.2.2. Immunohistochemical staining.....	22
3.2.2.2.1. Endogenous hydrogen peroxidase activity block.....	22
3.2.2.2.2. Antigen retrieval.....	22
3.2.2.2.3. Epitope blocking.....	23
3.2.2.2.4. Application of the antibodies.....	23
3.2.2.2.5. Detection of the primary antibody.....	23
3.3. Analysis of histological samples.....	24
3.4. Declaration of ethical considerations.....	24
4. RESULTS.....	24
4.1. Hematoxylin and eosin method.....	24
4.2. Immunohistochemistry method.....	25
5. DISCUSSION.....	30
CONCLUSIONS.....	33
ACKNOWLEDGEMENTS.....	34
REFERENCES.....	35
APPENDICES.....	43
<i>Appendix 1. IHC staining protocol.....</i>	<i>44</i>
<i>Appendix 2. Publications.....</i>	<i>45</i>
<i>Appendix 3. Non-exclusive licence for depositing the final thesis and opening it for the public and the supervisors' confirmation for allowing the thesis for defence.....</i>	<i>46</i>

LIST OF ABBREVIATIONS AND SYMBOLS

BBM - brush border membrane

DAB - 3,3'-diaminobensidine

G - goblet cell

GLUT - facilitated-diffusion glucose transporter

HE - hematoxylin and eosin

HIER - heat-mediated epitope retrieval

IHC - immunohistochemistry

LSAB - labeled streptavidin-biotin

OCFS - ostrich chick fading syndrome

PBS - phosphate buffer saline

SGLT – sodium/glucose cotransporter

TBS - tris buffered saline

INTRODUCTION

Glucose is the primary source of energy in all living organisms (Takata, 1996; Hussar *et al.*, 2016). In animals, it is transported across cell membranes by facilitated-diffusion glucose transporters (GLUTs) and sodium/glucose cotransporters (SGLTs) (Sala-Rabanal *et al.*, 2016; Sano *et al.*, 2020). Although well described in mammals, information about them is lacking in many species of birds, including the ostriches. In the small intestine – the main site of nutrient absorption - SGLT's and GLUT's work together to transport dietary monosaccharides from the intestinal lumen into the blood circulation (Takata, 1996; Sano *et al.*, 2020). The main glucose transporters in the small intestine are GLUT2, GLUT5 and SGLT1, of which SGLT1 alone is responsible for the uptake of glucose from the intestinal lumen into the enterocytes (Li *et al.*, 2004; Yoshikawa *et al.*, 2011). In humans and rats, a deficiency of SGLT1 in the small intestine leads to unabsorbed glucose, which in turn causes osmotic diarrhea and rapid death due to dehydration (Gorboulev *et al.*, 2012). A syndrome with the same clinical picture, called ostrich chick fading syndrome (OCFS), is encountered by many ostrich farmers, where up to 50% of under one-month-old chicks die (Terzich and Vanhooser, 1993; Cloete *et al.*, 2001). Even though the aetiology of OCFS is unknown, feeding practices might play an important role in the disease picture, since morbidity decreases when feeding is started after seven to 10 days of life compared to immediately after hatching (More, 1996; Shanawany, 1996). Scientific research about the expression of glucose transporters in ostrich chicks' small intestine is crucial for understanding young ostriches' absorptive capacity of glucose, and thus, their nutritional requirements. This information is especially important, as unabsorbed glucose might cause diarrhea also in the ostriches.

Ostriches, *Struthio camelus var. domesticus*, are important agricultural animals for their feathers, skin, healthy red meat, and medical purposes (El-Wahab *et al.*, 2012). They acclimatize well to different climates and have successful growth and reproductive performances, making them relatively easy birds to farm (Skadhauge *et al.*, 1984; Cooper and Horbanczuk, 2004). However, little scientific information exists about ostrich management; thus, many farmers have based their management practices, including feeding regimes, on research made on poultry, even though their nutritional and environmental requirements are different (Cooper and Horbanczuk, 2004). It has been described in literature, that the small intestine of ostrich's chicks continues to develop still months after hatching affecting the digestive capacity (Wang and Peng, 2008). In addition, GLUT 2 and -5 are poorly expressed in

newly hatched chicks' gastrointestinal tract, suggesting poor glucose and fructose absorption at young age (Hussar *et al.*, 2016). However, to the authors knowledge, no study has yet been conducted to assess the expression of SGLT1 in the ostriches' small intestine, thus information is lacking about one essential glucose transporter needed for glucose absorption.

As the knowledge about glucose transporters in the gastrointestinal tract provides important information about the body's ability to absorb monosaccharides, this cross-sectional study was conducted to immunolocalize SGLT1 for the first time in the duodenum and terminal zone of ileum in 1-, 14-, and 28-day old ostriches. Photos of the samples were saved on a computer for analysing and the expression of SGLT1 was described and compared between the different age groups and intestinal segments to assess the development of glucose transport in the small intestines of growing ostriches.

1. LITERATURE REVIEW

1.1. Overview of glucose transporters

Glucose transporters are membrane proteins that transport glucose and structurally similar substances across cell membranes. There are two major families of glucose transporters – the passive facilitated-diffusion glucose transporters (GLUTs) and the active sodium/glucose cotransporters (SGLTs) (Sala-Rabanal *et al.*, 2016; Sano *et al.*, 2020). There are three classes of GLUTs depending on the amino acid sequence of their proteins: class I facilitative transporters (GLUT 1-4), class II facilitative transporters (GLUT 5, 7, 9, 11), and class III facilitative transporters (GLUT6, 8, 10, 12 and the proton myo-inositol cotransporter GLUT 13) (Sano *et al.*, 2020). SGLTs contain at least six different isoforms (SGLT1-6), of which most research has been conducted about SGLT1 and SGLT2 (Wright *et al.*, 2011; Matsuo *et al.*, 2020).

GLUTs transport monosaccharides through facilitated diffusion towards their concentration gradient, whereas SGLTs are responsible for the active transport of monosaccharides against their concentration gradient with the help of sodium ions (Wright *et al.*, 2011; Sano *et al.*, 2020). According to Sala-Rabanal *et al.* (2016), GLUTs are found in abundance all over the body, whereas functional SGLTs are mainly expressed in the intestines and kidneys. However, GLUTs and SGLTs often work together in different tissues, for example, in the intestinal tract, to maintain glucose homeostasis (Yoshikawa *et al.*, 2011).

The phospholipid bilayer of the plasma membrane of the intestinal epithelium makes it impermeable to molecules like glucose (Takata, 1996). Therefore, glucose transporters are needed for the absorption of sugars from the intestinal tract into the blood circulation (Yoshikawa *et al.*, 2011; Sano *et al.*, 2020). SGLT1 and GLUT5 are found on the apical membrane of the epithelial cells, where SGLT1 is responsible for the transport of glucose and some galactose, and GLUT5 is responsible for the transport of only fructose into the enterocytes. After the initial transport of glucose and fructose, GLUT2 aids in the passive transport of the monosaccharides from the enterocyte into the hepatic circulation (Yoshikawa *et al.*, 2011).

In the kidneys, SGLT2, GLUT1, and GLUT2 work together to reabsorb glucose from the primary urine to regulate urinary glucose output. In the apical membrane of the proximal convoluted tubule, SGLT2 transports glucose molecules into the tubular cells, where GLUT1

and GLUT2 then transport them back into the blood circulation (Kanai *et al.*, 1994; Rieg *et al.*, 2014). In addition to having important physiological roles, glucose transporters also take part in many pathologies, for example, diabetes mellitus, and some cancers, thus the transporters might play important roles also when treating different diseases (Dyer *et al.*, 2002; Godoy *et al.*, 2006).

1.1.1. Facilitated-diffusion glucose transporters (GLUTs)

There are three classes of GLUTs depending on the amino acid sequence of their proteins: class I facilitative transporters (GLUT 1-4), class II facilitative transporters (GLUT 5, 7, 9, 11), and class III facilitative transporters (GLUT6, 8, 10, 12 and the proton myo-inositol cotransporter GLUT 13) (Sano *et al.*, 2020). GLUTs are found in abundance all over the body but are most important in the brain and liver tissues (Sala-Rabanal *et al.*, 2016).

