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Carcinogenic potential of processed meat and plant-based meat alternatives in the A/J Min/+ mice model (*Mus musculus*).

Karsinogent potensiale av prosessert kjøtt og plantebaserte kjøtterstattere i A/J Min/+ musemodellen (*Mus musculus*).

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Food Science

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Abstract

Colorectal cancer (CRC) is one of the most common cancers both worldwide, and in Norway, with high mortality rates. The risk of developing CRC has been associated with diet and lifestyle, where, according to IARC, red and processed meat are classified as probably carcinogenic and carcinogenic to humans, respectively. Multiple theories of the relationship between CRC and meat have been studied, however, the causality and underlying mechanisms are yet to be documented.

CRC arises mostly by somatic mutations (70%), with a mutation in the tumor suppressor gene adenomatous polyposis coli (*APC*) playing an initiating role of carcinogenesis. Some cases are however caused by germline mutations in the *APC* gene, such as the inherited condition familial adenomatous polyposis (FAP). Multiple intestinal neoplasia (Min/+) mice model has previously been established as a human model for studies on colorectal carcinogenesis.

The overall aim of the present study was to investigate the carcinogenic potential of experimental diets prepared from commercially available processed meat –and plant-based meat alternatives in the A/J Min/+ mouse model. An inbred colony of A/J mice heterozygous for the Min trait (Min/+) were included in this study and were randomly recruited into 4 experimental groups at 4 weeks of age. The experimental diets were based on commercial processed meat -and plant-based meat alternative products (hot dog, hamburger and vegan burger). The mice were exposed to experimental diets for 9 weeks before termination. Lesions in the small intestine (SI) and colon were scored after staining and fixation using a light microscope.

We found a carcinogenic initiating potential of the hot dog in the SI. The reference diet exhibited a promoting effect on the SI and colon, as well as an initiating potential on the colon. However, a promoting potential on the colon was also found in the vegan diet. In the colon, gender-specific differences in carcinogenic potential were found. The hot dog diet and the reference diet exhibited an initiating potential in males and females, respectively. Moreover, the vegan diet showed a promoting potential in males.

Altogether, we revealed a carcinogenic potential of not only processed meat products but also processed meat alternatives. However, further research is needed to understand the complex interplay of dietary, genetic and lifestyle factors on carcinogenesis and the underlying mechanisms.

Abbreviations

ACF	Aberrant crypt foci
ANOVA	Analysis of Variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
B6	C57BL/6J
CDCA	Chenodeoxycolic acids
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CpG	Cytosine-phosphate-guanine
CRC	Colorectal cancer
CVD	Cardio-vascular diseases
DMC	Dunn's Multiple Comparison
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen receptor
FAP	Familial adenomatous polyposis
FELASA	Federation of European Laboratory Animal Science Association
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
HCA	Heterocyclic amines
HDI	Human Development Index
IARC	International Agency for Research on Cancer
K-ras	Kristen rat sarcoma viral oncogene homolog
KW	Kruskal-Wallis
LCA	Lithocholic acids
LOH	Loss of heterozygosity
MDA	Malonidaldehyd
Min	Multiple intestinal neoplasia
MMR	Mismatch repair

MSI	Microsatellite instability
NMBU	Norwegian University of Life Sciences
NOC	N-nitroso compounds
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline solution
PUFA	Polyunsaturated fatty acids
RO	Revered osmosis
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
SI	SI
TCF	T-cell factor
TP53	Tumor protein 53
Tukey HSD	Tukey honest significant difference
UNDP	United Nations Development Programme
UPF	Ultra processed food
WHO	World Health Organization
WRCF	World Cancer Research Fund
Wnt	Wingless integration 1

Contents

1.0 Introduction

1.1 Cancer

Cancer represents the greatest global health concern, accounting for almost 10 million deaths in 2020 (WHO, 2022). Breast, lung, colon and rectum and prostate cancers are among the most common and most lethal cancers worldwide, caused by a combination of genetic predisposition and lifestyle factors (Ibid.)

The definition of cancer is currently being applied as a collective term for diseases which involves uncontrolled cell division. The uncontrolled division of cells can affect and invade nearby tissue (National cancer institute, 2021). Cell division is a common phenomenon, and usually occurs in every cell in the human body. Cell division occurs as a regenerative step for defective cells, creating new cells, or replacing old cells. However, genetics, age or cell damage caused by lifestyle factors (radiation, virus-infections, chemicals, diet, alcohol consumption, smoking, stress, physical activity levels, sleeping habits and gut microbiota) may lead to mutations in the DNA (alterations of the DNA-sequences that can occur in the process of replications, insertion or deletion of bases in the genome) (National human genome research institute, 2023; WHO, 2022). Mutations in the DNA may further lead to uncontrolled cell multiplication and growth of abnormal cells. The abnormal cells may pile up and form lumps of tissue, known as tumors.

Tumors are usually characterized as one of two main categories - benign or malignant (Boutry *et al.,* 2022). Benign tumors emerge from the mutation of a single cell which allows it to grow and divide beyond its normal range and possibly affect surrounding tissue and the host as a whole organism. However, the ability to metastasize (invade resident tissue and mobilize to other organs) is non-existent in benign tumors, which separates them from their counter pole, malignant tumors.

Malignant cell growth can be characterized by the ability to avoid growth-inhibitory signals, insensitivity to apoptosis, unlimited replication, preserved angiogenesis and spreading to other organs causing metastatic cancer (Hanahan & Weinberg, 2000; Boutry *et al.,* 2022). This chain of reactions is a part of an event called carcinogenesis (Conlin, 2005).

1.1.1 Carcinogenesis - The stages of cancer

There are several existing theories of the cellular development of cancer. According to the multistep theory of carcinogenesis, developed by Armitage and Doll (1954), the development of cancer can be divided into three steps: initiation, promotion and progression. These three steps are frequently mentioned when explaining the carcinogenesis. Further, mutations in three gene types seem to be responsible for these steps, namely proto-oncogenes, tumor suppressor genes and stability genes (Vogelstein & Kinzler, 2004). Proto-oncogenes regulate normal cell differentiation and homeostasis, but when altered, their function becomes over-active (American Cancer Society, 2022). A mutated (activated) proto-oncogene is called an oncogene and leads to uncontrolled cell growth. Tumor suppressor genes down-regulate cell replication or induce apoptosis (programmed cell death) when needed during DNA-replication. Alterations (inactivation) of tumor suppressor genes leads to unregulated DNA-replication, and clonally expansion of abnormal cells which normally would undergo apoptosis. Stability genes, or DNA repair genes, are responsible for repairment of defects or abnormalities that occur during DNA-transcription, also triggering apoptosis if repairment is not possible. Changes in stability genes leads to unrepaired damage and uncontrolled cell growth in the DNA (Ibid.)

The initiation process relies on two independent events, (1) accumulation of irreversible mutations and epigenetic alterations of the DNA caused either by a carcinogen or spontaneously, leading to inactivation of tumor suppressor genes and activation of oncogenes, and (2) clonally expansion of the altered genes (Greten & Grivennikov, 2019; Duesberg & Li, 2003). During promotion (the second stage), a contributor of tumor growth such as inflammation, oxidative stress or immune deficiency stimulates angiogenesis and mobilization of cells which deploys tumor-supporting functions and causes an expansion of the lesion. In the final stages of carcinogenesis, the lesion progresses into a malignant tumor that inhibits the ability to grow uncontrolled and metastasize.

Several studies acknowledge the stage of metastasis as an individual, fourth step of carcinogenesis (Greten & Grivennikov, 2019; Duesberg & Li, 2003; Vogelstein & Kinzler, 2004). During metastasis, an increase in locomotion of the cell as well as production of matrix-degrading proteases causes a seeding and growth (invasion) of cancer cells to other tissues (Vogelstein & Kinzler, 2004).

1.2 GI tractus in humans and mice

The gastrointestinal tractus is roughly divided into two parts; the upper- and lower gastrointestinal tract (Treuting *et al.,* 2018a). The function of the gastrointestinal system includes transport, absorption of vitamins and other nutrients, and digestion. For both humans and murines the upper intestinal tract consists of all parts from mouth to small intestine (SI), which includes oral, oesophagus, stomach, and the SI. The SIs anatomy can be further classified into three parts: the proximal (duodenum), middle (jejunum), and distal part (ileum) (Figure. 1A). Furthermore, the SI is the longest part of the GI tractus in both humans and murines. The surface area lining of the SI is composed of finger-like projections called villi and evaginations called crypts (Figure. 2A). The villi differ from species to species, but for all species the length of the villi declines from duodenum to ileum. However, the villi in rodents are taller in the duodenal and jejunal parts, and humans have shorter villi than murines. The villi-structures increase the surface area of the SI and will assist in increasing the absorption of nutrients. For all species the crypts can be situated at the base of the villi. Murines and rodents have, compared to humans, raised foci. When these raised foci appear together in groups they may be referenced to as gut-associated lymphoid tissue (GALT). These cells involve lymphoid aggregates (Ibid.).

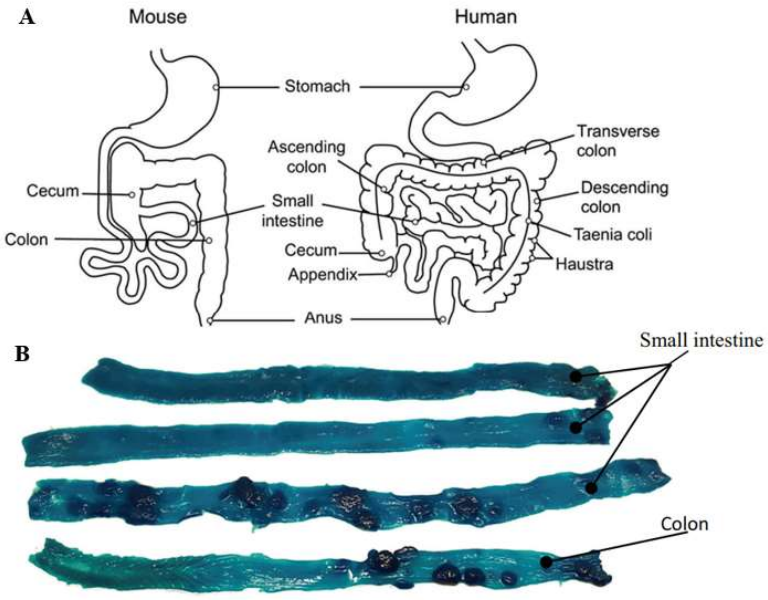

Figure 1. The Gi tractus of murines and humans (A), and the SI ***and colon of a murine with tumor growth (B). Adapted from (A) Nguyen et al. (2015), and (B) Sødring et. al (2016).***

Cecum is located between the upper and lower intestines and play a pivotal role in fermentation of food in mice, but not in humans, where colon is the main fermentation chamber (Fig. 1) (Treuting, 2018b).

Humans, murines and other rodents share similar anatomy of the lower GI-tractus (Treuting *et al.*, 2018b). They have the commonality that the colon does not include any villi but have colonic mucosal folds. In rodents the folds differ from each section, whereas humans only have transverse folds. The human colon can be classified into right and left colon, whereas the colon of rodents is classified into proximal, mid, and distal colon. The colon of humans and rodents is composed of four different cellular layers: the mucosa, submucosa, muscularis propria and serosa (Doherty, 2010). The mucosa is located at the surface of the lumen, consisting of multiple invaginations called crypts, associated with a flat surface of columnar epithelium (Levine & Haggitt, 1989). The crypts contain stem cells, both in humans and rodents, cells that are unique in their way of generating daughter cells with identical abilities of cell proliferation (Treuting *et al.*, 2018b; Barker, 2014) (Fig. 2B). The daughter cells migrate and terminally differentiate into epithelial cells, until they are replaced by newly differentiated epithelial cells. The stem cells, which can be found near the base of the crypts, have a high turnover rate. The cells will divide and renew every 3 – 5 days in humans, and 2 or 3 days in rodents, which makes the mucosa one of the tissues with the most expeditious turnover. However, the high turnover makes the stem cells vulnerable for mutations.

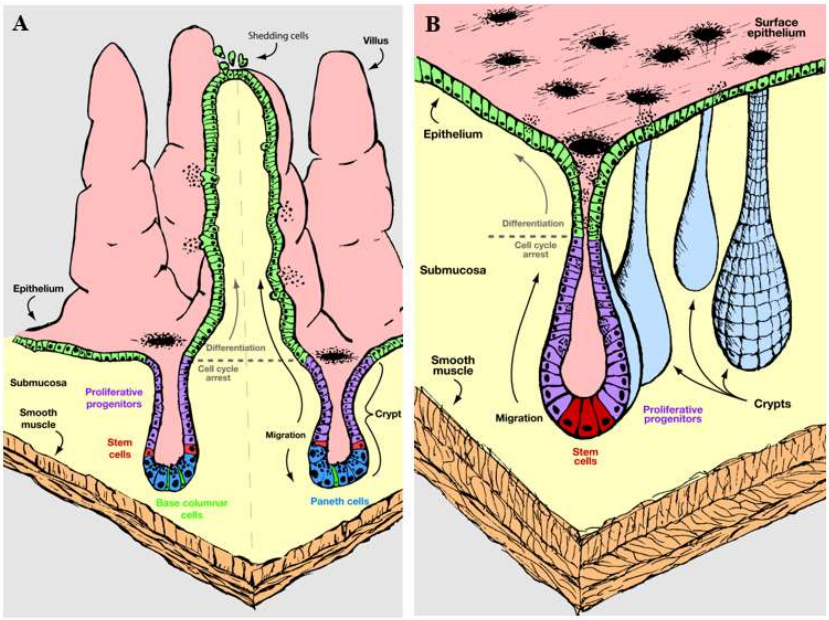

Figure 2. Structure of the SI (A) and the colon (B). In the SI, the stem cells are located right above the Paneth cells, near the bottom of the crypt. In the colon, however, the stem cells reside at the crypt bottom. Proliferating progenitor cells make up the remaining parts of the crypt in the SI, up to the crypt-villus junction where differentiation markers are expressed. In the colon, the progenitor cells reside at two thirds of the crypt, the rest being occupied by differentiated cells. Adapted from Sancho et al. (2004).

1.3 Colorectal cancer

Colorectal cancer (CRC) is one of the most common forms of malignant cancers with high mortality rates (IARC, 2022). The disease is responsible for between one and two million new cases each year and accounted for more than 930,000 deaths in 2020. This makes CRC the third most common type of cancer worldwide, with second to highest mortality rates (WCRF, 2022). Highest incidence rates of CRC are found in developed countries, such as European countries and Australia/New Zealand. According to a study by researchers from the IARC (2022), CRC is estimated to increase to 3.2 million new cases and 1.6 million deaths by 2040 (Morgan *et al.,* 2022). Most of these cases are expected to be seen in countries with high human development index (HDI), with Norway as second to the top of the list, after Switzerland (Morgan *et al.,* 2022; UNDP, W.Y). According to the WCRF, Norway is listed as number three over countries with highest overall rates of CRC in 2020, whereas Norwegian women are world leading (WCRF, 2022). Norwegian women are prone to suffer from CRC and CRC-related

deaths in a higher degree than Norwegian men, and the cause for this is unknown. This poses many important and interesting questions regarding health, lifestyle, genetics and gender-specific factors especially towards Norwegian women, but also men. With these statistics, studies on preliminary and underlying factors of causing CRC, is at high necessity.

