Final report

Introduction

Displacement of the abomasum is a frequent condition in dairy cattle which occurs due to abomasal hypomotility and gas accumulation. In most of the cases the abomasum displaced to the left side, although right displaced abomasum (RDA) also reported [1,2]. Left displaced abomasum (LDA) is a common dairy cattle disease occurring in 1-7% of the Holstein-Friesian population and in many other breeds. The LDA is commonly associated with high yielding and intensively fed dairy cows in early or late lactation, but the disease can be found in beef cattle populations as well [1,3]. Effective prediction would be necessary as LDA increases veterinary costs of dairy farms and decreases life expectancy with restricted milk production [4].

The multifactorial cause of the LDA is presumably based on genetic factors affecting the initiation and maintenance of the migrating motor complex (MMC). The heritability estimates mostly measured in Holsteins fluctuate between 0.2 and 0.5 [2–5]. LDA can occur at any time of the lifespan, however 80% of the displacements are observed within one month of partition [6]. Reduced abdominal motility is the primary etiological cause of the LDA. Some breeding technology (like calving problems, overfeeding in the dry period, decreased food intake) are risk factors which strongly affect the incidence of the disease. Hypocalcaemia, metabolic alkalosis and concurrent diseases such as ketosis and metritis may also led to the development of the disease [7]. The occurrence of LDA may be reduced, but not completely eliminated by the avoidance of the risk factors. Some risk factors have been identified to play role in the evolving of the displacement of the abomasum like calving problems, twin births, overfeeding in the dry period, decreased feed intake and high body condition before calving [7].

The migrating motor complex (MMC) is considered as a cyclic interdigestive reflex which travels along the gastrointestinal tract to propel and empty those contents. The MMC comprehends three or four phases vary upon species. Phase III is the most characteristic one and contains intense contractions. The whole MMC cycle ranges between approximately 90-120 min. Among other peptides - like ghrelin (GHRL), gastrin (GAST), serotonin (SRT) - motilin (MLN) is considered as an endocrine regulator of the MMC [8]. In 1966 Brown et al. published a paper in which they described that the activity of denervated and transplanted pouches was elevated after the administration of alkaline solution. Two alternative explanations have emerged to interpret this phenomenon. One was that the alkaline solution contributes to the secretion of a stimulatory humoral agent. In those days they were not able to name this so-called humoral agent [9]. After some years the same research group successfully isolated this agent and they named it MLN after the observation that this polypeptide stimulates motor activity [10]. MLN is a small, 22 amino-acid peptide hormone encoded by the MLN gene located at bovine chromosome 23. This hormone plays an important role in the regulatory system of the interdigestive motility. It is produced by the endocrine cells of duodeno-jejunal mucosa and its plasma level increase during the interdigestive periods. The peaks in the MLN plasma level led to cyclical peristaltic contractions in the fasting periods in order to gastric emptying of the gut content (phase III of MMC). It is clear that there is a strong correlation between the expression patterns of MLN and the emergence of LDA. Interestingly a single nucleotide polymorphism (SNP) was found in the first non-coding exon of the bovine MLN gene. This SNP is considered as a crucial influencer of the relation between the MLN gene and a transcription factor called NKX2-5. By the result of this mutation the MLN expression is decreased by 89% compared to the wild type individuals. This SNP and the alteration of MLN expression may play an important role in bovine LDA through the motility of the abomasum [4].

The motilin-motilin receptor system seems to be the main genetic factor behind the LDA phenotype [3,4,11]. The characterisation of the bovine motilin receptor is absent in the literature, although in many species, like dog, guinea pig, kangaroo rat, it is already published [12,13]. Also, to investigate the motilin-motilin receptor complex and its ligand's relationship we choose to establish a motilin knockout rabbit model.

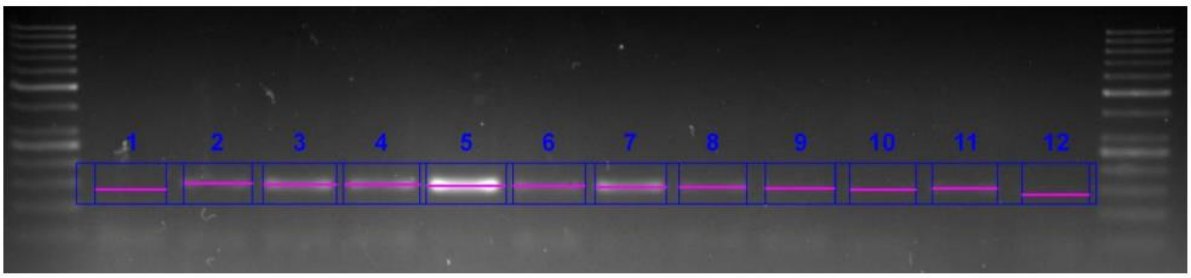
In summary, the project had two main objectives: firstly, to characterize the motilin receptor in the bovine digestive system, and secondly, to knock out the motilin gene in rabbits.

Characterisation of motilin receptor in bovine gastrointestinal tissues:

Tissue samples were obtained from both control and diseased animals, with six animals in each group. Collected tissue samples encompassed various segments of the gastrointestinal tract, including the rumen, reticulum, omasum, abomasum, pylorus, duodenum, jejunum, ileum, appendix, colon, and rectum. Following collection, the samples underwent a cold phosphate-buffered saline (PBS) wash before being preserved in RNALater solution to mitigate nucleic acid degradation. DNA extraction from abomasum tissue samples was carried out utilizing the phenol-chloroform method, specifically for genotyping purposes. Total RNA was extracted from all cattle tissues using RNAzol Reagent (RNazolRT Molecular Research Center) followed by DNase treatment and RNAqueous-Micro Kit RNA isolation kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's recommendation. The RNA integrity was verified using Agilent Bioanalyser 2100 (Agilent, Palo Alto, CA, USA). High-Capacity cDNA Reverse Transcription (Thermo Fischer Scientific) kit was used for cDNA synthesis according to the manufacturer's instructions. To assess reverse transcription fidelity, a PCR was conducted employing endogenous control primers targeting the RPS15A gene. Multiple primer pairs targeting the motilin receptor were designed utilizing Primer3 software. **Subsequently, all designed primer pairs underwent comprehensive testing, with individual primers evaluated in diverse combinations and PCR conditions.**