GLUTs, especially class I facilitative transporters, are important for maintaining glucose homeostasis, thus defects in their function might cause detrimental effects in the body (Matsuo *et al.*, 2020). GLUT2 is expressed in the liver, but according to a study by Guillam *et al.* (1998), hepatic glucose metabolism, thus glucose homeostasis, is not affected by the absence of the transporter. This means that even though GLUT2 plays an important role in the hepatic regulation of blood glucose, there are also other pathways affecting it. However, the transporter might be important in the disease mechanism of liver cancers since an over-expression of GLUT2 is described with the disease. An increased expression of GLUT5 has also been described in approximately one-third of liver carcinomas, as well as in other cancers such as breast and colon adenocarcinomas and seminomas of testes (Godoy *et al.*, 2006; Sala-Rabanal *et al.*, 2016).

GLUT1 and GLUT3 are important for glucose transport across the blood-brain barrier. GLUT1s are found in the cerebral microvascular endothelial cells and in neuroglial cells, and GLUT3s are found in the neurons of the hippocampus, cerebral cortex, hypothalamus, and thalamus (Hou *et al.*, 2007). In a study by Hussar *et al.* (2012), GLUT1 receptors were also expressed in the olfactory epithelium. In the brain, these receptors are not regulated by insulin, but rather by other factors such as blood glucose levels. In rats with chronic hyperglycemia, both GLUT1 and GLUT3 are, however, downregulated and when the hyperglycemic conditions are fixed, the expression of both transporters increase. The downregulation is probably a protective mechanism to avoid cell damage in the brain cells from excessive glucose (Hou *et al.*, 2007).

From the class II facilitative glucose transporters, GLUT5 and GLUT11 are genomically very similar to each other compared to the other transporters of the same class. Even though there are genetic similarities, GLUT5 is a fructose-specific glucose transporter, whereas GLUT11 can transport both fructose and glucose (Doege *et al.*, 2001). Expression of GLUT5 is seen in the intestinal tract and many tumors with high demands for fructose as energy (Godoy *et al.*, 2006; Yoshikawa *et al.*, 2011), whereas GLUT11 is expressed in many tissues including the brain, placenta, heart and skeletal muscle, liver, kidneys, pancreas, and lungs. GLUT7 transports glucose and fructose in the small intestine, colon, prostate, and testes (Doege *et al.* 2001; Li *et al.*, 2004).

In the gastrointestinal tract, GLUTs and SGLTs work together to absorb dietary sugars. The small intestine is the main site of nutrient absorption, but its phospholipid bilayer of the plasma membrane makes it impermeable to molecules like glucose, thus glucose transporters are necessary for the absorption of monosaccharides from the intestinal lumen into the blood circulation (Takata, 1996; Sano *et al.*, 2020). There is intense expression of GLUT1, GLUT2, GLUT5, and SGLT1 in the gastrointestinal tract (Yoshikawa *et al.*, 2011; Hussar *et al.*, 2017). However, the expression of GLUT1 is insignificant in the small intestines as it is mostly expressed in the stomach and large intestines (Yoshikawa *et al.*, 2011). In the ileum, there is also some expression of GLUT7, thus it probably has a role in the absorption of glucose and fructose at the end of carbohydrate digestion. In the small intestine, the absorption of sugars starts with SGLT1 and GLUT5 transporting monosaccharides from the apical membrane of the enterocyte inside it, where GLUT5 mediates mainly the uptake of fructose. After the initial absorption of monosaccharides, GLUT2 transports the glucose and fructose from the enterocyte into the hepatic circulation (Li *et al.*, 2004; Yoshikawa *et al.*, 2011). In ostriches, GLUT2 and GLUT5 have been described to be poorly expressed in the newly hatched chick's duodenum and ileum, but the expression starts to get stronger at approximately one week of age (Hussar *et al.*, 2016).

In female mammals, GLUT1, GLUT3, GLUT4 and SGLT1 are found in the endometrium (Korgun *et al.*, 2001; Salker *et al.*, 2017). According to von Wolff *et al.* (2003), the expression of GLUT1 in the endometrium of healthy women increases rapidly during the phase of decidualization, a phase essential for embryonal implantation, whereas women with idiopathic fertility have been observed to have less expression of GLUT1 in the endometrium. The increased expression of GLUT1 is probably rather related to the process of decidualization than the effect of gestational hormones or cytokines, thus there is a possibility that decidualization

happens because of increased expression of GLUT1 and not *vice versa*. This would mean, that the expression of GLUT1 in the endometrium is crucial for decidualization and implantation to happen, and therefore crucial for gestation to continue.

1.1.2. Na⁺/glucose cotransporters (SGLTs)

Glucose transporters of the sodium/glucose cotransporter (SGLT) family are expressed in many different tissues of the body including the intestines, kidneys, brain, liver, and lungs (Díez-Sampedro *et al.*, 2003; Sala-Rabanal *et al.*, 2016). There are six isoforms of SGLTs (SGLT1-6), of which SGLT1 and SGLT2 have been the most studied and are primarily expressed in the intestines and kidneys, respectively (Chen *et al.*, 2010; Matsuo *et al.*, 2020; Sano *et al.*, 2020).

Through active transport, SGLT1 transports one molecule of glucose in exchange for two sodium molecules, but it can also transport sodium passively across cell membranes in the absence of glucose. The passive transport of sodium ions is able to cause an osmotic gradient between cell membranes, causing SGLT1 to also act as a water channel (Loo *et al.*, 1999). For example, in the salivary glands of rats, SGLT1 is partly responsible for salivary production through its action as a water channel (Sabino-Silva *et al.*, 2009). In addition, it also transports urea both passively and actively (Leung *et al.*, 2000). In the hippocampus and cerebellum of humans, SGLT1 acts not only as a glucose transporter, but also as a water, sodium, and urea transporter and is important for energy homeostasis, food regulation and learning (Wright *et al.*, 2011). In the hypothalamus, SGLT1 can depolarize glucose-sensitive neurons causing cell signaling (O'Malley *et al.*, 2006). According to Elfeber *et al.* (2004), an upregulation of SGLT1 happens in the brain after a hypoxic event, suggesting it having an important role during brain infarcts and might, therefore, be important when treating them. In oocytes, SGLT1 transports both glucose and water into the cells (Wright *et al.*, 2011). It might also play an important role the fertility and gestation of female mammals, as smaller litter sizes and birth weights have been observed in mice with weaker expression of SGLT1 in the endometrium (Salker *et al.*, 2017).

The expression of SGLT1 in the intestinal tract is regulated by different factors, including genetics, hormones, and carbohydrate contents of feed (Batchelor *et al.*, 2011; Sano *et al.*, 2020). SGLT1 is responsible for the initial absorption of glucose and galactose from the intestinal lumen into the enterocyte. In humans, SGLT1 deficiency in the small intestine causes glucose-galactose malabsorption characterized by severe acidic, osmotic diarrhea and dehydration due to unabsorbed dietary sugars and sodium (Gorboulev *et al.*, 2012). The diarrhea will resolve immediately when glucose and galactose are removed from the diet. However, the condition leads to death unless the abovementioned monosaccharides are removed from the diet

due to severe dehydration caused by the continuous diarrhea (Scheepers *et al.*, 2004; Wright *et al.*, 2011). On the contrary, increased amounts of SGLT1 can protect the intestines against infections by protecting the epithelial cells from cell barrier defects and from bacterial lipopolysaccharide-induced cell death, thus SGLT1 might have important immunological roles as well (Yu *et al.*, 2005).

In the kidneys, SGLT2 is expressed on the apical membrane of the early proximal convoluted tubule, whereas SGLT1 is expressed on the distal convoluted tubule (Kanai *et al.*, 1994; Vallon *et al.*, 2011; Gorboulev *et al.*, 2012). SGLT2 is responsible for up to 97% of the glucose reabsorption, whereas SGLT1 reabsorbs the remaining glucose, approximately 3%, from the primary urine (Rieg *et al.*, 2014). Changes in the expression of SGLT1 dose not change the kidneys' ability to reabsorb glucose enough to cause glucosuria, as is the case with decreased expression of SGLT2 (Kanai *et al.*, 1994). Inhibition of SGLT2 in the kidneys is associated with increased food and water intake due to increased excretion of glucose and water through urine, which the body tries to compensate for. However, this inhibition can be used to treat hyperglycemia in diabetic human patients. Chronic hyperglycemia destroys pancreatic tissue, and thus, treating the hyperglycemia with SGLT2 inhibitors leads to increased pancreatic function and the possibility for the pancreas to start producing insulin again (Jurczak *et al.*, 2011).