CRC can be categorized into two substantial types, sporadic and hereditary, depending on the origin of the initial mutation (Fearon & Vogelstein, 1990). Sporadic CRC is accountable for 70% of all CRC cases and is caused by point mutations, where mutations occur in single somatic cells (Mármol, 2017; Vogelstein & Kinzler, 2004). A highly common pathway of sporadic CRC is the chromosomal instability (CIN) pathway, which accounts for 80 % of sporadic CRC. About 5 % of all CRC cases are due to inherited mutations that occur in one allele of the mutated gene, where Familial Adenomatous Polyposis (FAP) is one of the most common types.

1.3.1 Colorectal carcinogenesis

Colorectal cancer is one of the best studied forms of cancer regarding genetic mutations and involving mechanisms and has been used to formulate genetic models of carcinogenesis (Luebeck & Moolgavkar, 2002; Karakosta *et al.,* 2005). Mutations in three gene types builds the foundation of colorectal carcinogenesis, namely the tumor suppressor genes *adenomatous polyposis coli* (*APC*) and the *transformation related protein 53* (*TP53*) gene, as well as the oncogene *Kirsten rat sarcoma viral oncogene homolog* (*K-ras*) (Vogelstein & Kinzler, 2004). The *APC* gene encodes for a large protein with a significant role in intercellular adhesion, cytoskeleton stabilisation, cell-cycle regulation and apoptosis (Conlin *et al.,* 2005). A defect of the *APC* gene is suggested to enable uncontrolled replication of oncogenes, hence promoting carcinogenesis. It is also suggested that mutations in the *APC* gene arise in the early stages of carcinogenesis, and therefore play an important, gate-keeping role in the stages of cancer of both FAP and most sporadic CRC (Yamada *et al.,* 2002). The TP53 gene encodes for a nuclear phosphoprotein that binds to the DNA to activate DNA repair mechanisms during transcription. An inactive TP53 gene would therefore allow damaged cells to continue within the cell cycle, and later clonally expand. *Kirsten-ras* (*K-ras*) is an oncogene that help regulate cellular proliferation, and thereby allowing uncontrolled cell growth when activated.

Development of colorectal cancer is often explained by three specific pathways; chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and microsatellite instability (MSI) (Pino & Chung, 2010). Each cell in an organism contains clusters of DNA called chromosomes, which divides during cell differentiation (National Cancer Institute, W.Y; O'Connor, 2008). The CIN pathway is caused by mutations and/or defects in chromosome division, leading to instability in chromosomal numbers, or loss of heterozygosity (LOH) (Pino & Chung, 2010) (Fig. 3). LOH is a common form of allelic imbalance by which heterozygous somatic cells become homozygous because one of the two alleles get lost. FAP develops through the CIN pathway, which is often called the gatekeeper pathway due to the inactivation of gatekeeper genes, such as tumor suppressor genes (e.g., *APC* in CRC) that inhibits tumor growth (Macleod, 2000). The CIMP pathway is epigenetically caused by hypermethylations of CpG islands in the promotor region in the DNA, which silences tumor suppressor genes (Pino & Chung, 2010). The MSI pathway, also called the caretaker pathway, is a form of sporadic CRC that is characterized by nucleotide instability (Macleod, 2000). In CRC, caretaker genes include mismatch repair (MMR) genes, which by loss of functions, increases the DNA mutation rate and the chance of gatekeeper functions being lost.

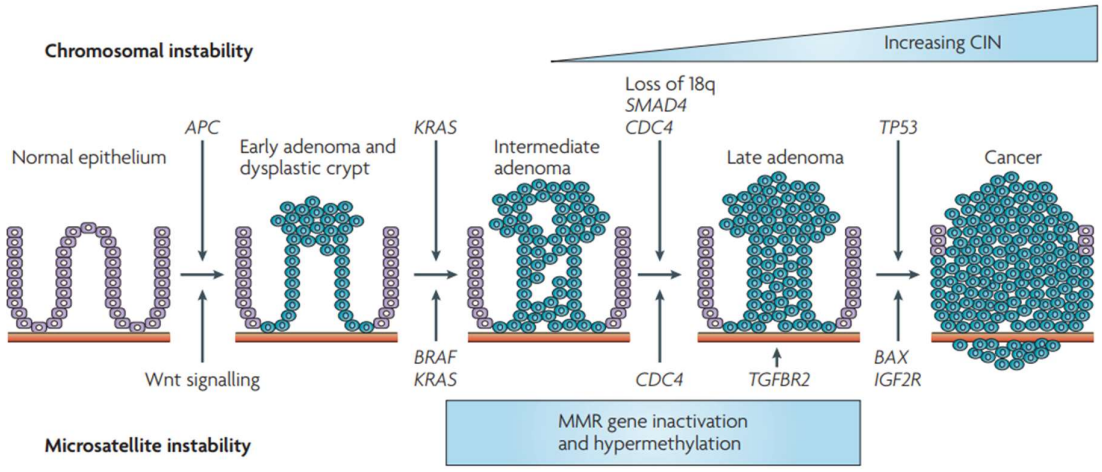

Figure 3. Development of CRC through the Chromosomal instability (CIN) pathway. Inactivation of the APC gene initiates the development, followed by an activation of oncogenes, and further inactivation of tumor protein 53 (TP53). Thereby transforming the epithelium into cancer. Adapted from Walther et al. (2009).

1.3.2 The canonical Wnt pathway and the *APC* Gene

The Wingless-related integration site (Wnt) signalling pathways are a collective term for signal transduction pathways that regulate crucial embryogenetic processes in eukaryotes, such as tissue patterning, cell polarity, cell fate specification, proliferation and migration (Routledge & Scholpp, 2019; Cadigan & Nusse, 1997). The canonical β-catenin-dependant pathway is a subgroup of the Wnt signalling pathways and involve regulation of β-catenin levels in the cell by either phosphorylation or accumulation of β-catenin (Fig. 4). The tumor suppressor gene *APC* plays an important role in the canonical β-catenin-dependant pathway, being a key component in a large "destruction complex" that regulates β-catenin. An activated Wnt signalling pathway includes the binding of a Wnt-ligand to a Wnt receptor (Frizzled receptor) at the cell-membrane, which signals a translocation of the destruction complex to the cell membrane region near the Wnt receptor (Bienz & Clevers, 2000). The translocation inhibits the *APC* complex to phosphorylate β-catenin, thereby increasing the β-catenin levels in the cell. The β-catenin further translocate into the nucleus and binds to a T-cell factor (TCF) protein, triggering transcription of Wnt target genes in the DNA. The transcription of the Wnt target genes finally stimulates cell growth and proliferation.

When Wnt-signalling is inactivated, β-catenin is phosphorylated by the destruction complex, leading to a ubiquitination and ultimately degradation of β-catenin (Bienz & Clevers, 2000) (Fig. 4). This process down-regulates the β-catenin levels in the cytosol, participating to normal cell proliferation, survival, differentiation and migration.

However, mutations in the *APC* gene leads to accumulation of β-catenin in the cytosol, even though the canonical Wnt signalling pathway is inactivated (Cadigan & Nusse, 1997). A mutational inactivation of *APC* stabilizes the large destruction complex by mimicking Wnt stimulation, and thereby inhibiting ubiquitination and degradation of β-catenin. This in turn increases the β-catenin levels, and the expression of *Wnt* target genes (Cadigan & Nusse, 1997; Giles *et al.,* 2003). During embryogenesis in mice, *Wnt* gene products are expressed all over the intestinal tract, whereas in healthy, adult humans, expression is thought to exist towards the bottom of the colonic crypts (Lickert *et al.,* 2001; Holcombe, 2002). Although it is believed that Wnt signalling have proliferating effects of stem cells in the human intestinal tract, this has not been formally proved (Gregorieff, 2005). However, the proliferative effects of the expressed *Wnt* genes on progenitor cells in colonic crypts is linked to initial premalignant

lesions, such as small polyps and aberrant crypt foci (ACF), seen in colorectal carcinogenesis of FAP patients and most sporadic cases (Giles *et al.,* 2003; Holcombe, 2002).

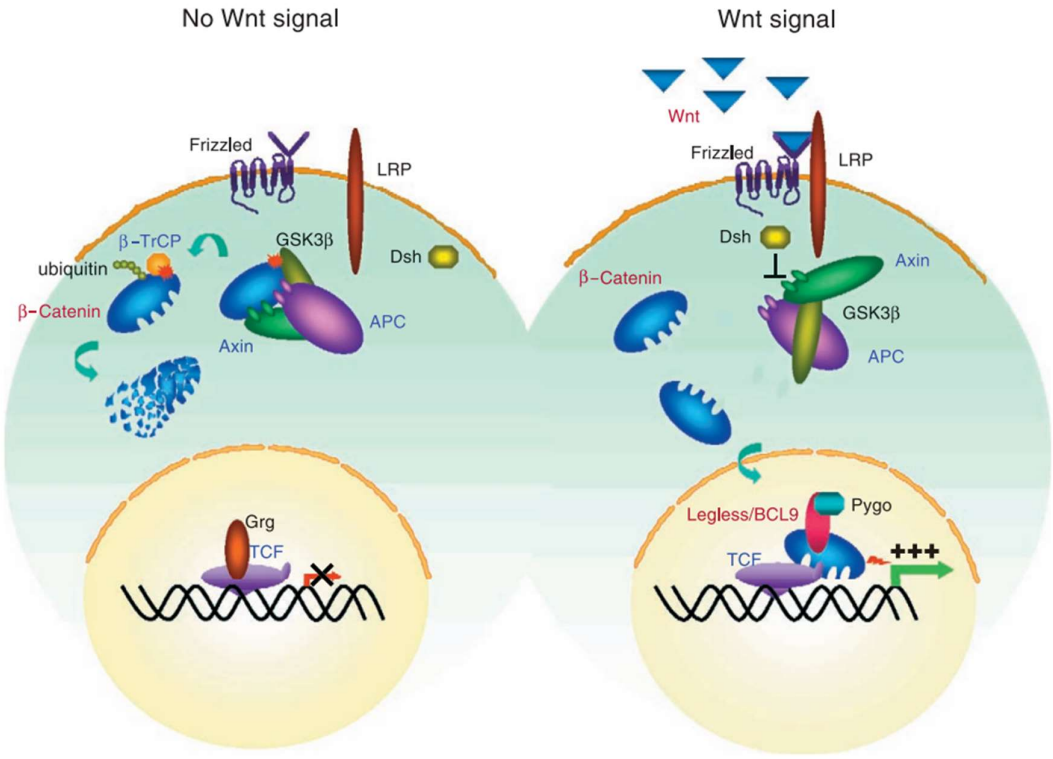

Figure 4. The Wnt signalling pathway. Left: an inactive Wnt pathway with the absence of a Wnt ligand regulates β-catenin levels in cytosol by phosphorylation and degradation by the APC complex. TCF target gene transcription is repressed. Right: Wnt-ligand binds to the receptor and the APC complex is translocated, granting accumulation of β-catenin. β-catenin moves into the nucleus, promoting transcription of TCF target genes. Adapted from Giles et al. (2003).

1.3.2.1 Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis (FAP) is an inherited disorder caused by a germline mutation in the tumor suppressor gene *APC*, which due to an inactivation of the tumor suppressing functions of the gene, leads to a formation of several possibly malignant colonic polyps (Màrmol, 2017; Fodde, 2002). The polyps develop during adolescence, and if left untreated, the adenoma will in all cases develop into CRC (Samadder *et al.,* 2014). The inherited mutation only occurs in one allele of the gene, meaning that a point mutation in the other allele will lead to an emergence of the carcinogenesis.

A so-called two-hit hypothesis was developed by Knudson (1971), explaining how both alleles in a tumor suppressor gene, such as *APC*, must be inactivated to induce cancer growth (Knudson, 1971). As for FAP patients, already predisposed with one mutated allele, only "one-hit" is required for cancer growth (Fodde, 2002). An *APC* gene with one functioning and one defect allele may lead to what is known as LOH and is one of the initial steps of developing CRC.

1.3.3 A/J Min/+ mice as a model for colorectal cancer studies

Prospective intervention studies on cancer and cancer development in humans would be unethical, as stated by the declaration of Helsinki from 1964 (Ashcroft, 2008). Human studies are often performed as prospective studies, which have a lot of confounding factors connected to it, such as nutritional factors, and lifestyle factors. Furthermore, especially concerning nutrition studies, it is problematic to perform intervention studies as CRC is a multifactorial disease that takes years to manifest from initial exposure. The strongest association are usually found in migration studies, like the study performed by Marchand & Kolonel (1992). The researchers investigated Japanese migrators to Hawaii and discovered a higher incidence of CRC among the participants after relocation (Marchand & Kolonel, 1992). However epidemiological studies can only find association factors, causality and dose-response relationships are found in animal studies.

As a result, murine models have been developed as an analogue for human research on CRC. The murine models have been applied extensively in medical and biomedical research as there are similarities between humans and rodents in anatomy, genetics, and physiology (Nguyen *et al.*, 2015). Comparative genomics have shown that humans and lab mice (*Mus musculus*) share 90% of corresponding regions in their genome (Chinwalla *et al.*, 2002). The authors also found a 99% homology between the mice and human genome.

According to Yamada *et al.* (2002), the most commonly used murine model was the C57BL/6J Min/+ (B6 Min/+), however their study found that Min/+ mice lost heterozygosity and developed tumors mainly in the SI (Yamada *et al.*, 2002). Furthermore, other studies show that these mice have a lower incidence of tumors in the colon, and that the adenomas rarely develop into an invasive carcinoma (Sødring *et al.,* 2016). The A/J mice strain was introduced as possible replacement as this strain is more sensitive towards carcinogens that are specific to the colon. Sødring *et al.* (2016), describes how the A/J Min/+ mice have an incidence of 100%

for carcinomas in the colon, when the mice were older than 30 weeks. Also, the same article describes how, for the first time in A/J Min/+ mice, the flat abberant crypt foci (fACF)(preneoplastic lesions) transitioned to adenomas, and subsequently carcinomas (Sødring *et al.*, 2016).

A common promotor of carcinogenesis in A/J Min/+ mice and humans, is the mutation of the *APC* gene (Sødring *et al.,* 2016). Similarly to humans, the *APC* gene in the Min/+ mice has a heterozygous truncation mutation at codon 850, which spontaneously induces intestinal adenomas. Hence, the A/J Min/+ mice model is often used as a human model for colorectal carcinogenesis.