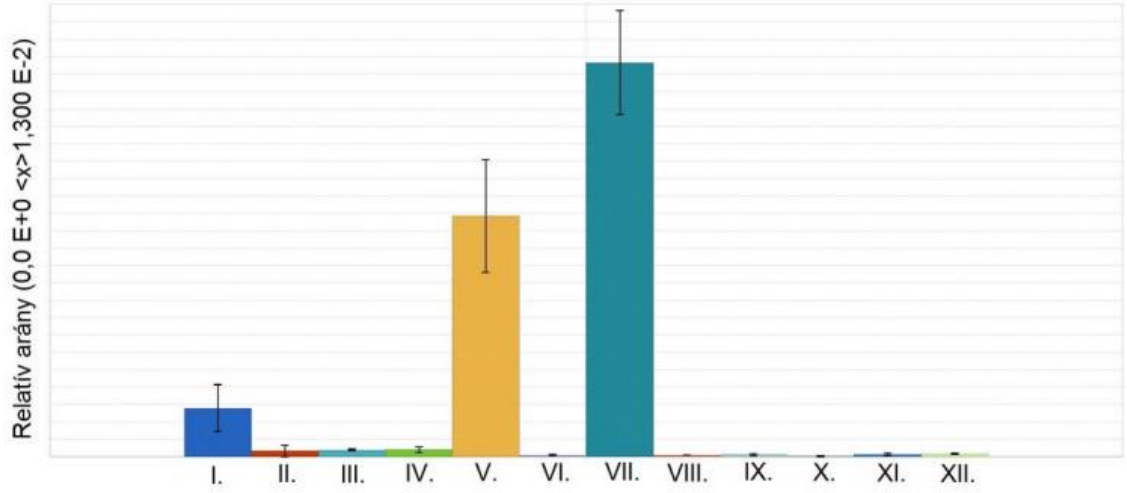
RPS15A F	GAATGGTGCGCATGAATGTC	gDNS: 101; cDNS: 101
RPS15A R	GACTTTGGAGCACGGCCTAA	
MLNR 1F	CTTCCCTTCGACTTGTACCG	gDNS: 1563; cDNS: 767
MLNR 1R	ATGGAGGCGCTCAGATAGAA	
MLNR 2F	GTGGTGACTGTGCTGCTGAT	gDNS: 1838; cDNS: 1042
MLNR 2R	CTGACGTCTGCACGTTGG	
MLNR 3F	GTGGTGGTTCTGGCGTTTAT	gDNS: 233; cDNS: 233
MLNR 3R	CCTCTCTGTCTGGACCGTCT	

Densitometric measurements of RT-PCR samples run by gel electrophoresis were performed. According to the results obtained from RT-PCR utilizing the motilin receptor-specific primer pair 3F-1R, we observed an ascending trend in MLNR expression from the rumen. The signal intensity exhibited a continuous increase in strength from the rumen through the reticulum, omasum and abomasum. Conversely, a decline followed by a subsequent increase in signal strength was noted at the pylorus, progressing towards the intestine. Notably, starting from the jejunum, the intensity plateaued at a lower level compared to the other examined tissues. The image below shows the densitometric evaluation of a sample. **Through densitometric measurements, we assessed the level of MLNR expression across the tissues of the bovine digestive organ system.**



1. rumen, 2. reticulum, 3. omasum, 4. omasum laminae, 5. abomasum, 6. pylorus, 7. duodenum, 8. jejunum, 9. ileum, 10. appendix, 11. colon, 12. rectum

q-RT-PCR was executed with 5x HOT FIREPol® EvaGreen® (Solis BioDyne) master mix. The reaction followed the manufacturer's program settings (RPS15A 0.5 µl; 3F-1R 0.7 µl) with 1 µl of cDNA in a final volume of 15 µl. In addition to the RPS15A endogenous control, a primer pair specific to the motilin receptor (3F-1R) was utilized for amplification. The q-RT-PCR results show that the motilin receptor is expressed in all samples. Through the assessment of MLNR relative expression in comparison to the endogenous control RPS15A, noteworthy values were observed in the rumen, abomasum, and duodenum samples. Conversely, the expression levels in the remaining samples were either lower or negligible. The image below shows the q-RT-PCR evaluation of the samples. **Through q-RT-PCR analysis, we validated the gene expression levels of MLNR in various tissues, revealing its heightened expression in the rumen, abomasum, and duodenum tissues.**



I. rumen, II. reticulum, III. omasum, IV. omasum laminae, V. abomasum, VI. pylorus, VII. duodenum, VIII. jejunum, IX. ileum, X. appendix, XI. colon, XII. rectum, n=5.

We also examined which genes are expressed in the tissue of the bovine abomasum and compared the transcriptome. To identify the factors affecting LDA, we used single end sequencing from the abomasum of 5 healthy and 5 LDA affected animals. We were collecting tissue samples during the essential surgical procedures of LDA diseased animals conducted by trained veterinarians, other samples acquired at the slaughterhouse. The reads were aligned to the bovine UMD3.1 (GCA_000003055.3) reference genome Total RNA was extracted from all cattle tissues using RNAzol Reagent (RNazolRT Molecular Research Center) followed by DNase treatment and RNAqueous-Micro Kit RNA isolation kit (Invitrogen, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer’s recommendation. The RNA integrity was verified using Agilent Bioanalyser 2100