SGLT3 is a non-functional transporter, and thus, does not transport glucose. Instead, it mediates membrane potentials in response to increased glucose concentrations (Díez-Sampedro *et al.*, 2003; Bianchi and Díez-Sampedro, 2010). It is expressed in cholinergic neurons of the intestines and neuromuscular junctions of acetylcholine receptors of skeletal muscles. As the motility of the small intestine is regulated by cholinergic neurons, glucose might play an important role in mediating peristaltic movements after feeding by depolarizing the cell membranes through the glucose-sensitive SGLT3 (Díez-Sampedro *et al.*, 2003). The expression of SGLT3 is affected by obesity, leading to a downregulation of its expression in the small intestinal epithelium (Soták *et al.*, 2021). On the contrary, the expression of SGLT1 in the duodenum is upregulated with obesity, causing increased glucose absorption from the small intestine in overweight animals. Increased absorption of dietary glucose is further related with increased release of gastric inhibitory polypeptide and glucagon, and decreased release of glucagon-like-peptide-1, which together causes hyperglycemia, and thus, hyperinsulinemia, which can predispose to diabetes mellitus type two (Nguyen *et al.*, 2015). It is also known that diabetic patients have an increased capacity to absorb dietary sugars from the intestinal tract

due to increased expressions of SGLT1, GLUT2 and GLUT5, and thus, the glucose transporters are of great importance in the disease mechanism of obesity and diabetes mellitus (Dyer *et al.*, 2002).

Little is known about the function of SGLT4. According to Tazawa *et al.* (2005), it can transport mannose, fructose, and glucose and is expressed mostly in the intestines, kidneys, and liver, thus it might play an important role in the body's sugar metabolism. Intense expression of SGLT4 is seen in the intestines and kidneys, moderate expression in the liver and weak expression in the stomach, trachea, and brain.

SGLT5 is a mannose and fructose transporter mainly found in the kidneys (Grempler *et al.*, 2012). It is located on the epithelial tissues of the glomerulus and on the convoluted tubules, where it reabsorbs fructose from the filtrate and primary urine. SGLT5 probably works together with GLUT2 in the kidneys, by first transporting fructose into the tubular cells through the apical membrane, after which GLUT2 transports the fructose through the basolateral membrane back into the blood circulation. A decreased expression of SGLT5 causes increased excretion of fructose through urine, thus the transporter is important for fructose reabsorption (Fukuzawa *et al.*, 2013).

SGLT6 is expressed in the brain and small intestine. In the brain, expression is described intracytoplasmic in neuronal cells within axons and dendrites, whereas it in the small intestines is expressed in the cells of lamina propria and myenteric plexuses (Baader-Pagler *et al.*, 2018). There is strong expression in many brain regions, the most intense being in the substantia nigra and spinal cord (Chen *et al.*, 2010). Due to the high expression of SGLT6 in the brain, there has been suggestions of it playing a role in the regulation of food intake. However, according to a study by Baader-Pagler *et al.* (2018), no link has been found between the expression of SGLT6 and food intake in mice.

1.1.2.1. SGLT1 localization in mammals

SGLT1 is mainly expressed in the small intestine (Li *et al.*, 2004; Yoshikawa *et al.*, 2011; Gorboulev *et al.*, 2012). However, it has also been described to be expressed in other parts of the body including the kidneys, large intestine, brain, and the uterus (Sano *et al.*, 2020; Yoshikawa *et al.*, 2011 Yu *et al.*, 2010).

For a long time, it was thought that SGLT1 was only found in the intestines and kidneys (Turk *et al.*, 1994). In newer studies, it has been reported to be expressed also in other tissues, for example, in the brain, T-lymphocytes, pancreas, and the liver (Sano *et al.*, 2020; Wright *et al.*,

2011). In the brain, SGLT1 is expressed in the cerebral cortices, amygdala, hippocampus, and hypothalamus (Yu *et al.*, 2010). In the brain of rats, expression has been described in the neurons and luminal membrane of the brain capillaries (Elfeber *et al.*, 2004). According to Sala-Rabanal *et al.* (2016), there is expression also in the liver, skeletal muscles, and cardiomyocytes of mice. However, the role of SGLT1 in glucose utilization is smaller than for other glucose transporters, thus its expression in different tissues does not mean it is physiologically important.

In the small intestine, SGLT1 is intensely expressed on the apical membranes (brush border) of the villous epithelial cells (Elfeber *et al.*, 2004; Yoshikawa *et al.*, 2011). In mice, the expression stays intense from the duodenum to the end of ileum. On the intestinal villae, the most intense expression is seen from the crypt-villus junction until the middle of the villae. There is no expression in the crypts, and decreased expression is observed also higher up on the villae until the tips (Yoshikawa *et al.*, 2011). Similar expression of SGLT1 has been described in dogs and cats, who also have the most intense expression in the middle of the villae and no expression in the crypts (Batchelor *et al.*, 2011). SGLT1 has not been observed to be expressed in the capillaries of the small intestine (Elfeber *et al.*, 2004). However, significant expression of SGLT1 has been found on the luminal surface of the colonic enterocytes in mice. The expression is intense in the proximal colon and rectum, suggesting that glucose absorption might happen throughout the intestinal tract in some species (Yoshikawa *et al.*, 2011). The expression of SGLT1 has also been observed in the colon of pigs, humans, and chicken (Bindslev *et al.*, 1997; Yoshikawa *et al.*, 2011).

In the kidneys, SGLT1 is expressed in the more distal part of the proximal convoluted tubule (Vallon *et al.*, 2011). It is also expressed in acinar and myoepithelial cells of parotid salivary glands of rats, being partly responsible for salivary production (Sabino-Silva *et al.*, 2009). Elfeber *et al.* (2004) has reported, that expression of SGLT1 is also present in the acinar cells of the submandibular salivary glands of rats. In female mammals, expression of SGLT1 has been described in the endometrial epithelium, reaching its peak intensity during the time of embryonal implantation (Salker *et al.*, 2017). After parturition, expression has been noted in the lactating mammary glands (Carbó and Rodríguez, 2023).

1.2. Ostriches as agricultural animals

Ostriches, *Struthio camelus var. domesticus*, are flightless birds belonging to the family Ratites. The family includes, in addition to the ostriches, emus and rheas (Basuny *et al.*, 2017). Ostriches originate from Africa, and the first commercial ostrich farm was established in South Africa in the 1860's, where they were farmed for their feathers. Ostrich farming started gaining popularity all over the world because of the growing demand for their healthy meat, skin, and feathers (El-Wahab *et al.*, 2021). As ostriches can acclimatize well to different climates, it is possible to farm these animals all over the world (Skadhauge *et al.*, 1984). There is relatively little scientific information about ostrich management, thus many farmers have based their management practices on research made on poultry (Cooper and Horbanczuk, 2004).

Ostriches have very successful growth and reproductive performance (Cooper and Horbanczuk, 2004). However, one significant problem with ostrich farming is high chick mortality rates, with almost 50% of the ostriches dying within the first month after hatching (Cloete *et al.*, 2001). The high mortality rates might partly be caused by an inability to absorb dietary sugars from the feed (Hussar *et al.*, 2016). It is known, that the secretion of the digestive enzymes amylase, lipase, and trypsin increase with age in the ostriches. At four days old, all the abovementioned digestive enzymes are secreted in smaller amounts compared to when being seven days and older, thus the young chicks' capacity to digest carbohydrates, fats and proteins is not fully developed at the time of hatching (Noy and Sklan, 1995). The mortality rate of the chicks varies between different farms. In some farms, chick mortality can be under 30%, whereas in other farms it is almost 90%. Due to the big variations, farm management possibly plays an important role in chick survival (Terzich and Vanhooser, 1993). According to Verwoerd *et al.* (2008) the most common cause of ostrich chick mortality is the bacterial pathogen *Escherichia coli*, whereas Terzich and Vanhooser (1993) state, that ostrich chick fading syndrome (OCFS) is the main cause. OCFS is characterized by depression, diarrhea, anorexia, and death within 3-5 days. The etiology of OCFS is unknown but no specific pathogen has been associated with the affected chicks (More, 1996). However, the clinical picture of OCFS is very similar to what humans and rats experience when affected by glucose-galactose malabsorption. According to More (1996), the main causes of death in the chicks are lower-limb deformities and OCFS. The third most common cause of death was Salmonellosis, though all deaths reported were from one single farm where domestic birds had contact with wild birds. Young ostriches are, however, very susceptible to many stressors, including nutritional,

environmental, and social stress, which can, together with management practices, significantly affect the development of both contagious and non-contagious diseases (Verwoerd *et al.*, 2008).