1.4 Carcinogens

Compounds or agents that may lead to cancer are classified as carcinogens (American cancer society, 2023). These compounds may change or do damage to the DNA, which in turn may lead to uncontrolled cell division. Examples of carcinogenic factors can be ultraviolet or radioactive radiation, asbestos, or lifestyle factors, such as tobacco, diet and physical inactivity. International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization (WHO), made a classification of carcinogens based on the hazard of developing cancer whereas there are 4 different groups, 1 – carcinogenic, 2a – probably carcinogenic, 2b possibly carcinogenic, 3 – not classified. Approximately 100 compounds or exposures are classified in group 1, which includes both tobacco and processed meats (IARC, 2018).

Not all people who are exposed to carcinogens will develop cancer, as this is largely dependent on individual factors such as dose, duration of exposure, genetic susceptibility, and other lifestyle factors, such as diet (Mármol, 2017).

1.4.1 Diet and cancer

The diet and lifestyle of an individual may be instrumental in the development of cancer in humans, especially for those that are genetically predisposed (WCRF, 2018). World Cancer Research Fund (WCRF) has reported that certain types of food may reduce the risk of developing cancer, whereas others may enhance the risk of developing cancer (Ibid.). While

fermented dairy products is suggested to reduce the risk of breast cancer, fiber and calsium are proposed to reduce the risk of developing CRC (Kaluza *et al.* 2021; WCRF, 2018). Red and processed meat have been associated with promotion of cancerous growth (Ibid.). The associations between processed red meats and cancers are assessed by expert panels, and IARC has from thereof classified processed meat as a group 1 carcinogen (IARC,2018; WCRF, 2018).

The dietary pattern termed "western" was defined by Terry *et al.* (2001) as a diet that involves high amounts of red and processed meats, saturated fatty acids and refined sugars and carbohydrates. The western dietary pattern has been recognized as an associated factor in development of different lifestyle diseases, such as Diabetes mellitus type 2, metabolic syndrome, and different forms of cancer (Kim & Je, 2018; Schwingshackl *et al.,* 2017).

1.4.1.1 Red and processed meats and IARC/WCRF

Red and processed meats have been established as one of the foods that may be carcinogenic to humans, and has been classified as a class 1 carcinogen (IARC, 2018). Red meat was placed in group 2 A (probably carcinogenic to humans). Although both the IARC and WCRF have found associations between red- and processed meats and CRC, the mechanisms behind and causality are still unclear.

There are different definitions of what defines red and processed meats, and the term is applied incoherently throughout epidemiological research. The IARC and WRCF define read meat as muscle from mammals, whereas processed meats are categorized as meat that has been processed through for instance salting, curing, smoking or other enhancement processes to either improve preservation or flavour (IARC, 2018). The Norwegian cancer association (Kreftforeningen, W.Y.s), products such as bacon, sausage, and salami are classified as a processed meat products.

The report "Development of Norwegian diet" from the Norwegian health directorate states that the consumption of red meats in the Norwegian population have increased in 2021 (Norwegian Health Directorate, 2022). According to the same report, based on wholesale numbers, the consumption of red meats has increased from 50,5 kg to 53 kg per person. Furthermore, a population study conducted by Parr *et al,* (2013), Norwegian women between 41-70 years were found to have an increased risk CRC, when the participants consumed high amounts of

processed meat (Parr *et al.,* 2013). The association was found to be the strongest between sausage and CRC.

Meat is a collective term that is applied for the animal protein that derive from mammalian muscle. Generally, meat is classified into red meat and white meat depending on which animal, and which parts of the animal that are applied (Cross *et al.,* 2012). Chicken, turkey, and fish, as well as different species of fowl, could be defined as white meat. The myoglobin levels in these meats are lower than red meats, and thus the meat appears white. Red meat appears red, and is usually described as beef, veal, lamb, and occasionally pork. However, pork and duck has been difficult to categorize since the appearance of duck and pork change from pale to red post-cooking. It is also interchanged frequently in several studies and is therefore hard to categorize precisely. The hazard analysis from 2018 made by IARC defined the red meats as beef, pork, lamb, mutton, horse, goat and veal (IARC,2018). The classification comes as a result of the amounts of heme-iron, the oxygen-binding component that is present in the Myoglobin of the sarcoplasm. Red meats are rich in myoglobin, and red muscle fibres.

There are multiple theories as to why red meats are classified as carcinogenic to humans, whereas the one that is most extensively researched is the connection between red meat and heme-iron. A study by Pierre *et al.* (2004) found that a diet of beef and black pudding increased the colonic load, as well as malondialdehyde (MDA). The exposure led to a dose-dependent promotion of ACF in the colon. The same authors proposed that the effect was linked to peroxidation, as heme induces peroxidization of polyunsaturated fatty acids (PUFA). The concurrent heme and fat ingestion leads to generation of lipid peroxyl radicals, which can alter and sever DNA bases. Another theory regarding heme, is that N-nitroso compounds (NOC) of many which are known carcinogens, is stimulated by heme-iron (Cross *et al.,* 2003). There has also recently been a theory regarding gut microbiota, and how consumption of heme leads to change in microbiota (Ijssennagger *et al.,* 2015). This leads to a damage of the gut epithelium, and consequently hyperproliferation of the damaged cells. Another theory is dietary fat. Dietary fat, and especially the saturated fats, have long been known to be linked to obesity and cardio-vascular diseases (CVD) (Briggs *et al.,* 2017). Red meats are rich in monounsaturated fatty acids, including Oleic (C18:1), Palmitic (C16:0) and Stearic acid (C18:0) (Valsta *et al.,* 2005). The theory that has gathered most momentum is lipid peroxidation products, which is a product of bacterial consumption of bile acids, and consequently linked to toxic and carcinogenic secondary bile acids (Ajuzh *et al*., 2014).

There have also more recently been theories concerning gut microbiota. The colonic microbiota is the bacterial cells that resides inside the colon and contributes to digestion by fermenting insoluble fibre and other dietary components that have not been fully digested (Vipperla & O ´keefe, 2016). The microbiome refers to the collection of genomes from all the microorganisms in the environment. Microbiota, on the other hand, usually refers to microorganisms that are found within a specific environment. Microbiota can refer to all the microorganisms found in an environment, including bacteria, viruses, and fungi (Dalal *et al.,* 2021). The microbiota lays a crucial role, not only for digestion but also by producing metabolites, which may affect the body positively or negatively. Dietary factors, such as low variety of nutrients, could lead to dysbiosis. Dysbiosis is considered as a negative disturbance of the microbiota and could be the result of for instance use high intake of antibiotics, and as mentioned, different dietary factors. There are many suggested mechanisms that would explain how pivotal the microbiota would contribute to colorectal cancer. Many of these theories are multifactorial, but some suggest that diet contributes to dysbiosis in colon, which may enhance pro-inflammatory metabolites (Ibid.). Research on humans with CRC show that the fecal microbial load and composition were different from the healthy humans (Balamurugan *et al.,* 2008). This leads to a damage of the gut epithelium, and consequently hyperproliferation of the damaged cells.

Furthermore, there have been theories around protein, meat-related mutagens, bovine virus infections and N-glyconylneurmanic acid (Portune *et al.*, 2016; Cross & Sinah, 2004; Zur Hausen & De Villiers, 2015; Samaraj *et al.* 2015). However, these theories are not further explored in this study.

1.4.1.2 Ultra processed food, and plant-based meat alternatives

The link between red/processed meat products and health concerns, as well as sustainability, animal welfare and other causes, has made many consumers demanding more plant-based options. Soy based products, like Tofu, has long been popular in the Asian countries, but have become a staple food in many households (Ishaq *et al.*, 2022). However, over a longer period, these products have been lacking in the organoleptic and sensory attributes and has therefore not been an adequate substitute for meat products. In more recent years, several advances within the industry, and better understanding of the ingredients, the meat substitutes has

improved. In fact, the market for plant-based meat products, also termed meat analogues, have been rapidly expanding, and is expected to grow to around 235 million USD by the year 2032 (Fact.Mr, W.Y). The most employed meat-substitute ingredients are soybeans, legumes and cereals, which are formulated to mimic the sensory and organoleptic attributes of meat. The products are usually shaped like hot dogs, nuggets, hamburgers, and slices to increase the appeal to the omnivorous consumer (Ishaq *et al.,* 2022). The research on these studies is scarce, but a review by Ishaq *et al.* (2022) mentions a study where usage of *in vitro* digestion models that compared plant-based foodstuffs and meat, resulted in slower digestion of lipids in the SI (Zhou *et al.,* 2021). Since many of the plant-based products also contain anti-nutrients that hinder digestion, such as tannins, lectins, phytates and trypsin inhibitors, it is important to investigate the impact of processing, and *in vivo* trials of nutrient absorption from these products.

Many of these plant-based meat-substitutes can be defined as ultra processed. Ultra processed food (UPF) is a recent term that has been gaining traction by the mass media for quite some time. Even though there are some foods that often are mislabelled as ultra processed (hamburger), and other foods that are labelled as ultra processed (hot dog), the definition is not yet well-established. The term is used interchangeably and often mislabelled among consumers, mass media and even in research. The classification known as NOVA has divided the foods into 4 groups based on degree of processing, whereas group 1 is minimally or not processed, and group 4 is ultra processed (Monteiro *et al.,* 2019). The term is being described by Monteiro *et al.* (2019), as formulations or foods that are the result of industrial processing and uses industrial ingredients or formulations (Ibid.). This would be emulsifiers, preservatives or other compounds added to the food to enhance or change functional and sensoric properties. Often ultra processed foods are seen as cost-effective, as many additives are cheaper and promote longer shelf life in different products. This results in cheaper products for the consumer. Nevertheless, ultra processed foodstuffs have been linked to cancer and obesity in several studies. Even so, more research is required on the topic to be able to investigate the underlying mechanisms of the impact of ultra processed foods on health (Fiolet *et al.,* 2018; Mendonça *et al.*, 2016; Schnabel *et al.,* 2018)

1.5 Research questions and objectives

Diet and nutrition are key factors closely related to causation and prevention of numerous cancer forms (World Cancer Research Fund and American Institute for Cancer Research, 2007). Existing data on CRC development reveal a complex interplay between diet, lifestyle factors, genetic predisposition and microbiota. How processed meat products are involved in initiation, promotion or progression of CRC are not yet known. Research efforts are needed to better understand cancer risk factors, as well as improving prevention policies.

The primary objective of the present study was to investigate the carcinogenic potential of experimental diets prepared from commercially available processed meat products -and plant-based meat alternative in A/J Min/+ mice. The following secondary objectives were defined:

1. To assess the number, size and load of lesions in SI and colon in A/J Min/+ mice exposed to the experimental diets;

2. To investigate gender differences in number, size and load of lesions in SI and colon in A/J Min/+ mice exposed to the experimental diets;

3. To explore the influence of the experimental diets on biometrical parameters, i.e., body weight, liver weight and length of SIs and colon.

We hypothesize that:

1. the processed meat products have a higher carcinogenic potential than the plant-based meat alternative and the reference diet in A/J Min/+ mice (Objective 1).
2. a higher processing degree of meat products, using nitrite, has a higher carcinogenic potential than processed meat products without nitrite (Objective 1).
3. the processed meat products have a higher carcinogenic potential than the plant-based meat alternative and the reference diet in A/J Min/+ mice females than in males (Objective 2).
4. diets dominated by processed meat products have a different influence on biometrical parameters (i.e., body weight, liver weight, length of SIs and colon) than diets dominated by plant-based meat alternatives (Objective 3).

2.0 Materials and methods

2.1 Ethical statement

The experiment was approved by the Norwegian Food Safety Authority (application ID: FOTS 23274) and conducted in accordance with national and local ethical guidelines at the Norwegian University of Life Sciences, (NMBU). The animals were kept at the Section for Experimental Biomedicine, NMBU Faculty of Veterinary Medicine, Ås, Norway, under specific pathogen free conditions according to recommendations by the Federation of European Laboratory Animal Science Association (FELASA; http://www.felasa.eu/).henhold)

2.2 Animals and husbandry

An inbred colony of A/J mice heterozygous for the Min trait (Min/+) were included in this study. Female A/J mice (wildtype +/+) in pairs were housed with one male A/J Min/+ mice to produce offspring with the Min/+ genotype. The offspring were weaned, separated by sex and marked by ear punches at 3 weeks of age.

Tissue from the ear punches were used to determine the Min/+ genotype as previously described by Sødring *et al.* (2015). In brief, the genotyping was performed at the animal facility laboratory by experienced technical personnel, using allele-specific polymerase chain reaction (PCR) on DNA extracted from ear punch samples. Visualization of the PCR product was achieved by gel electrophoresis using a 2,2% agarose gel (Lonza FlashGel Systems, Basel, Switzerland). Min/+ mice show a PCR product at 327 base-pairs, in addition to the +/+ (WT) allele consisting of 618 base-pairs (Dietrich *et al.,* 1993).

All mice were housed in NexGen Rat 900 individually ventilated cages (IVC) (Allentown Inc, USA) containing standard aspen bedding (J. Rettenmaier & Söhne GmbH + Co KG, Germany), white cardboard houses, brown paper nesting material, brown cardboard tunnels, wooden chewing sticks and transparent plastic tunnels (Scanbur A/S, Norway). Reverse osmosis (RO) water was available *ad libitum* from 250 mL bottles (Allentown Inc, USA). Bottles, RO water and cages (including bedding and nesting material) were changed once per week. Environmental enrichment and tops were changed once per month or when necessary. Breeding cages were fed Ssniff breeding feed *ad libitum* while offspring were fed a mixture of Ssniff

breeding and maintenance feed *ad libitum* from weaning until recruitment (Scanbur A/S, Norway). After recruitment, mice were fed AIN-93M feed (Scanbur A/S, Norway) *ad libitum* except for between 15:00 and 09:00 every Monday to Friday, at which the feed was removed to ensure feeding on the experimental diets. The AIN-93M feed was stored at 4°C until use. The animal room was kept on a 12:12 day:night light cycle with a transition period of 30 minutes. The temperature and relative humidity were 21.6 ± 0.4 °C and 52 ± 20%.

Mice included in the experiment were housed in groups of 5 mice per cage. The animals were monitored daily for any signs of discomfort due to intestinal cancer development (general appearance, behaviour, blood in faeces, rectal bleeding or rectal prolapse) and body weight were recorded weekly following a humane endpoint scoring form (see scoring form in Appendix E)

2.3 Experimental setup

This study is part of an exploratory pilot study. A/J Min/+ mice of both sexes (N=80) were randomly recruited into 4 experimental groups at 4 weeks of age (Fig. 5). From July to August 2022, the recruitments were performed continuously until 20 mice, regardless of sex, were included in each group. After recruitment, the mice were nocturnally exposed to experimental diets 5 nights per week (Monday to Friday) for 9 weeks between 15:00 and 8.00. The estimated daily intake of the experimental diets where maximum 5 grams per mouse. The weights of the frozen experimental diets were recorded before exposure and residues were removed and weighed the following day. The sampling at termination is included in section 2.1.4.