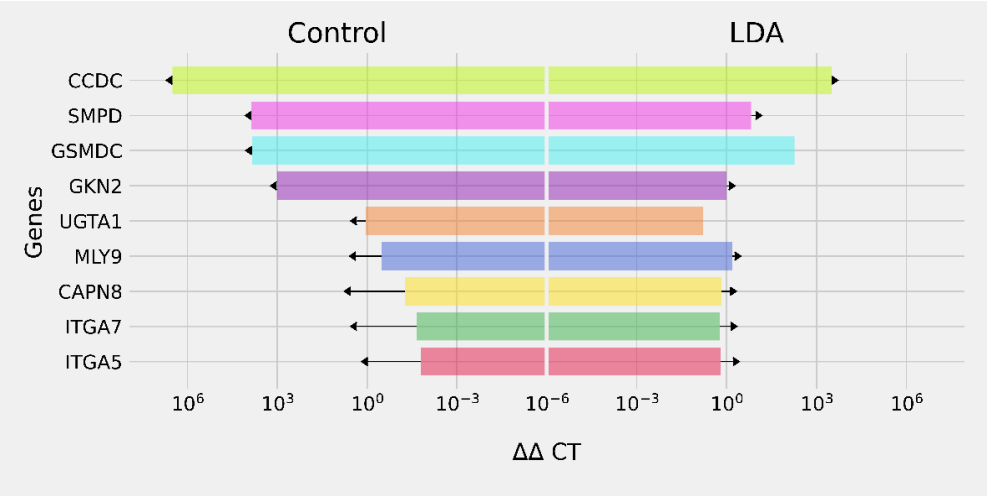
(Agilent, Palo Alto, CA, USA). The samples were sequenced by UD-Genomed Ltd. using an Illumina NextSeq500 analyser system.

For quality control, FastQC (v0.11.9) was used to make sure that only samples with quality score above 28 were involved in further investigations. Bos taurus genome fasta (http://ftp.ensembl.org/pub/release-105/fasta/bos_taurus/dna/Bos_taurus.ARS-UCD1.2.dna.toplevel.fa.gz) and the corresponding GTF file (http://ftp.ensembl.org/pub/release-105/gtf/bos_taurus/Bos_taurus.ARS-UCD1.2.105.gtf.gz) were acquired from the FTP site of Ensembl genome browser. Modified version of Tuxedo protocol was used to carry out the initial steps of RNA sequencing pipeline. The protocol uses HISAT2 (v2.2.1) to align reads to the given genome and uses HTSeq (v0.11.3) to count the number of reads mapped to each genomic location. Gene count matrix was constructed with Python Pandas (v1.1.3). All the steps in the downstream analysis were performed in R. Differential gene expression analyses was done with DESeq2 (v1.34.0). For Gene Set Enrichment analysis Clusterprofiler (v4.2.2) was used. Ensembl id to external gene name transformation was carried out using biomaRt (v2.50.3). **We evaluated the expression data derived from RNA sequencing of both healthy and diseased animals, pinpointing the genes that exhibited differential expression.**

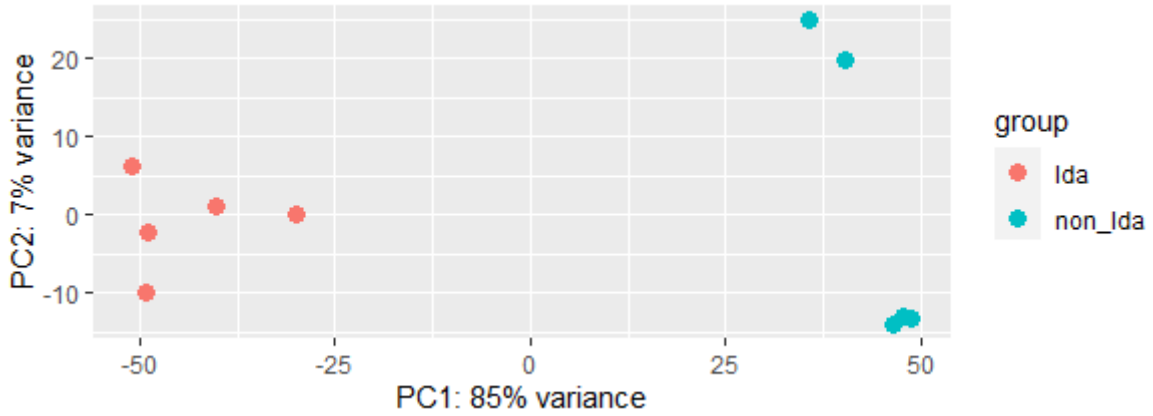
We choose the genes (CAPN8 (ENSBTAG00000003600.6), CCDC136 (ENSBTAG00000011002.6), GKN2 (ENSBTAG00000017199.6), GSDMC (ENSBTAG00000017478.6), ITGA5 (ENSBTAG00000013745.6), ITGA7 (ENSBTAG00000012897.6**)**, MYL9 (ENSBTAG00000011473.5), SMPD3 (ENSBTAG00000009639.5), UGT1A1 (ENSBTAG00000026181.5), RPS15A (ENSBTAG00000054615.1)) for validating the RNA sequencing data with q-RT-PCR.

Expression level of these genes were significantly different between the healthy and the diseased group. Ribosomal protein s15 (RSP15A) was used as an endogenous control. All primer were designed with the Primer3Plus online designing tool based on the cow sequences available from Ensemble to obtain different length fragments from RNA as DNA, to check the DNA contamination of the samples. 200 ng RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific) in a 20 µL reaction including random primers. Annealing and elongation incubations were conducted according to the basic protocol.