According to Shanawany (1996), ostrich hatchlings do not need additional feed during the first seven to ten days of life, since they can rely on the yolk sac for nutrients. It has been observed, that refraining from additional feed encourages the chicks to eat the yolk sac and drink more water, as well as decreases the occurrence of OCFS. Therefore, feeding practices might play an important role in the health of the young chicks. However, according to Cooper and Horbanczuk (2004), the chicks should always get additional feed from the very beginning of life for more balanced nutrition. Cooper and Horbanczuk (2004) state, that the reason for high mortality rates in young chicks is rather due to the wrong type of feed instead of the starting point of feeding, since many farmers rely on research results made on poultry and might therefore feed their ostriches incorrectly. It has been observed, that unbalanced breeder or chick ratios in ostriches increase the risk of inappetence, leading to death within the first few weeks of life, poor food conversion rates, decreased growth rates, leg deformities, lowered immunity, increased stress levels, and loss of feathers. For instance, ostrich chicks fed on all-maize diets often have poor body conditions and are paretic due to nutritional deficiencies. The chicks often suffer from deficiencies in selenium, vitamin E, and pantothenic acid and histopathology examinations reveal degeneration and necrosis of muscle fibers and arterioles. Even though feeding seems to play an important role, Cloete *et al.* (2001) describes high mortality rates also caused by stress alone.

In the nature, adult ostriches feed 64% of the time on gravel plains and 26% of the time on desert washes foraging green vegetation (Williams *et al.*, 1993). They mainly forage on forbs and climbing plants, graminoids, shrubs, succulents, and trees (Milton *et al.*, 1994). There is, however, little information about the behavior and feeding of ostrich chicks in the wild. According to Birdfact (2022), the mothers do not feed their hatchlings. For nutrition, the young chicks eat their yolk sacs after hatching and start following other birds from their flock after a few days of life when they have learned to walk. The older ostriches lead the chicks to the feed, where they instinctively start foraging at the age of one to two weeks.

Farming ostriches has economic benefits compared to chicken, which is already more affordable than bovine or sheep farming. From ostriches, in addition to eggs and meat, farmers benefit also from their skin and oils. The environmental impact is also lower in ostrich production systems compared to chicken production systems, even though the aquatic and

terrestrial ecotoxicity and human toxicity is higher due to high emissions of methane, nitrous oxide, and hydrogen sulfide (Ramedani *et al.*, 2019).

Ostrich meat has a relatively high nutritional value because of its low fat and cholesterol content and high content of good fatty acids, making it a relatively healthy red meat. The liver contains high levels of iron, four times more than the livers of chicken or turkey, thus it can be used as a source of iron in people suffering from iron deficiency (Majewska *et al.*, 2016). Ostriches are also valuable animals for their leather and eyes. Their eyes are used in corneal research making them important also in the medical field. As the ostrich eye is relatively large and its anatomical structures, especially the cornea, is similar to the human eye, ostriches are good candidates for corneal transplants for humans (Liu *et al.*, 2016).

Ostrich oil has been used in pharmaceutical and cosmetic industries in the treatment of eczema, contact dermatitis, burns, dry hair, bedsores, and muscle pains (Ramedani *et al.*, 2019; Eltom *et al.*, 2021). There is, though, little research about the oil's properties but the anti-inflammatory property of ratiite oils comes from γ -lactone. Emu oil is known to reduce inflammation in mice, and γ -lactone isolated from ostrich oil has been shown to decrease formalin-induced paw edema in rats more efficiently than ibuprofen suspension or diclofenac gel by significantly reducing skin thickness and inflammation (Eltom *et al.*, 2021).

1.3. Morphology of ostriches' small intestine

Relatively little is known about ostriches' organ systems, especially the gastrointestinal tract of young chicks (Bezuidenhout and van Aswegen, 1990; Dūrītis, 2011). It is, however, known that the gastrointestinal system of adult ostriches differs considerably from poultry and non-ruminant animals. The small intestine is relatively short compared to the large intestine, which can be up to three times longer than the small intestine (Cooper and Mahroze, 2004). According to Skadhauge *et al.* (1984), the duodenum of an adult ostrich is approximately 150 cm long, and the jejunum and ileum are 6-8 meters long, whereas Fowler (1991) and Bezuidenhout (1986) measured the duodenum to be 80 cm, jejunum 160 cm and ileum 400 cm long, thus variations in the small intestinal length of adult ostriches has been recorded. In the abdominal cavity, the duodenum forms a loop from right to left, with the pancreas in between them (Fowler, 1991; Cooper and Mahroze, 2004). The jejunum and ileum are coiled between the gizzard and pelvis. The submucosa is well-developed throughout the gastrointestinal tract and the intestinal villae of the small intestines are long, forming a labyrinth-like surface on the

mucosa. Duodenum causes a “duodenal reflux”, where ingested feed moves back from the proximal half of the small intestines, back into the stomach. This improves ostriches’ digestion due to the feed being exposed to duodenal and pancreatic enzymes up to three times (Cooper and Mahroze, 2004).

All small intestinal anatomical regions (duodenum, ileum, and jejunum) consist histologically of the mucosal layer (*tunica mucosa*) with submucosa, muscular layer (*tunica muscularis*), and serosal layer (*tunica serosa*) (Wang and Peng, 2008). The duodenum and jejunum are histologically very similar having a thick mucosal layer lining the innermost surface of the intestines. The mucosa is made of columnar epithelium, consisting mainly of enterocytes and goblet cells (Herrero, 2023). Besides these cell types, enteroendocrine cells have been described in the intestinal epithelium (Dūrītis *et al.*, 2021). The goblet cells are mucin-producing cells, important for epithelial cell protection and nutrient transport (Wang and Peng, 2008). They are very short-lived, with a life span of 2-3 days in mice, during which they undergo morphological changes constantly. The goblet cells found on the upper regions of the small intestinal villi are already mature mucin-producing cells, whereas ones lower on the villi are still immature (Specian and Oliver, 1991). In growing ostriches, the greatest amount of goblet cells can be observed in the duodenum and ileum when the ostriches are 45 days old (Wang and Peng, 2008). The apical membrane of the enterocyte contains numerous microvilli with digestive enzymes and nutrient transporters, thus is creating the functional brush border membrane of the small intestine. The enterocytes’ function is to absorb nutrients through the brush border membrane into it, and either utilize the nutrients or transport them further through the basolateral membrane into the blood circulation (Zhang *et al.*, 2019). At the base of the villus, intestinal crypts are located (Herrero, 2023). Paneth cells have not been described in ostriches’ intestinal epithelium even though found in other species of birds (Porter *et al.*, 2002). The ileal mucosa is similar to the more proximal segments of the small intestine, except there are fewer, thicker, and shorter villi. The submucosa is poorly developed and consists mostly of loose connective tissue. The muscular layer, which is thinner in the duodenum than in the jejunum, has an internal circular and outer longitudinal muscle layer. The serosa is the outermost layer and consists of mesothelium and loose connective tissue. The muscular and serosal layers are histologically similar in all small intestinal segments (Herrero, 2023).

The body weight of ostrich chicks and the weight and length of the small intestine increase most during days 1 to 90 of life. The relative weight of the whole small intestine peaks at day 41 of life, after which it starts to decline. However, the relative weight of the duodenum increase

fastest until 90 days of age (Cooper and Mahroze., 2004). The length and width of the intestinal villi also grow until 90 days of age; thus the absorptive ability of the small intestines is developing also during that time. In the duodenum and jejunum, the depth of the crypts increases until 90 days of age, whereas they start to get smaller at 45 days of age in the ileum (Wang and Peng, 2008).

2. AIM OF THE THESIS

This cross-sectional study was conducted to immunolocalize Na⁺/glucose cotransporter 1 in the epithelial cells of the duodenum and terminal zone of ileum in 1-, 14-, and 28-day old ostriches, and to compare the strength of expression between different age groups and between the small intestinal segments. Specifically, the study aimed to provide new information about the expression of SGLT1 in the small intestines of young ostriches, thereby improving our knowledge about their digestive capacity of dietary sugars.