The recruitment was done by about 4 weeks of age (28 days ± 6 days), 22 (minimum) and 29 (maximum) (Fig. 5). Both sexes are recruited but are kept in different cages. All mice that were recruited into the trial were put into separate research cages, and all mice were weighed, and samples of feces was collected in week 8 and week 13 to be used in a later study.

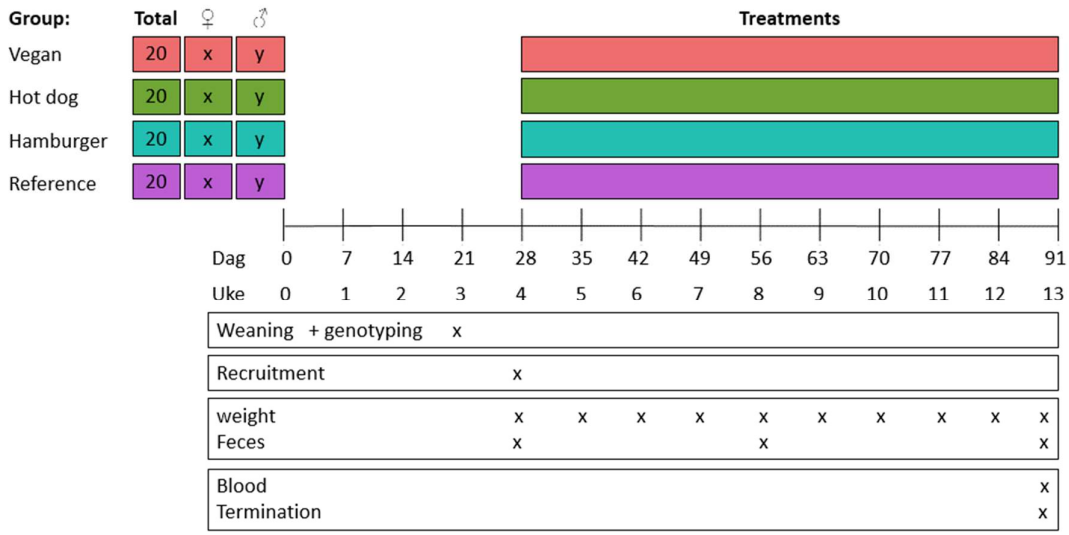

Figure 5. Experimental setup of the study. The total number of animals per group was 20. Vegan diet is illustrated as pink, the hot dog diet as green, the hamburger diet as turquoise, and the reference diet as purple.

2.4 Experimental diets

The experimental diets are based on commercial processed meat -and plant-based meat alternative products purchased from Oda.no and prepared at Animalia (Økern torgvei 13, Oslo, Norway). The diets were thawed at 2°C for 3 days prior to manual homogenization for the hamburger and vegan diets, and homogenization using an industrial meat grinder for the hot dog diet. Portions of 5 (+/- 0,5) grams were lightly compressed to meat balls and stored in plastic bags with a total weight of 25 (+/-1) grams per bag. The bags were vacuum sealed and heated in a water bath until a core temperature of 70°C was reached and subsequently cooled down in cold water for at least 10 minutes. The diets were transported to the Section for Experimental Biomedicine, NMBU (Ås, Norway) and stored at -20°C until the day of administration.

The nutritional content provided by the producer of the food products used in the diets are presented in Table 1. The nutritional content was also analysed using FoodScan, to compare to the values given by the producer. The nutritional values provided by FoodScan are listed in Table 2.

The ingredient list from the producers of the food products used in the experimental diets are shown in Table 3. The ingredients are listed from top to bottom, depending on amount (from highest to lowest).

Table 1. Nutritional content of the different food products used in the experimental diets, given by the producer. The nutrients are stated in grams per 100 grams of product.

	Hot dog	**Hamburger**	**Vegan burger**
Energy (kJ)	944.5	933	844
Energy (cal)	227.7	224.5	203
Fat (g)	18.5	17.1	16
Whereas saturated fatty acids (g)	6.7	7.7	7
Whereas monounsaturated fatty acids (g)	7.9	6.5	
Whereas polyunsaturated fatty acids (g)	2.4	0.8	
Carbohydrates (g)	5.2	2	2.8
Whereas sugars (g)	0.6	0.2	0.9
Protein (g)	10.1	15.6	12
Salt (g)	1.7	1.4	1.2

Table 2. Nutritional content of the food products from FoodScan. Grams are given per 100 grams.

	Hot dog	**Hamburger**	**Vegan burger**
Protein (g)	12.38	15.1	17.71
Fatty acids (g)	16.54	17.68	12.9
Water (moisture) (g)	64.21	62.74	62.26
Collagen (g)	2.3	2.83	2.69

Table 3. List of ingredients of the food products used in the experimental diets, listed by the producer.

Hot dog	Hamburger	Vegan burger
Meat of pork and beef (57%)	Coarse ground beef (85%)	Vann
Pork head meat	Water	Soy protein
Water	Starch	Vegetable oil (coconut oil, rapeseed oil)
Potato flour	Onion	Onion
Salt	Salt	Aroma
Spices	Pepper	Spices
Dextrose		Tomato
Stabilizer E451		Stabilizer (methylcellulose)
Antioxidant E315		Food coloring (beetroot)
Preservative E261		Mushroom
Preservative E326		Smoke aroma
Preservative E250		Food coloring (caramel)
Smoked with beech wood chips		Garlic

2.5 Sample collection

At 13 weeks of age (± 7 days) the mice were terminated. The body weight was recorded before the animals were anesthetized using isoflurane gas (Baxter, San Juan, Puerto Rico). Prior to cervical dislocation, blood was collected by cardiac puncture, using a 1 mL syringe with a hypodermic needle (23G, 16 mm) flushed with ethylene diamine tetraacetic acid (EDTA) disodium salt solution (Honeywell International Inc, Charlotte, USA). Blood was cooled on ice prior to centrifugation at approximately 6000 rpm for 10 minutes (Hermle Z160M, Hermle Labortechnik, Wehingen, Germany) and plasma was collected into a cryo tube, snap frozen in liquid nitrogen and stored in –80*C.

The gall bladder was carefully separated from the liver, the liver was excised, put in ice cold PBS and weighed. The left lobe was stored in one cryo vial and the remaining lobes in another cryo vial before snap freezing in liquid nitrogen and storage in –80*C.

The intestines were excised, and cecum was removed, and weighed. Cecum content was squeezed out into a cryo tube, while the tissue was put in a separate cryo tube, and frozen on liquid nitrogen. The SIs and colon were flushed carefully with ice cold phosphate buffered saline (PBS) before being cut open longitudinally and further rinsed in beakers with ice cold PBS. The SI was cut into three parts at approximately the same length. All intestinal segments were flattened, placed between two filter papers, and fixated in 10% formalin for approximately

24 hours. After fixation, the intestines were stained with methylene blue (0,1%) dissolved in formalin for approximately 25 seconds, rinsed in three consecutive baths of formalin and stored in 70% ethanol at 4°C.

The frozen feces, plasma, liver and cecum samples were collected for other projects and are therefore not further discussed herein.

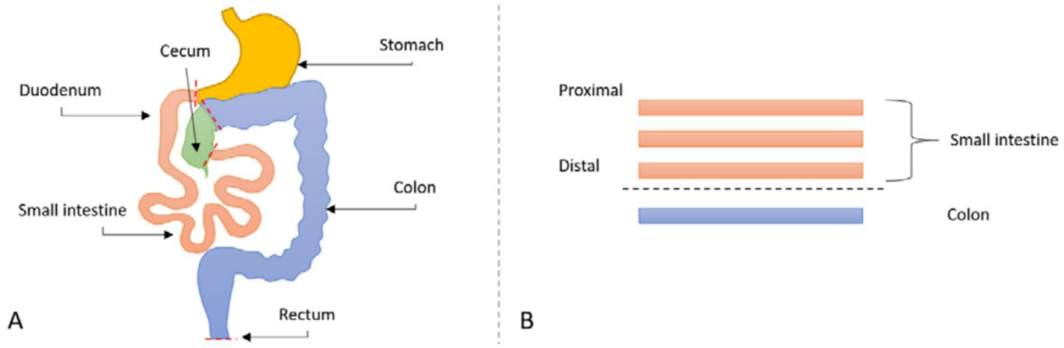

Figure 6. Simplified illustration of the Gastrointestinal tractus. The SI ***was separated from the stomach and cecum (indicates with red strippled line). The colon was cut from cecum and rectum for sampling (red strippled lines). B: Illustration of the preparation of the intestines.*** The SI ***was divided into three sections; proximal, midsection and distal. The colon was used intact. Adapted from Murphy (2020).***

2.6 Scoring of intestinal lesions

Colorectal carcinogenesis in the A/J Min/+ mouse model has been described previously (Sødring *et al.,* 2016). In brief, the initiating stage, characterized by mutations and arise of lesions corresponds to the "number" of lesions analysed in the present study. The promoting stage, where an expansion of the lesions is caused by a tumor growth contributor, relates to the "size" of lesions in the analyses.

A minimum of 24 hours after staining, the intestines were scored for lesions by surface microscopy using an inverted light microscope (ECLIPSE Ts2R, Nikon, Tokyo, Japan). Scoring was performed randomly and blinded to experimental groups by one observer for the colon and one observer for the SI. Lesions were separated from lymphoid organs and the diameter was measured with an eyepiece graticule in intervals of one cm along the length of

the intestine. Lesion size was calculated by assuming a completely circular shape. The total surface area covered by lesions was defined as lesion load. Colonic lesions were classified as either flat aberrant crypt foci (flat ACF; <30 crypts) or tumors (>30 crypts), as explained by Sødring *et al.* (2016). For the SI, the terms small lesions and large lesions were used, with the same classification as flat ACF, with <30 crypts for small lesions and >30 crypts for large lesions.

The fACF and tumor, as well as small and large lesions in the SI, was recognized by a colour difference from the normal epithelium. The fACF, tumors and lesions appeared as more turquoise in colour when compared to healthy epithelium (Fig. 7). Furthermore, the lesions were recognized by abnormally large crypts, which while laying on a flat surface may appear more like gyruses in appearance. The SI was, as described in previous sections, in proximal, middle and distal parts, and the lesions were divided into small and large. The small and large term for the lesions in the SI was applied for the same dimensions as the flat ACF and tumors in the colon.

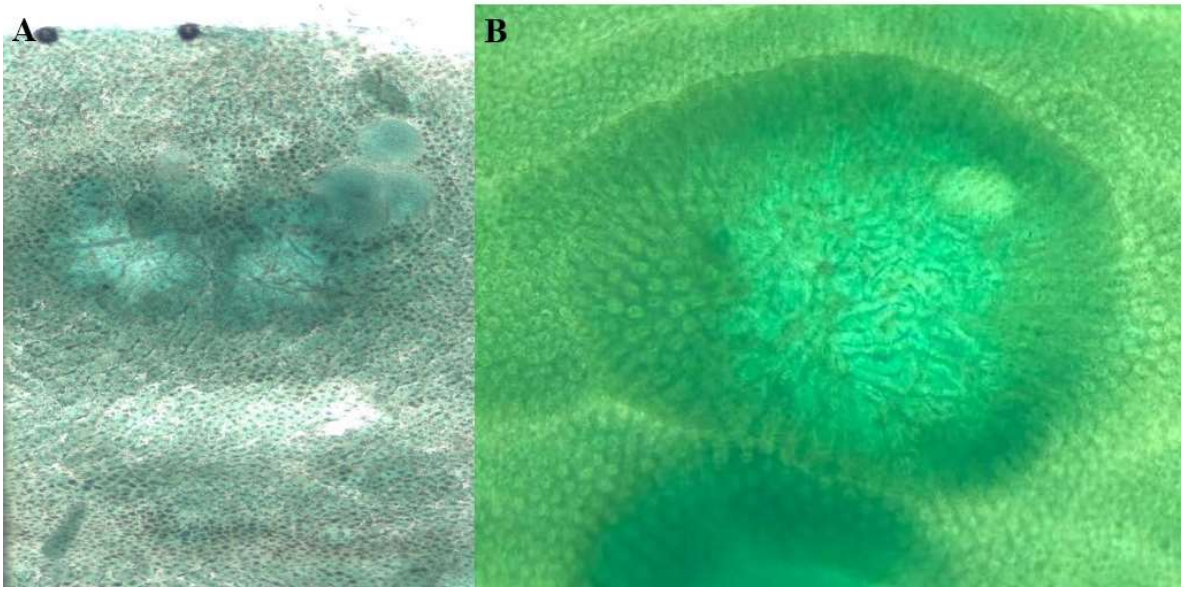

Figure 7. Surface morphology of lesions in the SI (A) and the colon (B). Photos: Haaseth, H., and Olsen, L.M.B.

2.7 Statistical analysis

All results are expressed as median values with 95% confidence intervals, and a significance level of 0.05 and borderline 0.08 were used in the analyses.

The lesions of the SI were divided into the categories small and large, whereas the colonic lesions were categorized as “fACF” of “tumor”. The statistical analyses of small/fACF and large/tumor number, size and load, and biometry, were conducted in R studio version (R-studio team, 2020) using the packages; Utils (R core team, 2022), stats (Core team, 2022), methods (R core team, 2022), grDevices (R core team, 2022), datasets (R core team, 2022), graphics (R core team, 2022), base (R core team, 2022), tidyverse (Wickham *et al.,* 2016), Dunn.test (Dinno, 2017), FSA (Ogle *et al.,* 2023), ggplot2 (Wickham, 2016). Variable fit to the normal distribution was tested using Shapiro-Wilk normality test (Royston, 1982; Shapiro & Wilk, 1965), histogram and Q-Q plot. Variance homogeneity within groups was checked by Barlett test. Variables with a satisfactory fit to the normal distribution (before or after log-transformation) and equal variance between groups were analysed using Analysis of Variance (ANOVA). If a significant result was obtained from the ANOVA, a Tukey HSD (honestly significant difference) post hoc test (Tukey, 1949) was conducted to investigate the differences between exposure groups, with and without gender stratification. Variables that did not fit to the normal distribution were analysed by Kruskal-Wallis (KW) test (Kruskal *et al.*, 1952) on non-transformed data. A Dunn's Multiple Comparison (DMC) (Dunnett, 1955) post hoc test with a Bonferroni correction was conducted if the Kruskal-Wallis's test yielded a significant result. All comparisons were performed with a statistical significance level of 0.05. Some results with significance level of 0.08, hereby known as borderline significant, were also included.

3.0 Results

The present study aimed to assess the carcinogenic potential, e.g. number, size (mm^2) and load (total area) of lesions in both the SI and colon, of a vegan ultra processed product (group 1), hot dog (group 2), hamburger (group 3), in A/J Min/+ mice following 9 weeks of dietary exposure. Additionally, AIN-93 was given to the reference group (group 4). The distribution of gender within each group is given in Table 4. In total, 20 mice were recruited to each group. One mouse in the hamburger group and one in the reference group were sacrificed prior to termination due to issues not related to the exposure, and therefore the hamburger and reference group includes 19 mice at termination.

Table 4. The total number of female and male mice recruited into the study, with the total number at termination in parentheses.