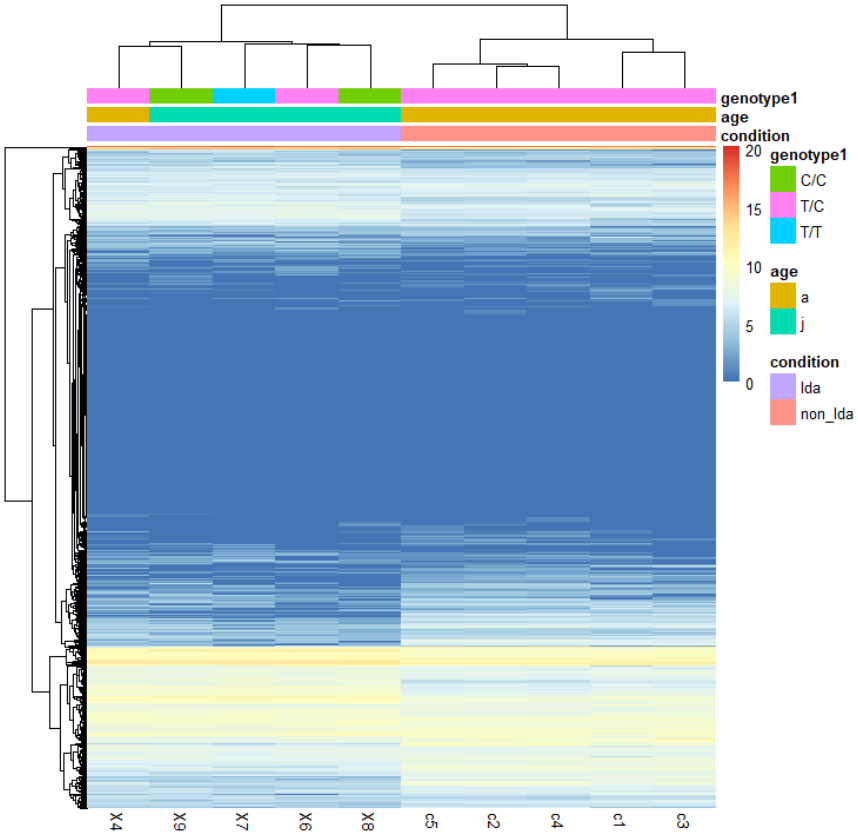
The q-RT-PCR reactions were performed in 15 µL final volume including the 5x Hot FirePol EvaGreen qPCR Plus master mix (Solis BioDyne) and the reactions were run on Roche LifeCycler 96 System. All samples were tested in triplicate form. The DNA amplifications were performed with My Taq™ Red Mix (Meridian Bioscience, Cincinnati, USA). Initial denaturation (95°C for 1 min) was followed by 35 cycles of PCR: denaturation at 95°C for 20 sec; annealing at 58°C, 60°C for 20 sec and extension at 72°C for 20 sec. The final extension was 72°C for 2 minutes. The figure below shows the q-RT-PCR data for 9 genes used as a validation of RNA sequencing. **Our analysis indicates that the expression outcomes obtained through q-RT-PCR exhibit no statistically significant variance in comparison to the RNA sequencing data.**



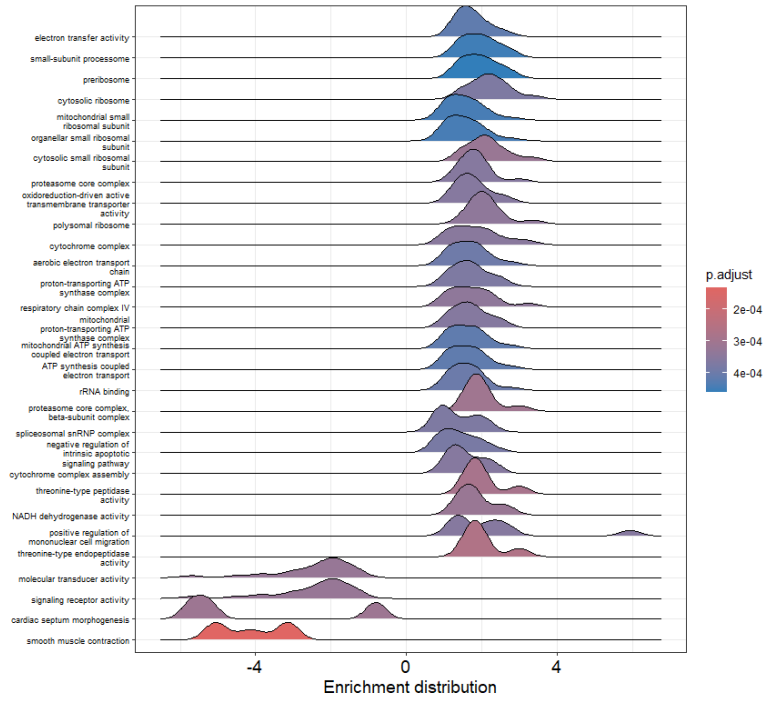
Principal component analysis was performed to determine whether the control and LDA affected individuals can be clustered based on their gene expression profiles. The principal component 1 (PC1) accounts for 85% of the total variation and hence control animals and LDA affected animals are grouped together based on their transcriptomic fingerprints. **The PCA analysis displays a clear separation between LDA affected and healthy animals.**



The same clustering pattern can be observed with heatmap visualization of the expression profiles, so there is a well-defined distinction between the control and LDA affected groups. There is no grouping displayed with regard to genotype or age. The heat map vividly illustrates the patterns of gene expression across healthy and LDA affected animals, we have no outliner samples with unusual expression profile. There is no observable difference in gene expression that would vary based on genotype or age. However, it can be noted that **all healthy animals had a heterozygous genotype (T/C), while among the diseased animals, in addition to heterozygotes, homozygous C/C and T/T variations are also present.**



An enrichment analysis was conducted, revealing distinct pathway patterns between LDA-affected and healthy animals. The analysis demonstrated an enrichment pathway term associated with smooth muscle contraction, molecular transducer activity, and signaling receptor activity has decreased expression levels in LDA-affected animals. Conversely, in the LDA-affected group there was az increast in included electron transfer activity, preribosome, cytosolic ribosome, and small-subunit processome pathways. **These findings suggest significant alterations in biological processes and molecular functions associated with LDA pathology, shedding light on potential regulatory mechanisms contributing to the observed differences between the two groups.**