3. MATERIAL AND METHODS

3.1. Animals and sampling

Intestinal specimens from the duodenum and terminal zone of ileum were collected from 15 female ostriches (*Struthio camelus var. domesticus*) raised on an African ostrich farm in Latvia (Ozolini AB). Ostrich eggs were incubated in an incubator (Euro Elektronik KL-72S) at the hatchery facility, and on day 39 of incubation, they were transferred into a hatching chamber (Euro Elektronik KK-24S) (Dūrītis *et al.*, 2021). Three days after hatching, the chicks were placed into a box with heated floor and were provided with commercial ostrich chick feed (Strus Premium-Strus 1) and water *ad libitum*. The ostriches were equally divided into three age groups: 1-, 14-, and 28-day-old ostriches, with five birds per group. From each bird, one duodenal and one ileal sample was collected. For the control, one sample from each group and each intestinal segment were collected.

All chicks were euthanized with xylazine, ketamine and pentobarbital. The chicks received one intramuscular injection of 0.5 ml of 2% xylazine and 0.5 ml of 10% ketamine to minimize the

pain during euthanasia. After the intramuscular injection, 0.5 ml of 20% pentobarbital was administered as an intracardiac injection.

3.2. Laboratory methods

In this study, methods of histology (tissue fixation, paraffin-embedding, cutting by microtome and staining) were used. The samples were stained by routine histology method with hematoxylin and eosin, and for the immunohistochemical staining, labeled streptavidin-biotin method (LSAB) was used. All samples were treated the same, except the negative controls which did not contain the primary antibody (Rabbit anti-SGLT1).

3.2.1. Tissue sample preparation

3.2.1.1. Fixation

Fresh tissue samples, 0.5-1.0 cm in diameter, from 15 ostriches' duodenum and terminal zone of ileum were fixed in 10% neutral buffered formalin solution for 48 hours at room temperature. The fixation protects the tissue from microbial contamination and prevents it from drying out.

3.2.1.2. Embedding

The samples were rinsed with phosphate buffer saline (PBS) until the fixative was removed. As paraffin is insoluble in water, the samples were dehydrated in a tissue processor (TISSUE-TEK II) before being embedded into paraffin according to the standardized tissue histological procedure (Carson, 1997):

- Samples were rinsed with PBS until the fixative (formalin) was removed
- Samples were dehydrated by immersing them in increasing concentrations of ethanol:
 - 10 minutes in 50% ethanol
 - 10 minutes in 70% ethanol
 - 10 minutes in 80% ethanol
 - 10 minutes in 95% ethanol
 - 10 minutes in 100% ethanol

To remove residual ethanol, the samples were immersed in ethanol-xylene and 100% xylene accordingly:

- Ethanol-xylene in a ratio of 2:1 for 10-15 minutes
- Ethanol-xylene in a ratio of 1:1 for 10-15 minutes

- Ethanol-xylene in a ratio of 1:2 for 10-15 minutes
- 100% xylene for 10-15 minutes 3 times

The tissue samples were embedded into paraffin to get solid blocks of wax. It was done in 60°C followingly:

- Xylene-paraffin in a ratio of 2:1 for 30 minutes
- Xylene-paraffin in a ratio of 1:1 for 30 minutes
- Xylene-paraffin in ratio of 1:2 for 30 minutes
- 100% paraffin for 1-2 hours
- 100% paraffin for 24 hours

The samples were ready to be cut once they had solidified into blocks of wax.

3.2.1.3. Microtome cutting

The blocks were cut using a microtome (Microm HM360) to form 7 µm thick slices of the small intestinal tissues. The cut slices were floated on Poly-L-Lysine-coated slides (Thermo Fischer Scientific, USA).

3.2.1.4. Deparaffinization and rehydration

The samples were deparaffinized, dehydrated and rehydrated in a graded series of ethanol to enhance staining of the histological slides, since paraffin impedes it. It was carried out accordingly:

- Three washes in 100% xylene for 5 minutes each time
- Two washes in 100% ethanol for 10 minutes each time
- Two washes in 95% ethanol for 10 minutes each time
- Two washes in distilled water for 5 minutes each time

3.2.2. Tissue sample staining

3.2.2.1. Hematoxylin and eosin staining

The samples were stained with hematoxylin and eosin. Hematoxylin, a basic dye, stains acidic structures purplish-blue, whereas eosin, an acidic stain, dyes basic structures pink. Harris hematoxylin, an alcohol-based stain, was used for the counterstaining of nuclei, giving a deep purple color. The staining was done following routine histology according to a protocol by Carson (1997) as follows:

- The samples were stained blue by immersing in Harris hematoxylin for 5 minutes

- They were washed under running tap water for 5-10 minutes
- They were rinsed with distilled water
- They were differentiated with acid alcohol (0.3%) for 1-3 seconds
- They were rinsed with water
- They were immersed in eosin for 1-2 minutes
- They were dehydrated in alcohol for 2 minutes in 95% alcohol
- They were dehydrated two times in 100% alcohol, 2 minutes each time
- They were cleared with xylene for 1-2 minutes
- Each sample was mounted with 1-2 drops of Canada Balsam
- They were covered with cover slides

3.2.2.2. Immunohistochemical staining

The samples were immunohistochemically (IHC) stained according to the labeled streptavidin-biotin (LSAB) method as following (Appendix 1):

1. Polyclonal primary antibody Rabbit anti-SGLT1
2. Biotinylated secondary antibody Goat anti-Rabbit
3. Streptavidin-conjugated peroxidase with DAB (3,3'-diaminobenzidine) as chromogen

All the antibodies were products by the company Abcam (UK) and the IHC staining was carried out according to the manufacturer's guidelines (Abcam, 2020).

3.2.2.2.1. Endogenous hydrogen peroxidase activity block

The samples were treated with hydrogen peroxidase to avoid non-specific background staining accordingly:

- Covering the samples with 3% hydrogen peroxidase for 10 minutes
 - Hydrogen peroxidase was diluted to 3% with tris buffered saline (TBS)
- They were washed two times in the buffer

3.2.2.2.2. Antigen retrieval

The samples were pre-treated with heat-mediated antigen retrieval technique to access the proteins used for IHC staining. Antigen retrieval was done by using heat-mediated epitope retrieval technique (HIER) followingly:

- Samples were immersed in 10 mM Tris/1 mM EDTA (pH 9) buffer for 20 minutes
- Immersed samples were kept for 18 minutes in sub-boiling temperature

- They were cooled down by keeping them 30 minutes at room temperature
- They were washed 3 times in the buffer

3.2.2.2.3. Epitope blocking

Epitope blocking was done to prevent the non-specific binding of the antibodies to other epitopes on the tissues. The protein block was applied in room temperature for 5 minutes on the samples, after which washed in TBS one time.

3.2.2.2.4. Application of the antibodies

The samples were immunohistochemically stained by LSAB method using polyclonal primary antibody Rabbit anti-SGLT1 (Abcam, UK) as primary antibody and ready-to-use biotinylated Goat anti-rabbit as secondary antibody. The secondary antibody was provided in the immunohistochemistry kit (Abcam, UK) and was used together with the primary antibody according to the manufacturer's staining protocol (Abcam, 2020):

- The primary antibody was diluted 1:1000 with TBS containing 1% bovine serum albumin
- Diluted primary antibody was applied to the samples and incubated in a moisture chamber at 37°C for 30 minutes
- The samples were washed four times in the buffer
- The secondary antibody was applied on the samples and incubated at room temperature for 10 minutes
- The samples were washed in the buffer four times

For the negative controls, identical samples not containing the primary antibody was used. For positive control, examples of human duodenum tissue stained for SGLT1 was available for comparison on the antibody producer's homepage (<http://abcam.com>).

3.2.2.2.5. Detection of the primary antibody

Biotinylated secondary antibody and streptavidin-conjugated peroxidase were used for antibody detection by using 3,3'-diaminobensidine (DAB) as chromogen, dying positively stained cells brown. It was done as follows:

- Streptavidin peroxidase was applied to the samples at room temperature and incubated for 10 minutes
- The samples were washed four times in the buffer

- 20 µl DAB chromogen was added to 1 ml of DAB substrate, mixed and applied on the samples. The samples were incubated for 10 minutes after applying
- The samples were washed four times in the buffer

3.3. Analysis of histological samples

Photos of the slides were taken with a camera (AxioCam, HRc, Germany) connected to the microscope Zeiss Axioplan-2 Imaging (Germany) and saved on the computer for analyzing. The samples were analyzed by eye evaluating the immunolocalization and strength of positive staining (weak, moderate, strong) for SGLT1 in the immunohistochemically stained goblet cells and the epithelial brush border membrane. The staining was compared between the intestinal segments and the different age groups.