Group	Female	Male	Total N
Vegan	**10**	**10**	**20**
Hot dog	**10**	**10**	**20**
Hamburger	**14 (15)**	**5**	**19 (20)**
Reference	**14 (15)**	**5**	**19 (20)**

3.1 Carcinogenic potential of the experimental diets

3.1.1 SI

Effects of the experimental diets on small intestinal lesions are presented in Fig. 8 and Table 5. Exposure to the hot dog diet (group 2) significantly increased the number and load of small lesions when compared to the reference group (group 4) (Tukey HSD, p =0.03, Dunn`s test, p=0.01) (Fig. 8A, Fig. 8C). In addition, exposure to the hot dog diet (group 2) showed borderline significantly increased load of small lesions when compared to vegan burger (group 1) (Dunn´s test, p = 0.06).

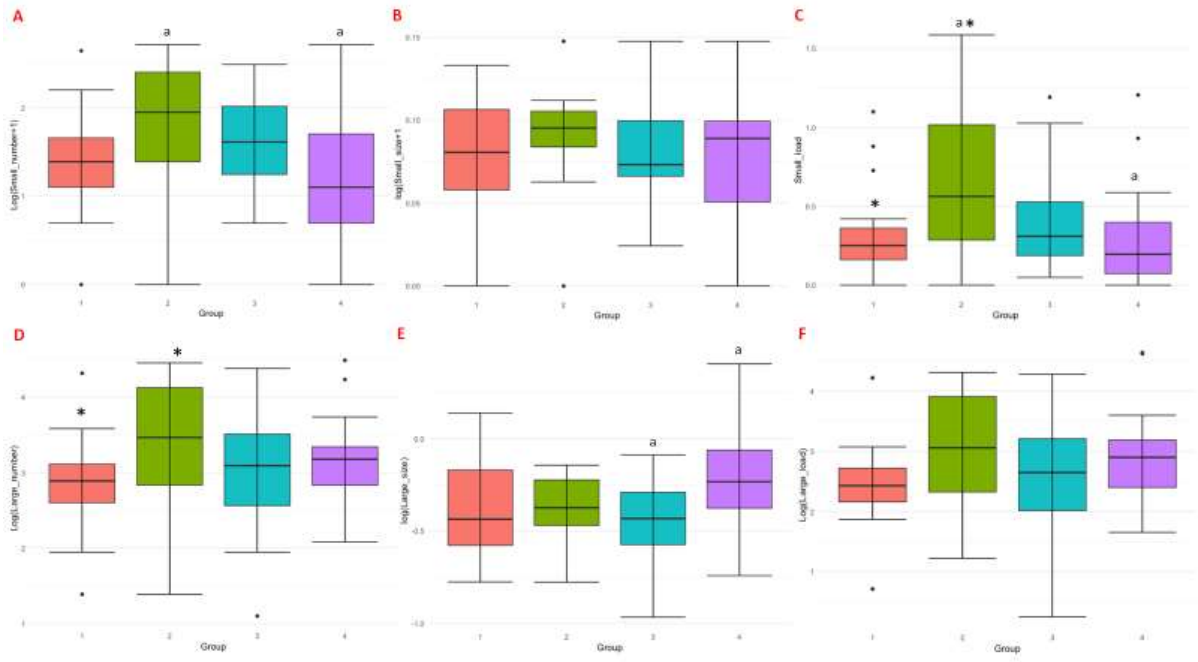

Figure 8. Carcinogenic potential of the experimental diets given as (A) number of small lesions, (B) size of small lesions, (C) load of small lesions, (D) number of large lesions, (E) size of large lesions, and (F) load of large lesions in the SI, (a/b/c = significance at chosen alpha level (p>0.05).* = borderline significance over chosen alpha level (p<0.1)). Group 1 (pink) = vegan diet, group 2 (green) = hot dog diet, group 3 (turquoise) = hamburger diet, group 4 (purple) = reference diet.

For the size of large lesions in the SI, exposure to the reference diet (group 4) significantly increased when compared to the hamburger diet (group 3) (Tukey HSD, p=0.02) (Fig. 8F), but not when compared to the hot dog diet (group 2) or the vegan diet (group 1). Interestingly, exposure to the hot dog diet (group 2) showed borderline significant increase in the number of large lesions when compared to the vegan diet (group 1) (Tukey HSD, p=0.06) (Fig. 8D). No significant differences were found for the size of small lesions or load of large lesions. No significant differences were found when numbers or load of small and large lesions were assessed together.

Table 5 presents the mean, median and minimum-maximum range of the number, size and load of lesions in the SI and the colon. The hot dog group was observed to have the highest median for number, size and load of small and large lesions, except the size of large lesions. For size of large lesions, the reference group was observed to have the highest median (M=0.79).

Table 5. Mean/median and range (min -max) of number, size and load of lesions in both the SI and the colon.

	Vegan	Hot dog	Hamburger	Reference
SI	Mean/Median [Min-Max]	Mean/Median [Min-Max]	Mean/Median [Min-Max]	Mean/Median [Min-Max]
Small number	3.65/3.00 [0.00-13.00]	6.40/6.00 [0.00-14.00]	4.73/4.00 [1.00-11.00]	3.26/2.00 [0.00-14.00]
Small size	0.08/0.08 [0.00–0.14]	0.09/0.09 [0.00–015]	0.08/0.07 [0.02–0,15]	0.08/0.09 [0.00-0.15]
Small load	0.32/0.25 [0.00-1.09]	0.65/0.56 [0.00-1.58]	0.40/0.31 [0.04-1.19]	0.30/0.19 [0.00-1.20]
Large number	20.35/18.00 [4.00–75.00]	38.25/32.00 [4.00–86.00]	28.63/22.00 [3.00–80.00]	27.58/24.00 [8.00–89.00]
Large size	0.71/0.64 [0.46-1.14]	0.69/0.68 [0.45-0.86]	0.65/0.64 [0.38-0.9]	0.84/0.79 [0.47-1.49]
Large load	14.68/11.37 [2.04–68.02]	27.69/21.40 [3.40–74.46]	21.24/14.14 [1.28-72.20]	26.40/18.17 [5.24–103.36]
Colon				
fACF number	9.10/5.00 [0.00-30.00]	19.85/8.50 [1.00-67.00]	9.94/4.00 [1.00-44.00]	35.95/20.00 [3.00-237.00]
fACF size	0.02/0.02 [0.00-0.07]	0.02/0.02 [0.004-0.03]	0.01/0.01 [0.005-0.03]	0.02/0.02 [0.01-0.03]
fACF load	0.19/0.10 [0.00-0.62]	0.31/0.20 [0.01-1.16]	0.14/0.05 [0.01-0.54]	0.66/0.29 [0.08-4.32]
Tumor number	0.80/0.50 [0.00-4.00]	1.40/0.50 [0.00-5.00]	0.58/0.00 [0.00-2.00]	1.11/1.00 [0.00-4.00]
Tumor size	0.24/0.09 [0.00-1.13]	0.92/0.14 [0.00-4.46]	0.67/0.00 [0.00-2.64]	0.77/0.26 [0.00-2.83]
Tumor load	0.40/0.09 [0.00-2.59]	2.96/0.14 [0.00-14.29]	1.01/0.00 [0.00-5.28]	1.42/0.26 [0.00-5.65]

3.1.2 Colon

Effects of the experimental diets on colorectal lesions are presented in Figure 9 and Table 5. Exposure to the reference diet (group 4) significantly increased both number and load of fACF when compared to the vegan diet (group 1) and hamburger diet (group 3) (Tukey HSD, $p<0.01$; $p<0.01$ and $p=0.03$; $p<0.01$, respectively) (Fig. 9), but not the hot dog diet (group 2). Furthermore, there was a significant increase in fACF size when exposed to the vegan diet and the reference diet when compared to the hamburger (group 3) (Tukey HSD, $p=0.01$ and $p=0.02$, respectively). However, no statistical significance was found for number, size or load of tumors (>30 crypts) between the groups.

The highest maximum number and load of fACF were observed in the reference group (group 4), whereas the highest maximum number of tumors was observed in the hot dog group (group 2) (Table 5). Noteworthy, the lowest fACF load was observed in the vegan diet.

In all the variables (number, size and load of fACF and tumor), the reference diet (group 4) was observed to have the highest median (Table 5). In contrast, the hamburger diet (group 3) was observed to have the lowest median in all variables.

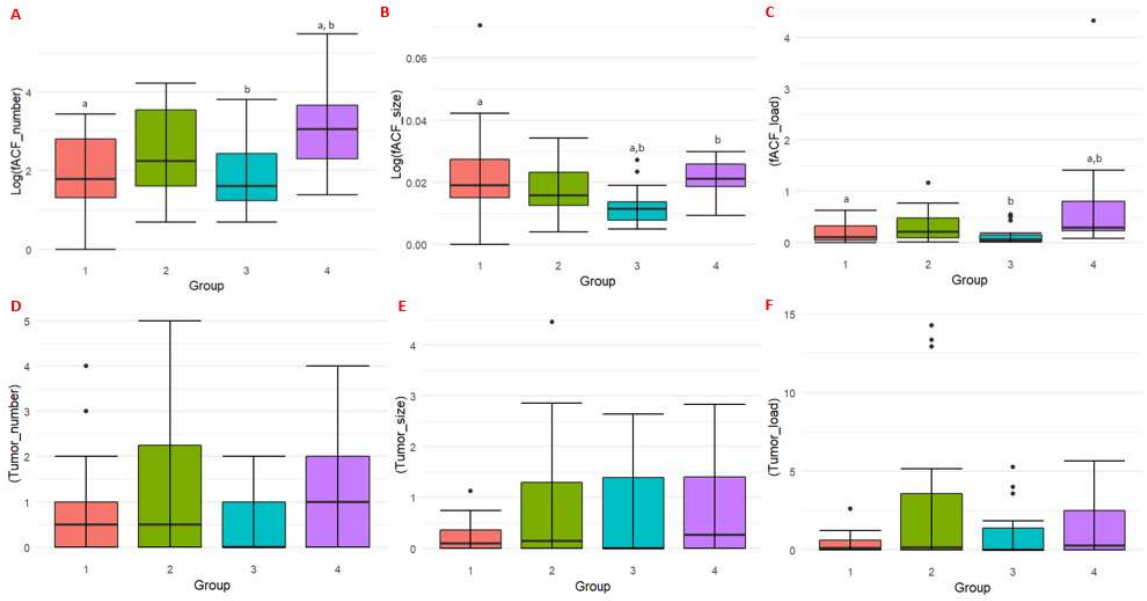

Figure 9. Carcinogenic potential of the experimental diets given as (A) number of fACF lesions, (B) size of fACF lesions, (C) load of fACF lesions, (D) number of tumor lesions, (E) size of tumor lesions, and (F) load of tumor lesions in the colon. ***(a/b = significance at chosen alpha level (p>0.05).* = borderline significance over chosen alpha level (p<0.1)). Group 1 (pink) = vegan diet, group 2 (green) = hot dog diet, group 3 (turquoise) = hamburger diet, group 4 (purple) = reference diet.***

3.2 Gender differences in carcinogenic potential of the experimental diets

3.2.1 SI

There were no significant differences in carcinogenic potential between the experimental -and reference diets when stratified by gender (Fig. 10). However, the highest median and maximum of number and load of small lesions were observed in the hot dog group for both females and males (Appendix B). In addition, the females exposed to the reference diet had the widest range

of number and size of small lesions. In the vegan group, males had a wider range in number, size and load of small lesions than the females.

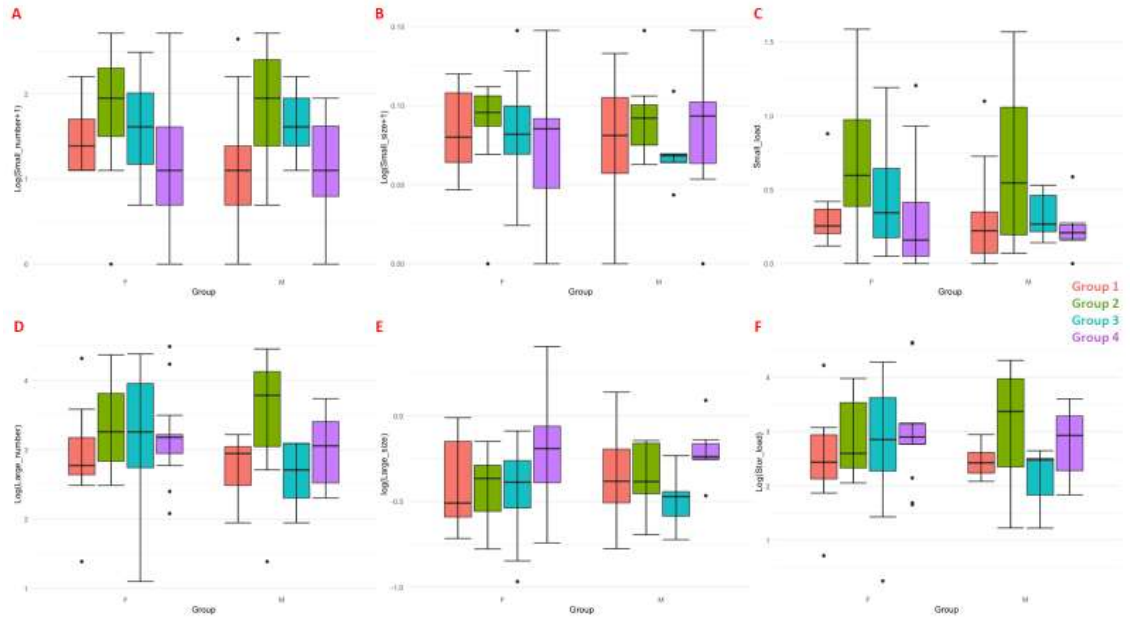

Figure 10. Carcinogenic potential of the experimental diets stratified by gender given as (A) number of small lesions, (B) size of small lesions, (C) load of small lesions, (D) number of large lesions, (E) size of large lesions, and (F) load of large lesions in the SI***, stratified by gender. (a/b/c = significance at chosen alpha level (p>0.05).* = borderline significance over chosen alpha level (p<0.1)). Group 1 (pink) = vegan diet, group 2 (green) = hot dog diet, group 3 (turquoise) = hamburger diet, group 4 (purple) = reference diet.***

Females in the hot dog group and hamburger group had somewhat comparable medians for the number of large lesions, however the hamburger group showed a wider range than the hot dog group (Appendix B). In males, the hot dog group had the highest median and maximum of number and load of large lesions. The males in the hamburger group did not display the wide range of number and load of large lesions as in females.

3.2.2 Colon

Carcinogenic potential of the experimental diets stratified by gender is given in Figure 11 and Appendix B. Females in the vegan group showed significantly lower number of fACF than females and males in the reference group and males in the hot dog group (Tukey HSD, p=0.02, p=0.02 and p=0.06, respectively).