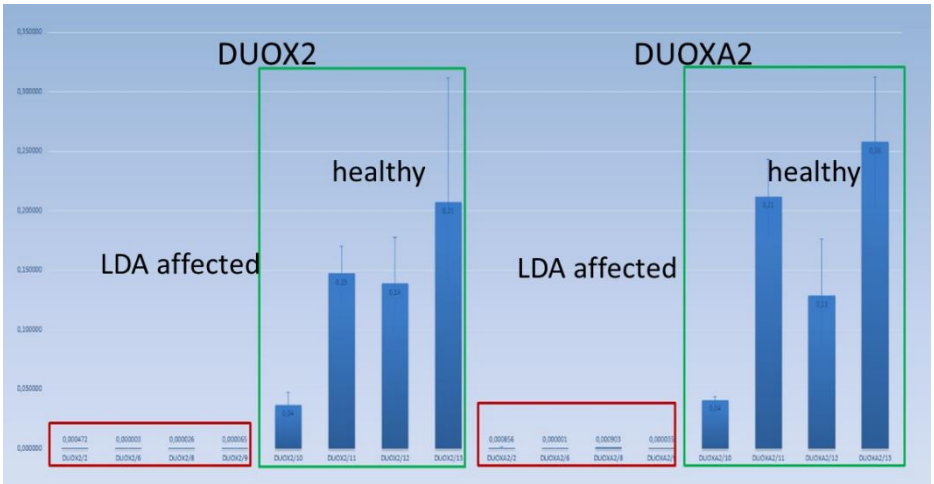
In the evaluation of RNA sequencing data, no distinctions were observed in the expression levels of motilin, motilin receptor, gastrin, or other hormones typically associated with presumed influence on intestinal motility, as described in the literature. Nevertheless, a notable contrast surfaced in the expression of dual oxidases affiliated with the NADPH oxidase family, prompting a more in-depth investigation into their role.

The NADPH oxidase/ Dual oxidase (NOX/ DUOX) family has seven members in mammals, NOX 1-5 and DUOX1-2, encoded by separate genes [14]. The function of those enzymes is producing reactive oxygen species (ROS), superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals in various tissues. Although the seven members of the DUOX family share their function in ROS production, each has its own specific property. DUOX1, DUOX2 primarily generate H_2O_2. The sustained level of oxidative stress can lead to significant cell damage causing apoptosis or inflammation and play a significant role in the pathogenesis of chronic inflammation, activation of wound healing and tissue fibrogenesis. Since the oxidative stress is an imbalance between ROS generation and antioxidant defence, DUOX family plays prominent role in the regulation of cellular pathophysiology. Both the up- and downregulation of NOXs and DUOXs may contribute to the development of numerous diseases [15,16].

DUOX1 and 2 (previously called ThOX1 and ThOX2, respectively) are not expressed in the cardiovascular system in mammals [17]. DUOXs were identified in the epithelial cells in the thyroid gland, lower gastrointestinal (GI) tracts, respiratory system [18,19] and in the urothelium [20]. DUOXA1 and DUOXA2 maturation factors are required for the endoplasmic reticulum to Golgi transfer and translocation to the plasma membrane of DUOX1 and DUOX2, respectively [21,22]. Both DUOX1 and DUOX2 produce H_2O_2 in the thyroid gland, which is essential for proper iodination and thyroid hormone synthesis. Mutations in the DUOX genes or their respective DUOXA partners, mainly in DUOX2/DUOXA2 genes, cause congenital hypothyroidism, both in adult patients [23,24] and children [25,26].

The q-RT-PCR reactions were configured following the same protocol as in prior experiments. We quantified the expression levels of dual oxidases in the stomach samples from 4 healthy and 4 LDA-affected animals, utilizing primers designed for the DUOX2 (ENSBTAG00000016234) and DUOXA2

(ENSBTAG00000016239) genes. Notably, **the expression in the diseased animals exhibited a significantly reduced value.**



Oxidative processes can lead to the formation of partially reduced, highly active oxygen metabolites. In mammalian species, superoxide and its derivatives are necessary to maintain proper homeostasis in the body, to moderate signal transduction, gene expression, and many other cellular processes. However, it is widely known that their excessive presence or contingent absence leads to the development of diverse diseases. As natural by-products of aerobic cellular metabolism, ROS maintain and regulate homeostasis and cell signalling. They are also essential for killing pathogens however, ROS can be harmful to host cells and tissues. Whether they act protective or detrimental, depends on the fine-tuning of the ROS production and disposal at the tissue, which is an evolutionarily conserved mechanism developed to be carefully balanced by generation and degradation of ROS. The ROS levels are strongly influenced by stress factors; the dysfunction of redox homeostasis results in increased production or inefficient elimination of ROS which causes oxidative stress. The sustained elevated level of oxidative stress can lead to significant cell damage causing apoptosis or inflammation and plays a significant role in the pathogenesis of chronic inflammation, activation of wound healing, and tissue fibrogenesis.

In addition to their role in redox processes, the ROS produced by DUOX enzymes supports the immune system in the defence against bacterial infections in the respiratory tract and lower tracts of the digestive system. In the respiratory airways and lower GI tracts, H2O2 generated by DUOXs supports host defence against bacterial infections as part of the innate immune response. DUOX enzymes play an essential role in the development of the immune response in the digestive system therefore loss-of-function mutations in the genes encoding the enzymes promote the progression of inflammatory bowel diseases in humans and mouse models. The reduced functioning of the local immune response of the intestinal system can also cause or show the development of intestinal diseases in cattle, including LDA with a less clear background. More information is needed in terms of both gene expression and microbiome for a more accurate understanding of the development and cause of the disease of bovine abomasum. Since **the change in DUOX2/DUOX2A gene expression also leads to intestinal changes in other species and destroys the body's resistance to bacterial infections, it may be a candidate gene for the development of the left displaced abomasum disease.**