3.4. Declaration of ethical considerations

The study was carried out in accordance with the Guidelines laid down by the European Community's Council Directive of 24th of November 1986 (86/609/EEC). The Ethical Committee of Latvian University of Agriculture has also approved the experiment. There is no conflict of interest.

4. RESULTS

4.1. Hematoxylin and eosin method

The histological analysis of the intestinal tissue samples stained with hematoxylin and eosin revealed the histological structures of the duodenum and terminal zone of ileum in ostriches. Both intestinal segments consist of three layers: the mucosa (*tunica mucosa*), muscular layer (*tunica muscularis*), and serosa (*tunica serosa*), as shown in figures 1 and 2, respectively. The mucosa, which makes up the villi of the small intestine (*villae intestinalis*), is the innermost layer lining the lumen. In the duodenum, the villi are noticeably longer and more numerous than in the ileum, where there are shorter and fewer in numbers. In figure 1, numerous goblet cells can be seen in the mucosal layer. The submucosa of the mucosa is poorly developed in

both segments of the ostrich small intestine and the muscular layer is noticeably thicker in the ileum compared to the duodenum.

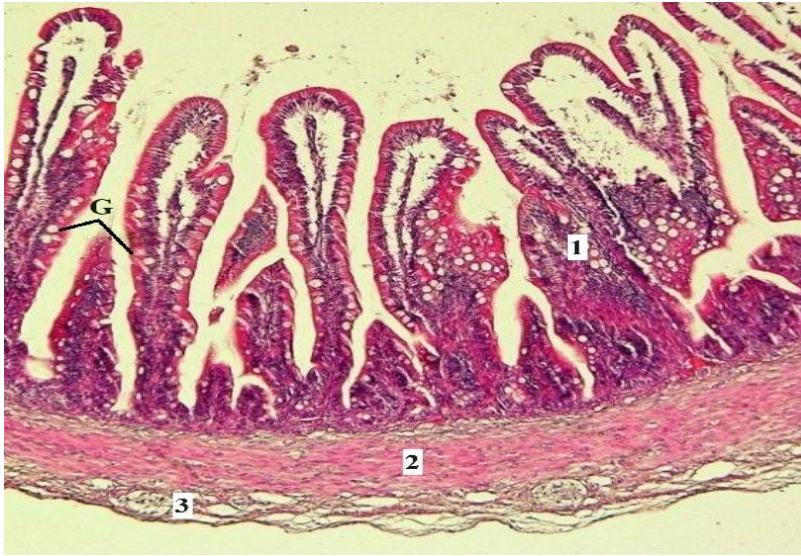


Figure 1. *Tunica mucosa* (1), *tunica muscularis* (2) and *tunica serosa* (3) of the ostrich duodenum. Goblet cells (G) nicely visible in the intestinal villi. Hematoxylin and eosin, 100x.

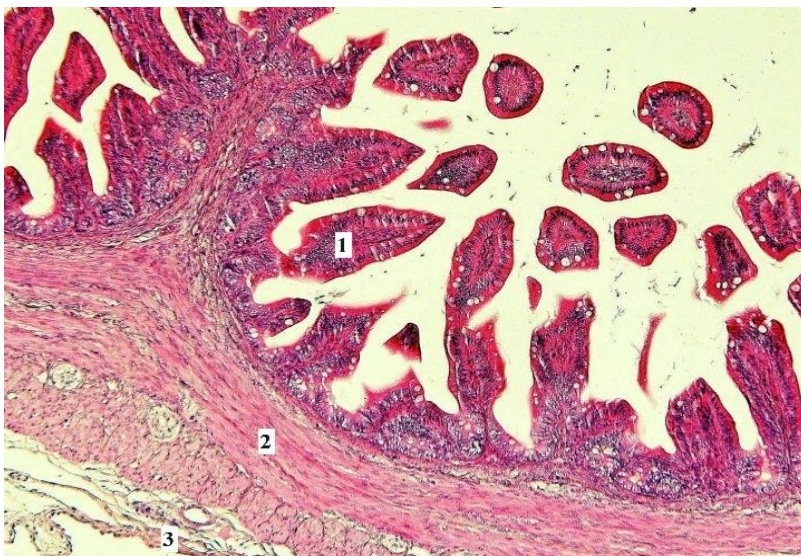


Figure 2. *Tunica mucosa* (1), *tunica muscularis* (2) and *tunica serosa* (3) of the ostrich ileum, Note the comparatively thick muscular layer. Hematoxylin and eosin, 100x.

4.2. Immunohistochemistry method

The results of the immunohistochemical (IHC) analysis revealed the immunolocalization and strength of the staining for SGLT1 in the epithelium of ostriches' duodenum and terminal zone

of ileum. SGLT1 was immunolocalized in the intestinal enterocyte's brush border membranes (BBM) and goblet cells (G).

The staining for SGLT1 in both the duodenum and terminal zone of ileum of one-day-old ostriches showed negative results in the goblet cells, whereas the brush border membranes of the epithelium were weakly stained (Figure 3 and Figure 4).

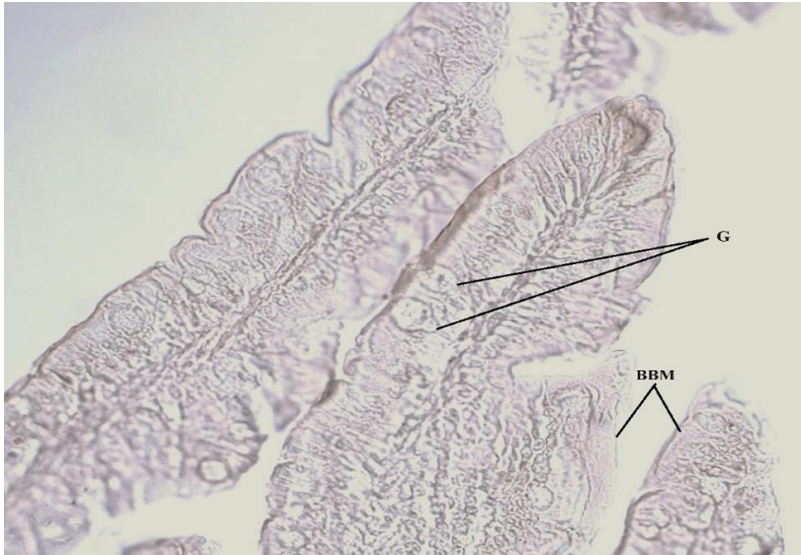


Figure 3. Weak staining for SGLT1 on the brush border membrane (BBM) of the duodenal epithelium of one-day-old ostrich. Goblet cells (G) are unstained. IHC, 200x.



Figure 4. Weak staining for SGLT1 on the brush border membrane (BBM) of the epithelium of the terminal zone of ileum in one-day-old ostrich. Goblet cells (G) are unstained. IHC, 400x.

In the duodenal epithelium of 14-day-old ostriches, the goblet cells remained mostly unstained, whereas the brush border membrane was weak to moderately stained for SGLT1 (Figure 5).

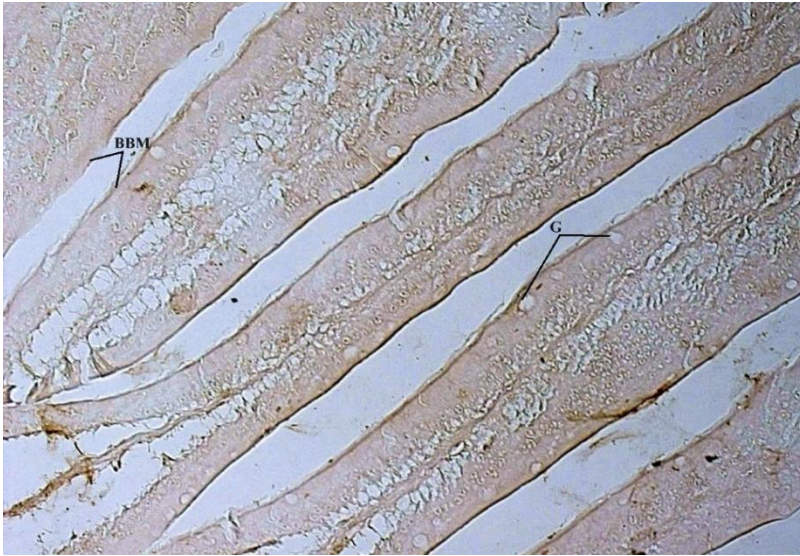


Figure 5. Moderate staining for SGLT1 only on the brush border membrane (BBM) of the duodenal epithelium of 14-day-old ostrich. Goblet cells (G) are unstained. IHC, 200x.