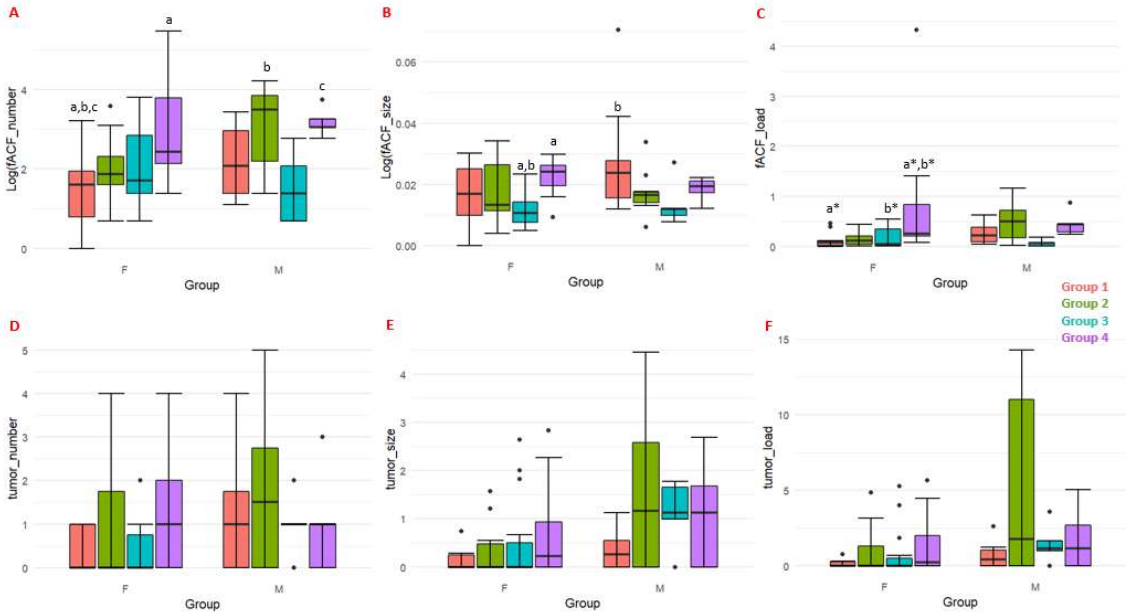

Figure 11. Carcinogenic potential of the experimental diets given as (A) number of fACF lesions, (B) size of fACF lesions, (C) load of fACF lesions, (D) number of tumor lesions, (E) size of tumor lesions, and (F) load of tumor lesions in the colon, stratified by gender. ***(a/b/c = significance at chosen alpha level (p>0.05).* = borderline significance over chosen alpha level (p<0.1)).*** ***Group 1 (pink) = vegan diet, group 2 (green) = hot dog diet, group 3 (turquoise) = hamburger diet, group 4 (purple) = reference diet.***

Size of fACF was significantly lower in females exposed to the hamburger diet when compared to females in the reference group and males in the vegan group. (Tukey HSD, p=0.05 and p<0.01, respectively) (Fig. 11).

Load of fACF was borderline significantly higher in females in the reference group when compared to females in the hamburger group and the vegan group (Dunn's test with Bonferroni adjustment, p=0.088 and 0.08, respectively) (Fig. 11). However, no significance was observed in the number, size and load of tumors.

We also observed that males exposed to the hot dog diet had the highest median in number and load of fACF and tumors (Appendix B).

3.3 Body weight, liver weight, and length of SI and colon

Table 6 displays the results of body weight, liver weight and length of SIs and colon following 9 weeks exposure to the experimental diets or reference diet.

3.3.1 Body weight

There were no significant differences in total body weight between the groups (p=0.93) or between gender (Anova, p=0.31 and p=0.46). However, we observed that the males in all groups had a higher mean than the females. Furthermore, the highest body weight was observed in the hamburger group for both genders (Male=29.0 g, Female=22.6 g).

Table 6. Body weight, liver weight and length of both SI and colon are presented with and without stratification on gender. Results are presented as mean and range [min-max values]. The results were analysed using Anova and Tukey HSD.

	Vegan burger	**Hot dog**	**Hamburger**	**Reference diet**	**P-value**
N (male/female)	10/10	10/10	5/14	5/14	
Final body weight, total (g)	23.4 [16.4-28.5]	23.9 [19.9-32.5]	23.9 [17.7-33.7]	23.5 [18.4-34.2]	Aov, 0.936
Final body weight, male (g)	26.2 [18.8-28.5]	26.4 [23.4-32.5]	29.0 [25.4-33.7]	28.2 [21.2-34.3]	Aov, 0.311
Final body weight, female (g)	20.6 [16.4-23.1]	21.4 [19.9-23.4]	22.6 [18.7-26.7]	22.1 [19.4-27.1]	Aov, 0.466
Liver weight, total (g)	1.09 [0.75-1.40]	1.17 [1.02-1.35]	1.11 [0.78-1.57]	1.04 [0.81-1.37]	Aov, 0.0951
Liver weight, male (g)	1.22 [0.92-1.40]	1.19 [1.02-1.35]	1.40 [1.17-1.57]	1.15 [0.93-1.32]	Aov, 0.0284
Liver weight, female (g)	0.97 [0.75-1.11]	1.16 [1.08-1.31]	1.04 [0.78-1.24]	1.00 [0.81-1.37]	Aov, 0.0045
Length of SI, total (cm)	37.65 [32.40 - 41.10]	35.53 [33.00 - 38.20]	36.37 [33.00 - 40.00]	32.96 [30.50 - 35.20]	Aov, 5.24e-11 Aov2, 4.4e-11
Length of colon, total (cm)	10.62 [9.40-11.80]	10.30 [8.50-11.40]	9.29 [8.50-10.30]	9.91 [8.40-11.70]	Aov, 8.44e-06 Aov2, 0.00459

***Aov: analysis of variance/Anova**

***Aov2: Two way Anova**

3.3.2 Liver weight

No significant difference in liver weight was found between the groups. The highest liver weight was however observed in the hot dog group (1.17 g) (Table 6).

When stratifying by gender, exposure to the hamburger diet significantly increased the liver weight in males when compared to the vegan diet, the hot dog diet, and the reference diet (Tukey HSD, p=0.08 (borderline significance level), p=0.04 and p=0.03, respectively). Female mice in the hot dog group had a significant increase in liver weight when compared to the vegan and reference diet (Tukey HSD, p<0.01 and p=0.01, respectively).

The male mice in the hamburger group were observed to have the highest liver weight (1.40 g), whereas the lowest liver weight was observed in females in the vegan group (0.97 g).

3.3.3 Length of SIs and colon

Length of SI and colon were measured after fixation (Table 6). The vegan group was observed to have the highest mean value with regards to length of SI (37.65 cm), while the reference diet had the lowest mean of all groups (32.96 cm).

The length of SI was significantly increased by exposure to the vegan diet when compared to the reference diet and the hot dog diet (Tukey HSD, $p<0.01$ and $p<0.01$, respectively) (Table 6). Exposure to the reference diet significantly decreased the length of the SI, when compared to hot dog diet, hamburger diet, and vegan diet (Tukey HSD, $p<0.01$, $p<0.01$ and $p<0.01$). The vegan group exhibited the highest mean in regard to length of SI, followed by the hamburger group (37.65 cm and 36.37 cm, respectively) (Table 6). When stratifying by gender a significant decrease in length of SI was revealed in the female reference group when compared to the male hamburger, hot dog and vegan diet, and females exposed to the hamburger, hot dog and vegan diet (Tukey HSD, females: $p<0.01$, $p=0.01$, $p<0.01$,males: $p<0.01$, $p=0.05$, $p<0.01$ respectively). Furthermore, a significant decrease of the SI was exhibited among the males exposed to the reference diet when compared to the male hamburger and vegan diet and female hamburger, vegan, and hot dog diet (Tukey HSD, females: $p<0.01$, $p=0.07$ (borderline), $p<0.01$, males $p<0.01$, and $p=0.01$, respectively).

Exposure to the vegan burger diet significantly increased the length of colon, when compared to the hot dog diet, the hamburger diet, and the reference diet (Tukey HSD, $p<0.01$, $p<0.01$ and $p=0.02$, respectively). Furthermore, exposure to the reference diet revealed a borderline significant increase when compared to the hamburger group (Tukey HSD, $p=0.06$). Length of colon was observed with the highest mean in the vegan group, followed by the hot dog group (10.62 g and 10.30 g, respectively) (Table 6). When stratifying by gender, a significant increase in length of colon was revealed in both males and females exposed to the vegan burger diet, when compared to the females exposed to the hot dog diet and the females exposed to the hamburger diet (Tukey HSD, females: $p=0.02$ and $p<0.01$, males: $p=0.08$ (borderline) and $p<0.01$, respectively). The females exposed to the vegan diet also displayed a significant increase when compared to the males exposed to the hamburger diet (Tukey HSD, $p=0.04$).

4.0 Discussion

The present study is part of an exploratory pilot where we assessed the carcinogenic potential of commercially available processed red meat and plant-based meat alternatives in the A/J Min/+ mouse model for colorectal cancer. The mice were exposed to experimental diets for 9 weeks. We also investigated gender differences in the carcinogenic potential and explored whether the processed red meat and plant-based meat alternatives influenced on biometrical parameters, such as body weight, liver weight and length of SI and colon.

4.1 Assessment of carcinogenic potential of the experimental diets

The hot dog diet significantly increased small number and load of lesions in the SI, when compared to the reference group (Fig. 8). Furthermore, the hot dog diet had borderline significantly increased number of large lesions in the SI when compared to the vegan burger, thus displaying an initiating potential in the SI. The carcinogenic effect of this processed hot dog in the SI corresponds to human studies where hot dogs have been found to increase risk of colorectal cancer (Parr *et al.,* 2018; WCRF, 2018; IARC, 2018). The hot dog used in our study is commercially available, has an array of different ingredients (Table 3) and could be classified as ultra processed. One of the ingredients that has been the most studied and questioned whether to have influence on the carcinogenesis is nitrite. Nitrite has been extensively evaluated by IARC and WCRF and is categorized in group 2A (probably carcinogenic to humans) (IARC, 2010). However, the evidence is scarce. Nitrite is known to be a precursor to NOCs, which are found to be highly carcinogenic in animal studies (Bogovski & Bogovski, 1981). However, Sødring *et al.* (2015) found that dietary nitrite had no effect on colonic carcinogenesis, and even suggested an inhibitory promoting effect in the SI. Nitrite is, as previously mentioned, a preservative and antibacterial agent used in foodstuffs to inhibit growth of *Clostridium botulinum* (Dellavalle et al. 2014). Nitrite is also beneficial for meat producers where it preserves or enhances red color in meat (Macdougal *et al.,* 1975). However multiple studies, including Sødring *et al.* (2015), hypothesize that nitrite is an initiator of carcinogenesis in mice, as found in the present study. Furthermore, in a review article by Crowe *et al.* (2019) five of 11 murine studies (*in vivo*) found a link between nitrite and promotion of CRC, yet three of the 11 studies found no link. Moreover, Cross *et al.,* (2003) reported that high levels of heme iron, nitrite and protein in combination increased endogenous formation of NOCs. On the other

hand, the hot dog also contains antioxidants, compounds which are established to have a protective effect on the formation of NOCs (Santarelli *et al.,* 2008). It seemed however, that the antioxidants did not play a protective role in our study. The complex relationships and interactions of the different compounds in processed meat and the carcinogenic potential should therefore be more extensively studied.

As described in the introduction, there are several possible mechanisms of processed meat inducing carcinogenesis. Heme has been discussed as a possible promoter of carcinogenesis. A study by Pierre *et al.* (2004) found that all the meat-based diets (blood sausage (high heme), chicken (low heme), and meat (medium heme) triggered the formation of fACF in the colon of Fischer 344 rats (Pierre *et al.,* 2004). Furthermore, they found a dose dependent effect, whereas the blood sausage (high heme) gave a higher effect than beef (medium heme). However, the same study also used AOM as an initiator, although this was only administered once, the carcinogenesis had been initiated by AOM and not the experimental diet. Another study performed by Sødring *et al*. (2015), showed no such effects of dietary hemin (a model of red meat) in the colon. Contradictory to Pierre *et al.* (2004), Sødring *et al.* (2015) found that mice fed with diets of hemin had significantly lower number of flat ACF in the colon than a nitrite-based diet. However, Sødring *et al.* (2015) did also establish a site-specific effect in the SI, where it stimulated growth of tumors. It could, however, be speculated that if heme was the cause of carcinogenesis, then the hamburger would also significantly increase the number or size of lesions. The hamburger group was one of the groups that did not lead to any significant increases in load, size or number (Fig. 8). It should therefore be questioned what the hot dog contains of components or other ingredients that can explain the present finding of initiating potential on carcinogenesis in the SIs.

An interesting reflection is that the processed diets in the present study have in common that they are both smoked or contain smoke aroma (Table 3). High processing temperatures have been linked to heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons (PAH) (Santarelli *et al.,* 2008). The same study states however, that smoke aroma will not provide contamination of PAH. HCAs are compounds formed by pyrolysis of amino-acids and creatine, which are shown to be ultimate carcinogens. PAHs are formed when inorganic compounds incompletely combust. PAHs and HCAs are most commonly found in smoked meat and fish, tobacco, and other products that have been exposed to high temperatures for a longer period of time. Even though both contaminant-groups are well-known carcinogens, several studies dispute that their levels present in processed food products are of CRC concern. Several other

meats, such as chicken, contain high amounts of HCA, but the same link to CRC is not established (Steppeler *et al.,* 2017). It is also established that the dosages used in exposure studies in murine studies are 1000 – 10000x what a human would be able to consume. However, Santarelli *et al.* (2008), also stated that the link between red, processed meat and colon cancer seems to depend on cooking methods rather than concentrations of PAHs and HCAs (Santarelli *et al.,* 2008). The experimental diets in the present study were heated in a water bath until a core temperature of 70°C was reached. This gentle cooking method was used in an attempt to reduce the formation of temperature-related contaminants enabling exploration of the carcinogenic potential of the protein source or processing procedures the specific diets represented. Therefore, we suggest that PAH and HCA have likely not been the most important drivers behind the initiation of lesions in the SI.

Another theory regarding the carcinogenic potential of red, processed meat and CRC is that the secondary bile acids may work synergistically with dietary carcinogens to promote the development of tumors (Ajouz *et al.* 2014). High-fat diets have been linked to increase secretion of bile acids (Hofman, 2004). Secondary bile acids, however, are produced by secondary fermentation of bile acids. As an example, Lithocholic acids (LCA), the secondary end product of chenodeoxycholic acids (CDCA), are found to be highly toxic in animal models (Ajouz *et al.,* 2014). Increased amounts of secondary bile acids cause the formation of reactive oxygen species (ROS), which may contribute to apoptosis of the intestinal cells by straining the antioxidant defense, genomic instability, and oncogenic growth, consequently, converting normal cells to cancer cells (ibid.). However, this would only account for the carcinogenic potential in the colon, as secondary bile acids are created by the colonic microbiota (Treuting, 2018b). Also, the vegan diet had the lowest fatty content of all the diets (Table 2). We cannot explain why an increase in fACF size was discovered when comparing the vegan diet and the reference diet to the hamburger diet (Fig. 9). However, secondary bile acids should be further investigated.