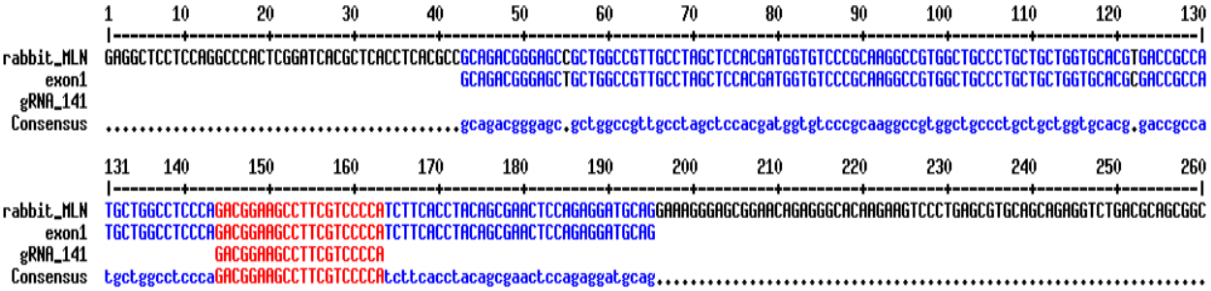
Despite the fact that we performed the above experiments and evaluated our results, we still did not manage to publish them. We tried to publish the manuscript in four newspapers (The Veterinary Journal, Research in Veterinary Science, Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics, Biology Open), but in response, two journal editor indicated that it did not belong to their field of science, despite that they contained articles related to genomics, in spite of all of these we have to wait months for the dismissive answer, so we are in a serious slippage regarding publication. Two of them accepted the topic of the article, but it did not find reviewers for months, so we finally got the manuscript back without a review. **The article is being reviewed in MDPI Animals journal, we received 3 positive reviews, and one reviewer requested changes regarding the analysis which we are working on.**

Knockout of the rabbit motilin gene:

The second main goal of the application was to create a motilin gene knockout rabbit line. To accomplish this, gRNAs targeting the first coding exon of the rabbit motilin gene were initially designed. Out of four gRNA sequences, we finally selected the two below for line establishment.

sgRNA name	Sequence	PAM	Position
g80	GCCGTTGCCTAGCTCCACGA	TGG	chr12: 24102114
g141	TGGGGACGAAGGCTTCCGTC	TGG	chr12:24102030

Female New Zealand White (NZW) rabbits of wild-type (WT) genotype, aged between 4 to 6 months, were subjected to treatment with pregnant mare serum gonadotrophin (PMSG, ProSpec, Israel, administered intramuscularly). After a period of 72 hours, donor does underwent insemination with wild type NZW bucks and received injections of human chorionic gonadotrophin (hCG, Choragon, Ferring GmbH, Kiel, Germany, administered intravenously). Simultaneously, recipient does were injected with a gonadotrophin-releasing hormone (GnRH) analogue (Receptal, Intervet International B.V., Boxmeer, administered intramuscularly). One-cell stage embryos were subsequently flushed from the oviducts of donor does approximately 19-20 hours post-hCG administration, followed by microinjection with a mixture of sgRNA-Cas9 mRNA. For the microinjection sgRNA (40 ng/µL) and Cas9 mRNA (300 ng/µL, Trilink Biotechnologies) were mixed on ice in 1:1 ratio and injected into the male pronucleus of the rabbit embryos. The microinjected one-cell stage embryos were either cultured to morula/blastocyst stage for genotyping or transferred to recipient does on one-cell stage. We used gRNA #g141 for the first microinjection experiment, which was successful.



The born offspring were tested using the T7 assay. The genomic DNA (gDNA) of tissue samples from the pups were digested with a standard proteinase K method and DNA was extracted with phenol–chloroform. The first exon of the motilin gene was amplified using PCR. The PCR fragments were digested by T7E1 for 60 min at 37 °C to detect the CRISPR-Cas9-induced mutations (T7E1 kit, M0302L, NEB, USA) and then analyzed by 1% agarose gel electrophoresis (Guschin et al. 2010). Mutations were further confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) of PCR fragments and TA-cloning.

Recipient mother	Transferred embryos	Newborns	Newborns with mutations
#1	21	3	2
#2	26	2	0
#3	24	4	1

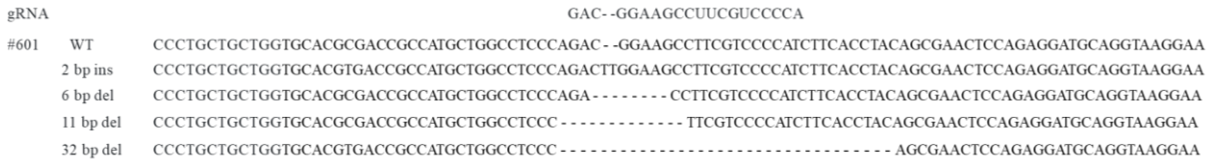
The superovulation, embryo washing, microinjection and the reimplantation of the embryos into the recipient mothers, required for genome editing, took place with extremely high efficiency. We transferred embryos into four recipient does, three of which gave birth. **Genome editing was successful in three (#601, #602, #603) of the 9 offspring born.**

ID	Mutation	Consequence
#601	allele A: 2 bp insertion (TT)	early stop codon
	allele B: 6 bp deletion(CGGAAG)	different amino acid sequence
	allele C: 11 bp deletion (AGACGGAAGCC)	early stop codon
	allele D: 32 bp deletion (AGACGGAAGCCTTCGTCCCCATCTTCACCTAC)	early stop codon
#602	6 bp deletion (CGGAAG)	different amino acid sequence
#603	11 bp deletion (AGACGGAAGCC)	early stop codon

Regrettably, the breeding of founders #603 and #602 proved unsuccessful. Founder #603, harbouring an 11 base pair deletion leading to a premature stop codon, held considerable promise as a candidate for line establishment. Additionally, the male sex of founder #603 had the potential to expedite the generation of F1 offspring significantly. Despite these advantages, male #603 exhibited an inability to reproduce, failing to yield any offspring through either insemination or natural mating.

A parallel scenario unfolded with the female #602 possessing the 6 base pair deletion. Despite reaching a mature age and maintaining overall good health, her breeding endeavours proved unsuccessful. The doe, unfortunately, could not achieve fertilization. These breeding difficulties are not unusual for laboratory rabbits. Breeding difficulties and the fact that we first wanted to breed founder #603 and then #602 caused a delay in the implementation of the application.