In comparison, the ileal epithelium showed moderately stained goblet cells and moderate to strong staining on the brush border membrane of the epithelium (Figure 6).

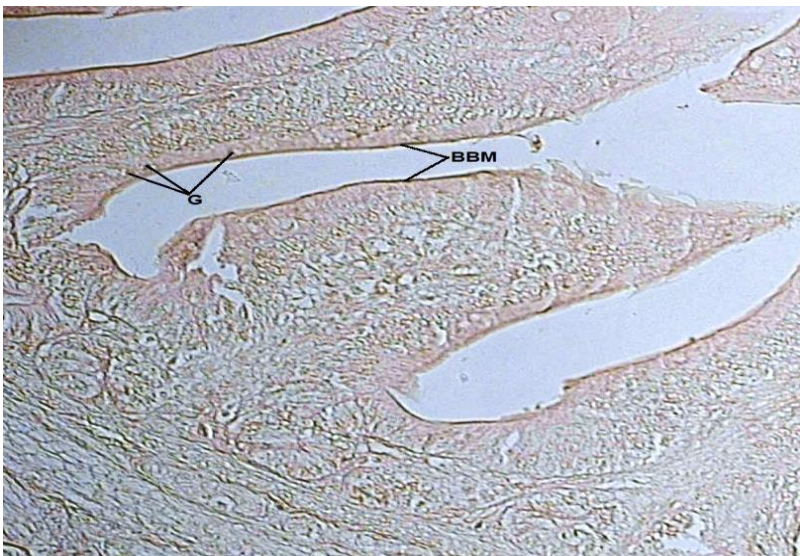


Figure 6. Moderate staining for SGLT1 in the goblet cells (G) and strong staining on the brush border membrane (BBM) of the terminal zone of ileum of 14-day-old ostrich. IHC, 200x.

At the age of 28 days, there was strong positive staining for SGLT1 in the goblet cells and on the brush border membrane of both the duodenal and ileal epithelium (Figure 7 and Figure 8).

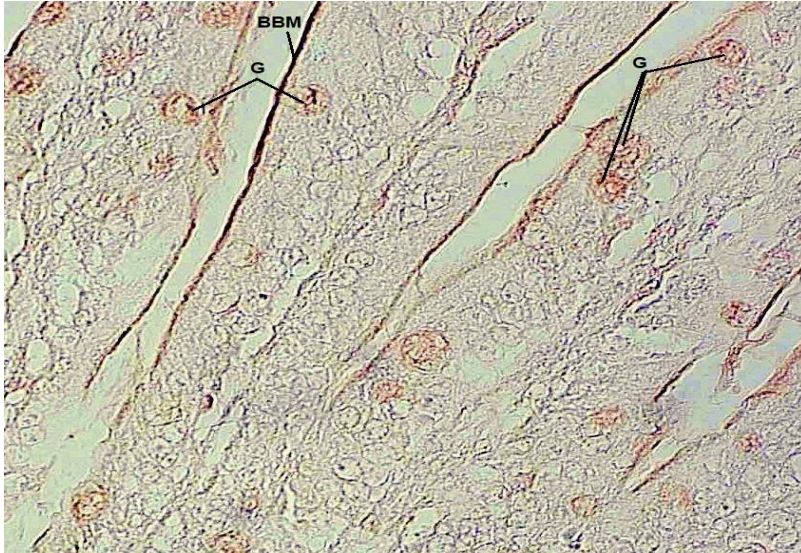


Figure 7. Strong staining for SGLT1 in the goblet cells (G) and on the brush border membrane (BBM) of the duodenal epithelium of 28-day-old ostrich. IHC, 400x.



Figure 8. Strong positive staining for SGLT1 in the goblet cells (G) and on the brush border membrane (BBM) of the epithelium of the terminal zone of ileum of 28-day-old ostrich. IHC, 400x.

The strength of expression of SGLT1 in the ostriches' small intestinal segments increased with age as presented in table 1. There were five samples that could not be interpreted due to the intestinal tissue being washed away during processing; one sample from one-day-old ostrich ileum, one sample from 14-day-old ostrich duodenum, and three samples from 28-day-old ostriches' ileum.

Table 1. The expression of SGLT1 in the epithelial cells of duodenum and terminal zone of ileum in one, 14-, and 28-day-old ostriches

Age group	Chick nr	Duodenum	Ileum
1 day	1.1	Weak	Weak
	1.2	Weak	Weak
	1.3	Weak	Weak
	1.4	Weak	Weak
	1.5	Weak	-
14 days	2.1	Weak	Moderate
	2.2	Moderate	Moderate
	2.3	Weak	Strong
	2.4	-	Moderate
	2.5	Moderate	Moderate
28 days	3.1	Strong	Strong
	3.2	Strong	Strong
	3.3	Strong	-
	3.4	Strong	-
	3.5	Strong	-

The results of the IHC-stained samples were compared with negative controls, where the staining for SGLT1 on the brush border membrane and the goblet cells of the intestinal epithelium was negative (Figure 9).

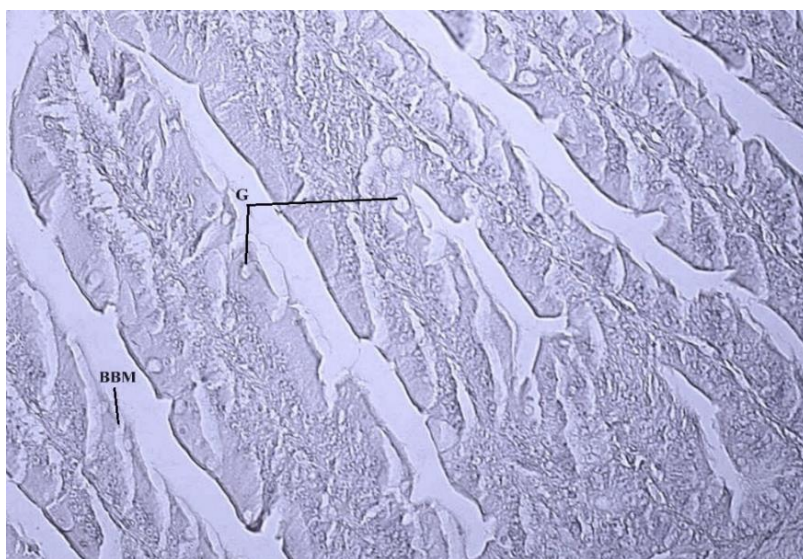


Figure 9. Control of duodenum of 14-day-old ostrich chick. Both the goblet cells (G) and the brush border membrane (BBM) of the epithelium are unstained. IHC, 100x.

5. DISCUSSION

The results of this study revealed the histological layers of the duodenum and ileum and immunolocalized SGLT1 on the mucosal epithelium of both intestinal segments. The histological analysis of the duodenal and ileal structures revealed the same observations as Herrero (2023) has described; three layers consisting of *tunica mucosa*, with poorly developed submucosa, *tunica muscularis*, and *tunica serosa*, with the muscular layer being thicker in the ileum than in the duodenum.

The positive IHC staining for SGLT1 was mainly observed on the brush border membranes of the enterocytes, but staining was present also in the goblet cells in the older age groups. These findings correspond with existing literature on small intestinal physiology, according to which the enterocytes' function is to transport nutrients through the apical and basolateral membranes (Zhang *et al.*, 2019). Thus, the presence of glucose transporters on the brush border membrane is crucial for the absorption of dietary sugars from the intestinal lumen.