The reference diet had significantly higher number and load of fACF (< 30 crypts), i.e., initiating potential on the carcinogenesis, in the colon when compared to hamburger and vegan diet (Fig. 9). Similar findings were obtained in Steppeler *et al.* (2017) where the reference feed (RM1) showed the highest tumor load when compared to different meat diets. This can correspond to our findings, however, Steppeler *et al.* (2017) used the RM1 as reference diet, which is seen to greater affect the carcinogenesis than the more newly developed AIN-93M feed (Fischer-Scientific, W.Y). Furthermore, Steppeler *et al.* (2017) did not investigate

processed meat, but dietary beef, pork, chicken and salmon. The standard rodent diet RM1 has been shown to cause significantly more fACFs and tumors in colon of the A/J Min/+ mouse model than the AIN-93M diet (Sødring *et al.,* 2015). Thus, in the present study, AIN-93M was utilized as reference diet and standard maintenance diet given to all animals in addition to the experimental diets. Since the dietary components in the standard rodent diets stimulate carcinogenesis in the A/J Min/+ mouse model, the reference diet may be regarded as a positive control (Sødring *et al.,* 2015). Interestingly, the hot dog diet was not significantly different in number and load of fACF from the reference, suggesting that the hot dog may have comparable initiating potential on the carcinogenesis as the reference diet (Fig. 9). However, this suggestion must be verified in future studies.

In addition, both the reference and vegan diet significantly increased fACF size, indicating a promoting potential, when compared to hamburger (Fig. 9). The reference diet also increased the size of large lesions in the SI when compared to the hamburger group. A promoting effect of AIN-93M on carcinogenesis in the A/J Min/+ mouse model has also been reported by Sødring *et al.* (2015). However, the higher promoting potential of the vegan diet compared to the hamburger was not expected.

The AIN-93M diet contained high amounts of carbohydrates and dietary fiber (Fischer-scientific, W.Y). Inulin-rich foods are known to be a preventative factor regarding colorectal cancer (Fernandez *et al.,* 2019). This is caused by the prebiotic effect to produce short chain fatty acids (SCFA). The microbiota resides in the intestines in humans with the main fermentation of dietary compounds in the colon, but in rodents the cecum is the main fermentation chamber (Treuting, 2018b). The microbiota is also responsible for *in situ* production of SCFAs, among these butyric- and propionic acids. Fiber rich foods, especially inulin, are closely connected to this prebiotic effect. Both butyric acids and propionic acids are manifested as protective agents which induce apoptosis in tumor cells in the colon. Fernandez *et al.,* (2019) investigated the link between fiber and induction of CRC by enriching common processed meat products with inulin. The results exhibit a statistical decrease of colonic polyps (49%) when comparing the inulin-rich meat products and the regular processed meat products. However, Moen *et al.,* (2016) investigated the link between dietary fiber and carcinogenesis in the A/J Min/+ mice. By using different doses of three diets (AIN-93M) enriched with 5% and 15% fiber, they found that mice fed with inulin had a 50% lower load of tumors than the other experimental diets. However, the same research also suggested a dose-dependent relationship between fiber and tumor load, as the AIN-93M feed with 15% (w/w) showed a significant

increase in tumor load. Our study did establish a significant effect of the AIN-93M feed, however we established mainly an initiating effect on carcinogenesis in the colon and not promoting effect (Fig. 9). A promoting effect of carcinogenesis was found when comparing the reference group and the hamburger group in the SI (Fig. 8), which is in opposition to Moen *et al.* (2016), who only found promoting effects in the colon. It could therefore be questioned whether the fermentation of the AIN-93M in the cecum produced metabolites with carcinogenic properties in the colon. Nonetheless, these findings require more investigation on the relationship of dietary fibers, microbiota, and carcinogenesis.

The gut microbiota, both in the SI and the colon, play key roles in the host adaptability to dietary variations, especially lipids. Repeated exposure to meat diets may lead to a permanent shift in the overall composition of the microbiota (Martinez-Guryn *et al.,* 2018). This shift in composition is referred to as dysbiosis and has been associated with several chronic inflammatory diseases and CRC. A variety of dietary substrates are used by these microbes to produce a range of metabolites, some of which are beneficial to the host, such as butyrate (Martinez-Guryn *et al.,* 2018). The microbiota has the possibility of acting as an inhibitory agent for carcinogenesis. However, a study by Sears & Pardoll (2011) proposes that microbiota also could act as a promoting agent for carcinogenesis. The study proposes the alpha-bug theory, which includes the theory that alpha-bugs live within the microbiota. The alpha-bugs can outperform bacterial species that show protective abilities against carcinogenesis. Thus alpha-bugs, by proliferating extensively, cause dysbiosis, ultimately leading to an enhancing effect of carcinogenesis. This hypothesis is supported by Bråten *et al.* (2017) by exhibiting a positive correlation between *Bacteroidetes* and tumor load. The same findings are stated in a study by Son *et al.* (2015) which found a significant association between abundance of *Bacteroidetes* and CRC in C57BL/6 *APC* Min/+ mice. Fernandez *et al.* (2019) also found a significant association between *Bacteroidetes*, but the findings were associated with the reduction of colonic polyps. Our results reveal significant differences for the reference when compared with the hamburger (Fig. 8 and 9). Microbiota analysis of feces was not examined in our study and must therefore be investigated at a later point in time.

An interesting aspect regarding the reference group is that this group was only exposed to this diet, hence, had a lower variety in their diet than compared to the other groups that were exposed to the experimental diets in addition to AIN-93M as maintenance diet. As stated by dietary recommendations worldwide and the WCRF (2018) the importance of a balanced diet can lead to prevention of many lifestyle diseases, including cancer (WCRF, 2018). Key *et al.*

(2002), and Doll & Peto (1985) states that diet is a cause of cancer that can be avoided by changing eating patterns (Key *et al.,* 2002; Doll & Peto, 1985). The same researchers state that between 35%-80% of all cancers could be attributed to dietary factors. Additionally, the AIN-93M diet is highly processed and could be classified as UPF. A definition of UPFs is hard to come by as there is a high variation in the uses of the term, however the organization NOVA has made a classification of UPFs as "foods that have ingredients which are mostly of industrial use, that concludes from industrial processes requiring special equipment" (Monteiro *et al.,* 2019). Hang *et al.* (2022) reviewed 3 prospective cohorts and found that intake of UPFs increased the risk of adenomas and polyps. Furthermore, exclusion of processed meats did not affect the risk. The results are similar to the findings in this study, whereas a significant increase of fACF size was found when the A/J Min/+ mice were exposed to the ultra processed vegan burger, compared to the hamburger (Fig. 9). The results highlight the importance of investigating the link between ultra processed foodstuffs and risk of carcinogenesis.

We hypothesized that the processed meat products have a higher carcinogenic potential than the plant-based meat products and the reference diet in A/J Min/+ mice. Indeed, we found a significant initiating increase of carcinogenesis in the SI when exposed to the hot dog diet compared to the reference diet. An initiating potential on the carcinogenesis was also found for the reference diet in the colon when compared to the vegan diet and the hamburger diet. However, the vegan diet and the reference diet also displayed a promoting effect as opposed to the hamburger diet in the colon. Such an effect of the reference diet was also found in the SI, compared to the hamburger diet. By affecting both initiating and promoting effects of both the SI and the colon, the reference groups had the highest carcinogenic potential, thus the hypothesis that diets with a high consumption of meat gives a higher carcinogenic potential must be partially rejected.

Another hypothesis was that a higher processing degree of meat products, e.g., added nitrite, has a higher carcinogenic potential than processed meat products without nitrite. In the present study the hot dog was the only commercially available processed product containing nitrite. The results displayed that exposure to the hot dog group exhibited a significant initiating effect of carcinogenesis in the SI, when compared to the reference group. The hot dog also exhibited a borderline promoting effect on carcinogenesis. However, the results did not reveal any significant results between the hamburger group and the hot dog group both in the SI and the colon. As mentioned, only the reference diet displayed an initiating effect of the carcinogenesis in the colon, whereas both the reference diet and the vegan diet showed a promoting potential.

Thus, the findings indicate a higher initiating effect of the hot dog in the SI, however, no conclusions can be drawn towards a higher carcinogenic potential of the hot dog compared to the hamburger. Concerning the colon, the results are in opposition to the hypothesis, disclaiming the proposed higher carcinogenic effect in processed meat products containing nitrite as opposed to processed meat products not containing nitrite. Due to the nature of the results, we would suggest more research to investigate the interrelation between red, processed meats, nitrite, and carcinogenic potential.

4.2 Gender differences

When stratifying by gender, an exposure to the vegan burger diet significantly decreased the number of fACF (< 30 crypts) in the colon in females, when compared to the females with the reference diet, thus exhibiting a lower potential to initiate the carcinogenesis (Fig. 11). A lower initiating potential of the vegan diet amongst females was also revealed when compared to the males of the hot dog diet and the males of the reference diet. Steppeler *et al.* (2017) also found that the reference diet had a higher impact of the carcinogenesis in A/J Min/+ mice than the meat-based diets. Interestingly, the AIN-93M consists mainly of carbohydrates, as well as soybean oil (Fischer-Scientific, W.Y). Soy is a legume known to be rich in natural estrogens, so called phytoestrogens, which has been suggested to have natural cancer-protective effects (Jargin, 2014; Adlercreutz & Mazur, 1997). The reference diet did however not display such an effect in the present study. The vegan diet, on the other hand, also based on soy, might have exhibited a protective role within the females. The observations of lower max and median levels in the vegan group amongst females in both the SI and the colon, further support the cancer-protective effects of the phytoestrogens (Appendix B). Hence, it can be speculated whether the composition and processing of the AIN-93M feed (reference diet) caused the increased gender-specific trend towards the females, and whether the possible anticarcinogenic effects of the phytoestrogens in the soy was more present in the vegan diet than the reference diet (Adlercreutz & Mazur, 1997).

The female hamburger group showed a decrease in fACF size as opposed to the males in the vegan group only (Fig. 11). Thus, it seemed to be a more promoting, gender-specific risk of carcinogenesis towards the males. Ditonno *et al.* (2021) suggests a role of two nuclear estrogen receptors (ER) in development of CRC, specifically ERα and ERβ, where ERα activates anti-

apoptotic pathways, and ERβ induces apoptosis (Ditonno *et al.,* 2021). Several studies using the *APC* Min/+ mice model propose the anticarcinogenic potential of ERβ (Giroux *et al.,* 2010; Weyant *et al.,* 2001 and Cho *et al.,* 2007). Giroux *et al.* (2010) treated both male and female mice with an ERβ-selective agonist, resulting in a significant decrease in SI polyp number and diameter (Giroux *et al.,* 2010). A study by Weyant *et al.* (2001) demonstrated the anticarcinogenic role of ERβ through upregulation of ERβ and downregulation of ERα in ovariectomized Min/+ mice (Weyant *et al.,* 2001). The experimental mice displayed the same number of lesions as the control group, thus exhibiting the protective role of ERβ. Another study by Cho *et al.* (2007) proposed however both ERα and ERβ as inhibitory modifiers of *APC*-dependent carcinogenesis, and that loss of ER signalling could induce CRC in postmenopausal women (Cho *et al.,* 2007). The role of estrogen receptors in CRC found in these studies could thereby explain the significant differences between the males and females found in our study, females having higher levels of endogenous estrogens. Moreover, the phytoestrogens in the vegan diet might have had an additional effect on the females.

A trend towards the reference group in females was observed for both the SI and the colon. In the colon, a significantly promoting effect of the reference diet was found when compared to the hamburger diet. Furthermore, the reference diet had highest maximum levels in most variables in both the SI and the colon (Fig 10 and 11). The A/J Min/+ mice, born with an inactive *APC* gene, is expected to develop cancer in most cases, however the arise of lesions can differ from the different ages (Sødring *et al.,* 2016). High maximum levels in small/fACF variables could translate into a triggering effect of the initiation of carcinogenesis, meaning that exposure to the experimental diet initiated the development of several, newly arisen lesions, consisting of < 30 crypts. Hence, exposure to the reference group seemed to cause an increase in initiated lesions in females. High max levels of large/tumor variables can relate to a supporting effect of the promoting stage of carcinogenesis, and that early onset lesions (and possibly congenital lesions) positively responded to the exposure of the diet by expanding to lesions of > 30 crypts. Based on the results from the large and tumor variables in the SI and colon, the reference group could have promoted the expansion of already existing lesion in females (Fig. 10 and 11).

Concerning the male groups, a trend towards the hot dog diet was found, showing initiating effects of carcinogenesis in the colon, as well as highest max levels in most variables in both the SI and colon (Fig. 10 and 11; Appendix B). The findings imply that intake of processed meat (hot dog) increased the carcinogenesis amongst males. Thus, a gender-specific

relationship between the reference group and the female mice, and the hot dog group and male mice was displayed.

Furthermore, it is recognized that the development of carcinomas occurs at earlier age and at a higher rate in men compared to women (Amos-Landgraf *et al.,* 2014). This corresponds to our findings, where the males exposed to the hot dog diet displayed a trend towards highest max and median values in all tumor variables (colon) when compared to the females (Appendix B). Moreover, expansion and promotion of early onset/congenital lesions is stated by Amos-Landgraf *et al.* (2014), to be more commonly seen in men than in women.

Another reflection is the influence of fat content on carcinogenesis, where Drasar & Irving (1973) suggests an association between a high dietary fat intake and development of cancer (Drasar & Irving, 1973). As the primary objective of this study was to investigate the carcinogenic potential of commercial processed meat –and plant-based meat alternatives in A/J Min/+ mice, it is interesting to reflect upon the effect of fatty acids, seen that a western diet is often characterized by high intake of processed meat and fats (Terry *et al.,* 2001). In the respect of the hot dog group amongst the males, fat content could possibly be associated to the carcinogenic potential found in this study, as the hot dog was richer in fat than of other macronutrients (Table 2). This is consistent to the findings of Wasan *et al.* (1997), where *APC* Min/+ mice displayed a significant increase in intestinal tumor when fed to a high fat diet (Wasan *et al.,* 1997). Another study by Miller *et al.* (1983) further supports these discoveries, finding that a diet high in saturated fats gave highest significance in relative risk of developing CRC (Miller *et al.,* 1983). However, these findings are not consistent with the results from the hamburger diet in the colon in our study, which seemed to have the lowest yield of lesions for both genders even though this diet had the highest fat content (Fig. 11; Table 2). Also, no significant differences were discovered between the males and females of the hamburger diet in our study, which does not correlate to the statement from Chakraborty & Wang (2020) that the association between a western diet and lifestyle and development of CRC are stronger in men than in women (Chakraborty & Wang, 2020).