The #601 male founder rabbit carried at least 4 different alleles, shown in the figure below.



Regrettably, the generation of genetically modified model animals through microinjection is concomitant with the emergence of mosaicism. The ability to establish the F1 generation from such founders is depend upon the degree of mosaicism present. The emergence of multiple alleles is inherent to the CRISPR/Cas9 technology, as the functionality of the CRISPR system introduced into single-cell embryos persists after the initial division. Consequently, several alleles are generated in the adult animal, which resulted in a mosaic pattern.

Fortunately, despite the mosaicism, we were able to create an F1 population with the required mutation from the offspring of founder #601. **We used the offspring of the F1 generation which carried a 2 bp insertion resulted in early stop codon to establish the population for the motilin knockout model.** Unfortunately, throughout the experiments, no discernible differences were noted in the nutritional patterns, weight, or weight gain of the animals in comparison to their wild-type counterparts. Remarkably, no phenotypic distinctions were observed in the motilin-deficient animals under investigation.

The anticipation of potential off-target sites for the sgRNA was conducted utilizing CRISPOR.org (http://crispor.tefor.net/). Subsequently, the nine most prominent potential off-target sites for MLN sgRNAs were selected for sequencing through PCR employing motilin-specific primers. The PCR products underwent analysis on a 1.5% agarose gel, followed by purification (utilizing the NucleoSpin Gel and PCR Cleanup kit, Macherey-Nagel, Germany) and subsequent Sanger sequencing. Sequence analysis and result evaluation were performed using Chromas software and the online blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). **No off-target events were detected in either the founders or the individuals within the established F1 population. This observation underscores the high efficiency of the CRISPR/Cas9 system, coupled with its specificity.**

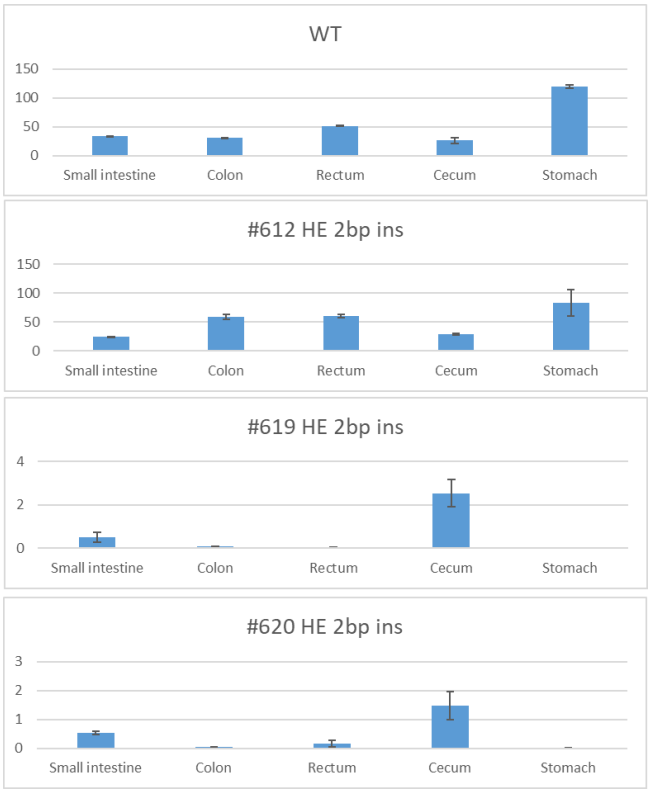
Off target ID	Locus/gene	Off-Target Sequence Compared to Target DNA	Position
MLN target		TGGGGACGAAGGCTTCCGTC	chr12:24102030
Off-target 1	exon:CORIN	TGGGGACGgAGGCTTaCGTC	chr2:36501315
Off-target 2	intergenic:SLC22A1-SLC22A2	aGGGGACGcAaGCTTCCtTC	chr12:149911229
Off-target 3	intergenic:ENSOCUG00000021067-SOX5	aGGGaACGAtGGCTTCaGTC	chr8:16017514
Off-target 4	intron:CALCOCO1	TGGaaACcAAGGCTTCCtTC	chr4:37500933
Off-target 5	intergenic:GJA8-GJA5	caaGGAgGAAGGCTTCCGTC	chr12:94002400
Off-target 6	intergenic:PREP-ENSOCUG00000024060	TGGaGACcAAGGCTaCCaTC	chr12:94001200
Off-target 7	intron:RALGPS1	TGGGGttGAgGGCTTCCtTC	GL018699:608910
Off-target 8	intergenic:NUP35-7SK	TGGGGAatAAGaCTTCaGTC	chr7:123492998
Off-target 9	intergenic:VANGL1-NGF	gGGGGAaGAAtGCTTCCaTC	chr7:123492998

In order to determine pre- and postprandial serum motilin levels, blood samples were collected into serum separator tubes from ear arteries at 3 different time points: after 12 h overnight fasting, then 1 and 2 hours after feeding. Blood samples were taken from a total number of 8 WT (4 males, 4 females) and 12 motilin heterozygote (6 males, 6 females) healthy NZW rabbits, between 3-6 months of age. ELISA method was used to measure motilin concentrations of serum samples (Rabbit motilin ELISA kit, Cusabio). Serum motilin concentrations were analysed using two-way repeated measures ANOVA (GLM, SPSS 27.0, IBM Statistics), where genotype effect (between subjects), time effect (within subjects) and their interactions were assessed. In case of a significant interaction, genotype effect was tested on each time point and time differences were tested in each group by Sidak post-hoc test. A probability value, $P < 0.05$ was considered statistically significant.