This study also revealed that the strength of expression of SGLT1 in the small intestine of ostriches increase with age, indicating that the ability to absorb glucose from the small intestinal lumen is not fully developed in the beginning of life. The expression of SGLT1 was stronger in the terminal zone of ileum than in the duodenum already at the first day of age, suggesting glucose uptake being better in the ileum than in the duodenum at the time of hatching. Even though the staining in the goblet cells remained weak until 28 days of age, the brush border membrane was intensely stained at 14 days of age in both intestinal segments. Previous studies have shown, that for glucose to be further transported from the enterocyte into the hepatic circulation, expression of GLUT2 is required, as it is responsible for the transport of glucose (and fructose) further from the enterocyte into the blood circulation (Li *et al.*, 2004; Yoshikawa *et al.*, 2011). According to Hussar *et al.* (2016), the expression of GLUT2 stays weak in the duodenum during the first 7 days of life, suggesting, in accordance with the results of this study, that glucose absorption in the duodenum is not fully developed in the first week after hatching. In the terminal zone of ileum, moderate expression of GLUT2 is already observed at 7 days of age (Hussar *et al.*, 2016). According to the findings of this study, SGLT1 has strong expression on the brush border membrane of the ileum at 14 days of age, indicating that glucose can be absorbed through the ileal wall in two-week-old ostriches. According to Wang and Peng (2004), intestinal villi continue to grow until 90 days of age, and the quantity of goblet cells increases

until 45 days of age, thus nutrient transport is still developing after the first month of life. Therefore, even though the expression of SGLT1 described in the small intestinal epithelium of this study is strong at 28 days of age, the absorptive capacity might still be relatively weak due to smaller villi and fewer goblet cells in the under one-month-old ostriches compared to older ones.

Many ostrich farmers are affected by high mortality rates in their ostrich chicks within the first month after hatching. The causes of these high mortality rates are probably multifactorial, but OCFS and intestinal pathogens have often been associated with the affected chicks (Terzich and Vanhooser, 1993). Mortality rates vary between different farms, and less OCFS has been described when feeding is started at seven to 10 days of age compared to feeding immediately after hatching (Terzich and Vanhooser, 1993; Shanawany, 1996). The results of our study suggest that glucose absorption in young ostriches might be inadequate, since the expression of SGLT1 is weak in the small intestine when under two weeks of age. Therefore, feeding practices might play an important role in the health of the chicks, since unabsorbed dietary sugars could potentially cause diarrhea, as is the case in rats and humans affected by glucose-galactose malabsorption (Gorboulev *et al.*, 2012). This would also explain why restraining from additional feed the first week of life decreases morbidity in the ostriches. In addition, the lesser quantities of amylase secreted in ostriches under 7 days of age, supports the suggestion of poor carbohydrate digestion during the first week of life (Noy and Sklan, 1995). Restraining from additional feed for the first week is also supported by ostriches' natural behavior in the wild, where they, according to Birdfact (2022), rely on their yolk sac until one to two weeks of age.

SGLT1 has, in addition to absorptive roles, also important immunological roles by protecting the small intestinal epithelium from cell barrier defects and pathological alterations caused by bacteria (Yu *et al.*, 2015). Our study did not evaluate pathological changes in the small intestinal tissue, nor the physiological capacity of the small intestines to absorb glucose molecules. In addition, information about SGLT4s role in the absorption of glucose from the small intestines is lacking even though it is found in abundance in the tissues, thus it is possible that it has an important role in the transport of dietary sugars (Tazawa *et al.*, 2005). Therefore, it is not possible to draw conclusions on whether the high mortality rates in young ostriches are caused by inadequate glucose absorption, intestinal pathogens, or something else. However, our results are highly suggestive of inadequate glucose absorption playing a role in the disease mechanism of the affected chicks due to the weak expression of SGLT1 in the small intestinal epithelium at young age.

A limiting factor of this study was the inability to evaluate five samples from the study, which could increase the risk of errors when drawing conclusions about the expression of SGLT1 since differences between individuals within the same age group might have been missed. In addition, the staining for SGLT1 was evaluated by eye, resulting in subjective analysis that can differ depending on the evaluator. However, the strength of expression was compared with the controls, different age groups, and intestinal segments, providing a reliable evaluation. Thus, the study gave valuable information about the immunolocalization of SGLT1 in the ostriches' small intestinal epithelium and its development during the first month of life providing an important basis for understanding the nutritional requirements of ostrich chicks.

CONCLUSIONS

Glucose transporters are crucial for maintaining glucose homeostasis in the body. They have essential roles in absorbing glucose from the gastrointestinal lumen into the hepatic blood circulation. SGLT1 is responsible for transporting glucose from the small intestinal lumen into the enterocytes, thus inadequate expression of the transporter will lead to unabsorbed sugars in the intestinal lumen. Many under one-month-old domestic ostriches suffer from watery diarrhea and high mortality rates, which could be caused by improper feeding regimes and practices. There is little scientific information about the correct starting point of feeding, but information about ostriches' gastrointestinal development and intestinal ability to transport nutrients provides knowledge about their feeding requirements. This study provided valuable information about the progressively increasing expression of SGLT1 in the small intestines of growing ostriches, suggesting that the intestines capacity to transport glucose is still developing at least one month after hatching. It is possible that the weak expression of SGLT1 during the first month after hatching contributes to the challenges experienced by ostrich farmers when feeding is started before the gastrointestinal tract has developed enough to utilize the nutrients from the feed. However, further species-specific research is needed to be able to optimize ostrich management practices and to improve the health of the ostrich chicks. Further research could be undertaken to investigate the immunoprotective roles of SGLT1 in the ostriches' small intestine, aiming to evaluate whether the weak expression of SGLT1 causes the intestinal epithelium to be more susceptible to cell barrier defects and pathological alterations in the young chicks compared to older ostriches.

ACKNOWLEDGEMENTS

I would like to acknowledge and give my warmest thanks to my supervisors Professor Piret Hussar and Professor Ilmārs Dūrītis for letting me participate in such and interesting study. I want to give a special thanks especially to Professor Piret Hussar for her patience, guidance, and great help during the process of writing my master's thesis, as well as her introducing me to scientific conferences.

I would also like to sincerely thank laboratory technician Mare Tamm and Cristin Allmang for their kind help in the laboratory works.

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APPENDICES

Appendix 1. IHC staining protocol

Deparaffinize and rehydrate formalin-fixed paraffin-embedded tissue sections:

- a. Incubate sections in three washes of xylene for 5 min each
- b. Incubate sections in two washes of 100% ethanol for 10 min each
- c. Incubate sections in two washes of 95% ethanol for 10 min each
- d. Wash sections twice in dH₂O for 5 min each

Add enough drops of Hydrogen peroxide Block to cover the sections. Incubate for 10 min. Wash 2 times in buffer.

Perform appropriate pretreatment:

- a. Add the appropriate antigen retrieval buffer (10 mM Tris/1 mM EDTA pH 9.0) to the microwaveable vessel with slides
- b. Place the vessel inside the microwave, maintain at a sub-boiling temperature for 18 minutes
- c. Cool at room temperature for 30 minutes
- d. Wash slide 3 times in buffer

Apply Protein Block and incubate for 5 minutes at room temperature to block nonspecific background staining. Wash 1 time in buffer

Apply primary antibody (Rabbit anti-SGLT1) and incubate for 30 min in moisture chamber at +37°C. Wash 4 times in buffer

Apply biotinylated secondary antibody Goat anti-Rabbit and incubate for 10 min at room temperature. Wash 4 times in buffer

Apply Streptavidin Peroxidase and incubate for 10 min at room temperature. Rinse 4 times in buffer. Add 20µl DAB Chromogen to 1 ml of DAB Substrate, mix by swirling and apply to tissues. Incubate for 10 min. Rinse 4 times in buffer

Add enough drops of Hematoxylin to cover the section. Incubate for 1 min. Rinse 7-8 times in tap water

Immerse slides in dH₂O two times for 5 min, dehydrate sections as described in Hematoxylin-eosin staining protocol and thereafter mount coverslips

Appendix 2. Publications


Hussar, Piret; Ahlstedt, Victoria Isabella; Järveots, Tõnu; Allmang, Cristin; Dūrītis, Ilmārs. *Eesti Arst, 102, Lisa 1: Tartu Ülikooli arstiteaduskonna aastapäeva teaduskonverents 2023, Tartu Ülikool, 12.10.23-13.10.23*. Ed. Siigur, U. jt. Tartu: OÜ Celsius Healthcare, 64–64.

Hussar, Piret; Ahlstedt, Victoria Isabella; Allmang, Cristin; Järveots, Tõnu; Dūrītis, Ilmārs. *Medicina, 60: International Scientific Conference on Medicine organized within the frame of the 82nd International Scientific Conference of the University of Latvia, Riga, Latvia, 05.04.2024*. Ed. Edgaras Stankevičius et al. Kaunas, Lithuania: Medicina, 150–150. (1).

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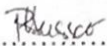
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