Genotype	Motilin concentrations (pg/mL) mean ± S.E.M., (min-max)		
	12h fasting (pre-prandial)	60 min	120 min
WT (n=8)	173,37 ± 30,98 (85,68 - 321,62)	197,85 ± 32,99 (105,57 - 344,11)	179,75 ± 31,38 (90,09 - 310,87)
MLN KO heterozygote (n=12)	196,69 ± 30,32 (47,90 - 311,60)	216,17 ± 31,91 (62,32 - 388,92)	200,00 ± 28,23 (56,85 - 317,70)

The rabbit serum motilin levels peaked 60 min after feeding and returned to the fasted level after 120 min in both groups, although the effect of time was not significant (P=0.064), which indicates that rabbit serum motilin ranges quite were similar to the human plasma motilin. **Unfortunately, we found no difference between the KO and wild-type phenotypes.**

Following the completion of the serum experiments, we decided to euthanize the animals to examine motilin gene expression at the tissue level. Utilizing q-RT-PCR, we assessed motilin gene expression in stomach, small intestine, colon, and rectum tissues from MLN KO 2bp insertion littermates, employing MLN-specific primers based on the previously outlined protocols. The outcomes revealed instances where, despite the presence of the 2 bp insertion, the expression of motilin remained comparable to that in wild-type animals. Conversely, we also identified offspring wherein the motilin level was significantly lower than in the wild type. The observed phenomenon can be explained by epigenetic mechanisms or individual differences. The q-RT-PCR results are depicted in the following figure.



To conduct a repeat of the experiments, a protocol must be devised to identify animals exhibiting lower MLN levels than the wild type. It is plausible that a multi-generational breeding approach may

be required to attain the desired motilin-deficient genotype. Alternatively, we have to investigate whether appropriate animals can be selected by individually measuring serum motilin levels.

Our findings which state that there is no phenotypic difference between MLN KO and wild-type animals, tentatively suggest that the presence of the hormone motilin may not be indispensable for the operation of the rabbit's digestive system, as its role could potentially be assumed by alternative molecules. While the experiments have been conducted, the unequivocally negative results pose challenges for publication. Considering this, there is an inclination to replicate the experiments using another line derived from #601, anticipating that this approach may yield phenotypic changes. Also, further exploration involving knockout animals subjected to factors contributes to the development of pathological condition, such as the presence of mycotoxins in the feed known to trigger inflammation or hypomotility, could provide additional insights. However, it is noteworthy that such investigations were not conducted within the scope of this study.

One valuable insight learned from establishing the MLN KO line is the requisite need for homozygous KO animals to conduct experiments assessing serum motilin or intestinal contractions. **Attempts were made to breed homozygous individuals and attain a sufficient population, yet encountered challenges wherein homozygous individuals were either not born within litters.** We had one homozygous animal, but it died before maturity. This observation suggests that the mutation may be sustained in a heterozygous form, while its manifestation as a homozygous genotype is presumed to lead to the development of a pathological phenotype.

In order to still be able to investigate the effect of knocking out the motilin-motilin receptor in rabbits, **we implemented a knockout of the motilin receptor in rabbits**, employing the same methods detailed previously. In brief, microinjection was utilized to introduce the components of the CRISPR/Cas9 system into the pronuclei of one-cell rabbit embryos, which included the gRNA designed specifically for the rabbit motilin receptor gene. Due to the extensive duration of the model animal production process, the development of this model is still in progress. However, we have successfully generated two founders. Founder #400 exhibits mosaicism, with its offspring presenting either a 28 bp deletion or a 2 bp deletion in the motilin receptor gene. Founder #401 introduces a 1 bp insertion into the motilin gene receptor. Preliminary findings indicate the feasibility of producing genome-edited F1 offspring, and no lethal phenotype has been observed thus far.

Since the beginning of the study, our understanding of the role of motilin in various animals has expanded. The prevailing consensus is that laboratory rodents lack genes responsible for producing motilin and/or its receptor, leading to an absence of functional responses to motilin. Despite this established view, ambiguity persists due to multiple reports highlighting the capacity of low concentrations of motilin to exhibit functional activity, especially within the gastrointestinal tract and the central nervous system of certain laboratory rodents. The absence of a universally accepted explanation for this anomaly raises concerns, particularly regarding the imperative to comprehend and address issues of reproducibility in animal research. Recently, a published article has delved into identifying suitable in vivo animal models for studying the motilin-motilin receptor system[27]. Notably, it has been observed that motilin may not play a functional role in the gastrointestinal system motility of guinea pigs[13], contrasting with its impact in rodents. However, the rabbit remains a significant model animal in this context, particularly given its remarkable similarity to humans. This indicates that the rabbit lines with knockout of motilin and the motilin receptor are topics of interest in the literature and are needed of further exploration. However, this requires more time than previously planned.

The application was hindered by many factors, having children, COVID, layoffs. In the period around the change of institution and after, it became extremely difficult to order the materials and equipment

needed for the experiments, causing long months of waiting. Nevertheless, the completion of the project's tasks was mostly hindered by the fact that the results were not as expected based on the literature data. Despite all these difficulties, I hope that we will be able to publish the results of the conducted experiments which describes gene expression levels in the abomasum of diseased and healthy animals in the near future, as the publication has already passed the first round of the review process. We aim to communicate our experiments related to the investigation of the motilin gene in rabbits by augmenting the alterations in motilin levels observed in wild-type animals with additional data. Furthermore, we plan to enhance the annotation of the motilin gene by sequencing the cDNA obtained from both wild-type and knockout animals and by using the existing rabbit whole-genome sequencing data accessible through open sources.

The project is experiencing delays beyond the initial timeline, attributed not only to challenges in the reproducibility of the founder animals but also to variations in motilin expression levels among the offspring. In light of the grant, it can be deduced that the establishment of an animal model entails substantial risk. In spite of exercising utmost care and employing state-of-the-art genome editing techniques, the precise feasibility and reproducibility of generating the intended animal model remain inherently unpredictable. Despite employing meticulous procedures in gene knockouts, such as the CRISPR/Cas9 system, it remains uncertain in advance whether the desired lineage can be successfully developed and if an adequate number of testable offspring and genotypes can be reliably produced.

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