In the case of the phytoestrogens and fats, Adlercreutz & Mazur (1997) states that a combination of a typical western diet consisting of high fat intake and phytoestrogens, may not be beneficial as to develop cancer, and that the cancer-preventing effects may be reserved to those of a low-fat diet. It can therefore be debated whether an intake of phytoestrogens through

soy either promotes or prevents carcinogenesis in humans, and if the effects are dependent on fat intake or other unknown factors.

To sum up, we hypothesized that the processed meat products would have a higher carcinogenic potential than the plant-based meat products and the reference diet in females than in male A/J Min/+ mice. We found that the hot dog diet amongst males and the reference diet amongst females had a significantly increased initiating potential of carcinogenesis when compared to the vegan diet amongst females. Our findings also revealed that exposure to the vegan diet for males and the reference diet for females gave a significantly higher promoting effect than the hamburger diet for females. Thus, the hypothesis must be rejected. We therefore emphasize the necessity of further research on the carcinogenic, gender-specific potential of red, processed meat versus processed meat alternatives.

4.3 The influence of the experimental diets on biometrical parameters

The last objective of the present study was to explore the influence of the experimental diets on biometrical parameters, i.e., body weight, liver weight and length of SIs and colon. No significant differences in body weight were found between the experimental diets, with or without stratification by gender (Table 6). However, the hamburger group had the highest mean value in both total bodyweight and for females and males separately. This could be related to the fat content of the hamburger, which according to the FoodScan results had the highest fat content compared to the hot dog and the vegan burger (Table 2). As stated by WHO (2021), an energy-dense diet with excessive amounts of fats and sugars, as well as decreased physical activity increases the risk of obesity (WHO, 2021). Obesity or other lifestyle diseases due to an energy-dense diet is also related to an increased risk of developing cancer, as previously mentioned (WCRF, 2018). Interestingly, the hamburger diet was observed to have a low carcinogenic potential in the colon (Fig. 9), suggesting that the fat intake did not display a significant role in the development of CRC in this study.

A large-scale study by Ma *et al.* (2013) states that the association between diet, obesity and CRC are stronger in men than in women, and Pischon & Nimptsch (2016) further states that the risk of developing malignant polyps in the colon is 70% higher in obese men than in women. The hamburger group displayed the shortest length of colon (9.29 cm) when compared to the other diets, but highest bodyweight for both males and females (M=29.0 g, F=22.6 g) (Table

6). Although speculative, it can be questioned whether the hamburger, and it´s components, may have disturbed the longitudinal growth of colon during adolescence, and thus also reduced the spread of crypts with mutations, consequently reducing the number of developing lesions.

Although no significant differences were found for total liver weight between the experimental diets, significant differences were found when stratifying by gender (Table 6). Exposure to the hamburger group significantly increased the liver weight amongst males when compared to the other groups. Increased body weight and obesity is associated with an increased risk of developing non-alcoholic fatty liver disease (NAFLD), a gastrointestinal metabolic disorder characterized by increased accumulation of triglycerides in the liver (Chakraborty & Wang, 2020). NAFLD is further correlated to several cancer types, amongst others, colorectal cancer (Chakraborty & Wang, 2020; Pischon & Nimptsch, 2016).

In addition, exposure to the hot dog diet significantly increased the liver weight of the female groups, when compared to the vegan diet and the reference diet (Table 6). A high liver weight could relate to the toxicity of the diet, as it is a frequently used measurement in toxicology (Cattley & Cullen, 2013). This could therefore apply for both the high liver weight amongst the males exposed to the hamburger diet and the females exposed to the hot dog diet. Liver weight often has a parallel relationship with body weight, thereby increasing with elevated accumulation of e.g., body fat (ibid.). Triglycerides may accumulate in the liver, increasing the liver weight, as a result of toxins inhibiting the hepatic function. We speculate whether the increased liver weight found in the present study is caused by the increased bodyweight, a possible toxic inhibition, or a relationship of both.

Exposure to the vegan diet gave the lowest mean value of body weight in both females and males (Table 6). This is not surprising considering that the vegan burger contained the highest levels of protein and the lowest levels of fat of the experimental diets, with 17.71 grams protein and 12.9 grams of fatty acids per 100 grams of product, respectively (Table 2).

All the experimental diets exhibited significantly shorter colon when compared to the vegan diet (Table 6). Similar results were found for the SI, where the vegan diet significantly increased the length when compared to the reference diet and the hot dog diet. Length of intestines and colon are known to be influenced by body weight (Hounnou *et al.,* 2002). In this study, the vegan group had the lowest overall bodyweight (23.4 g), but the longest intestines (SI=37.65 cm, C=10.62 cm). An interesting finding was also that exposure to the meat-based diets in females significantly decreased the length of the colon, when compared

to the vegan diet for both genders. The meat-based diets had higher amounts of fat, and lower amounts of protein than the plant-based diets. Literature states that animals adapt the length of the gastrointestinal tract to different diets as they need longer intestines and colon to digest fibre-rich food (Hunt *et al.*, 2021). It should be further investigated how a diet high in fibre and protein and low in fat influence on the A/J Min/+ mouse during growth stages and also whether there are gender specific regulations.

The reference group had significantly shorter SIs when compared to the vegan diet, hamburger diet, and hot dog diet (Table 6). In the colon, the reference diet had significantly shorter intestines when compared to the vegan group. However, the reference group had the second highest bodyweight and also exhibited a significantly increased number and load of fACF in the colon, when compared to the vegan diet (Table 6; Fig. 9). It is therefore questioned whether the growth of lesions inhibited the growth of intestines. As previously described (in section 1.2.1) the gastrointestinal tract has a relatively high turnover. In rodents cell renewal happens about every 2 - 3 days (Treuting, 2018a). But primarily growth happens during the phase of childhood and adolescence in mice. The mice in the present study were exposed to the experimental diet from childhood to adulthood, in a human perspective. This means that the A/J min/+ mice were exposed to experimental diets during a critical growth phase that may have affected the longitudinal growth. We can only speculate in whether the reference group, which had the shortest intestines, but highest number and load of colonic fACF, may have experienced inhibited longitudinal intestinal and colonic growth due to the defect crypt development from the *APC* mutations in the stem cells. Further research would be required to investigate the development and growth and functions of the intestines.

We hypothesized that diets dominated by processed meat products would have a higher influence on biometrical parameters than diets dominated by plant-based meat alternatives. The hypothesis must be partially rejected as the only biometrical parameter that was affected was the liver weight amongst males exposed to the hamburger group. The hamburger could be viewed as the product that was less processed in the study, even still it falls under the category of processed meat by IARC (IARC, 2018).

4.4 Strengths and limitations of the study

A strength of this study is the use of commercially available foodstuffs. Some other studies have investigated the relationship between commercially available products (Fernandez *et al.,* 2019), but in most intervention studies performed in murine models, the experimental diets are made of only one food component or mixed into standard rodent diet. As mentioned in section 1.2.4, murine models are used as analogues for human studies, however there is a question of the dose-response relationship when studying one singular component in human foodstuffs. Nutritional studies are complex, and often the food or nutrients of the human diet interact in ways that are not yet completely understood. It is therefore important to perform more studies on commercial foodstuffs and their carcinogenic effects, rather than singular components.

The results of our study point to a higher carcinogenic potential of the hot dog in the SI and reference feed and vegan diet in the colon. Unfortunately, no analysis of feces, or cecum content was performed, which would have given valuable information in regard to microbiota and potentially could further explain the causes of CRC in SI and colon.

A limitation of this pilot is the unequal distribution of males and females within each group. The recruitment of mice into the experimental groups was random but litterwise, the recruitment was performed until N=20 in each group, regardless of gender. This decision was based on time and budget limitations. The vegan and hot dog group had 8-10 mice per gender, however for hamburger and reference group there were only 5 males. The skewness of the gender may affect the results and may cause the effects to seem greater in the male group.

Another weakness regarding this exploratory study is the estimated daily intake of diets for each individual mouse. According to the Norwegian legislations, mice are not allowed to be housed singly and are therefore kept in groups. Due to housing of 5 mice per cage, we could not control the exact intake of feed per mouse per day, thereby making it difficult to assess the level of exposure required to initiate carcinogenesis. However, the mice were weighed once per week to follow their weight gain and eliminate the animals that were not eating the experimental diets. In this study we did not observe large variations in body weight within each cage (data not shown), indicating that all mice were eating the experimental diets more or less equally distributed between the individuals. It is also complex to translate the intake of experimental diets in this study to human conditions. The mice were exposed to the diets 5 nights per week, which is somewhat overestimated with regards to the health authorities' recommendations on daily/weekly intake of processed meat. Nevertheless, this study focused

on exposing the mice to commercially available products to mimic the exposure to potential carcinogens in relevant levels. Additionally, the differences in toxicokinetics (absorption, distribution, metabolism, excretion) in mice compared to humans may influence on the toxicodynamics (e.g., effects) and thus a higher exposure level may be needed to achieve a human relevant effect. Overall, with these considerations taken into account, we believe this study has used a relevant exposure model.

4.6 Reflections

The processed hot dog had significant effects on carcinogenesis in the SI. The ramifications of IARC`s monographs of placing processed meat in group 1, may have a major impact on dietary recommendations all over the world. As mentioned in the introduction, the Norwegian Health Directorate recommends small amounts of red and processed meat, maximum 500 g per week. Most Norwegians do, however, eat more meat per week than recommended. A rough calculation shows that the average Norwegian consumes about 1,1 kg per week (Norwegian health directorate, 2022). However, IARC conducts hazard analysis, not risk assessment, and thus, does not take the exposure dose into consideration (IARC, 2018). IARC has assessed the relative risk of developing CRC by eating processed meat to 1.18, which means that people who eat 50g processed meat per day have an 18% chance of developing CRC compared to those who do not eat processed red meat. In comparison, tobacco, which is also a group 1 carcinogen, has a relative risk of about 1.70. Moreover, the cancer research organization in UK estimates that about 13% of bowel cancers are caused by processed meat, whereas 72% of lung cancer cases are caused by tobacco smoking (Dunlop, 2015). The hazard analysis performed by the IARC has resulted in a debate on the risk related to consuming red and processed meat. However, it is important to consider the ramifications on a larger scale. Health organizations worldwide are now recommending restricting people's intake of red and processed meat and replacing it with vegetarian or vegan options. This has given rise to a market of meat-analogue products, such as the vegan burger that has been tested in our study. It could be speculated that more people would replace meat-based products with such vegan analogues in hopes of being healthier. Even though the vegan diet in our study yielded a lower bodyweight in the mice, which in humans is considered as a protective factor of carcinogenesis, the group exhibited increased size of fACF in the colon, which suggest a promoting potential on the carcinogenesis

(Fig. 9). It is therefore of importance to further explore the carcinogenic potential of the ingredients and the vegan products themselves.

The meat industry has been challenged for a longer period, not only because of the proposed associations between cancer and red and processed meat estimated by IARC, but also because of sustainability reasons. FAO estimates that about 70% of the agricultural land is currently being used for animal production, which accounts for about 18% greenhouse emissions (FAO, 2006). This has caused a major decline in the reputation of meat and meat-based products. However, to assess whether a diet is sustainable or not, it is important to assess the topographical potential and climatic conditions of each country. According to the World Bank, only 2.7% of Norway is arable land, in addition only 30% of these lands can be used for cultivating vegetables of high quality (Matdepartementet, 2021). The rest is used for cultivation of animal feed, as Norway has a very complex topography. There are also complexities regarding urbanization, whereas the best soil for cultivating vegetables is in the southern part of Norway, close to the capital. Urbanization and expansion of the urban areas have reduced the area of productive soil that could be used for cultivation. By speculating in how a further restriction of meat and processed meat would affect Norway, it is clear that this would cause challenges towards achieving self-sufficiency. Norway is completely self-sufficient regarding meat, dairy and eggs, however the overall score of self-sufficiency in Norway is only at 45,9% (Norwegian health directorate, 2022). For vegetables and other vegetable products, Norway is less self-sufficient and relies on import. This emphasizes the importance of meat in the Norwegian diet and how in terms of both sustainability reasons and nutritional reasons it is important to continue investigating the causality and underlying mechanisms behind the associations between meat and cancer.

The hot dog diet could be classified as ultra processed. As previously mentioned, there is no clear definition of what ultra processed foodstuffs are, but they are generally recognized as being food with industrial or synthetically made ingredients (Monteiro *et al.,*2019). Both the vegan burger and the hot dog could, based on the NOVA classification, be characterized as ultra processed. However, as ultra processed foodstuff is a fairly recent topic, more research on the relationship of ultra processed food and carcinogenic potential is required. Processed meat has long been under investigation, but the increasing popularity and demand for vegetarian and vegan options causes a need for more research. The terms *processed* and *ultra processed* are relatively new and have arisen from how the food has been handled. It is also about new ingredients and how processing may influence the products. There have been several

controversies regarding processed foods because of the number of additives, generally known as E-components. Many of these compounds are used more or less to meet the ever-growing need of a more enlightened consumer. For instance, low fat products often contain emulsifiers to make the texture mimic a full-fat product. This stems directly from the consumers demand of a healthier product, but with identical organoleptic/sensory properties as the full-fat variant. The same applies for the ultra processed products used in this study. Nitrite is used in the hot dog to preserve both color, quality and shelf-life, because it is a known fact that the consumers do not want discolored products or products that will deteriorate from 1 day after purchase. Furthermore, antioxidants in the hot dog are used to inhibit the effect nitrite has on the human body. In addition, the hot dog contains calcium to inhibit the effects of heme (Table 3). The vegan burger also contains different additives, in which the mixture, to our knowledge, is not explored extensively.

The incidence of dysbiosis, IBD and IBS are increasing, and it is questioned whether the present increase in intake of ultra processed foods important influencing factors for gastrointestinal illnesses (Mendonça *et al.,* 2016) are. Further investigations need to focus on the relationship between whole foods and interactions between compounds, not just single components, to unravel key drivers of carcinogenesis. This would give a further understanding of how we can adapt our diet and nutrition and reduce the cancer risks.

Another dilemma by restricting meat intake is that meat in general is a very good source of protein and vitamins. The Eat-lancet report from 2019 highlights that to shift to a more sustainable diet, 50% of the meat intake must be reduced by 2050, and plant-based diets must increase (Willett *et al.,* 2019). The same trend is seen in dietary recommendations around the world (Norwegian health directorate, 2022). Although meeting dietary needs of protein by excluding meat would be possible, meeting the dietary needs of essential amino acids would be more challenging. Meat and other animal derived products are generally termed as “high-quality proteins”, the term is used because these products contain all the essential amino acids (phenylalanine, valine, leucin, threonine, tryptophane, isoleucine, methionine, histidine, leucine) (Milton, 1999). A mixture of different plant-based proteins (legumes and cereals) is needed to achieve an amino acid profile similar to animal-based proteins (Day, 2013). Furthermore, Bohrer (2017) explains that humans on plant-based diets would be at risk of developing deficiency in heme-iron, zinc, B-vitamins and the mentioned essential amino acids (Bohrer, 2017). Platel & Srinivasan (2016) also mentions that plant-based foods have a lower bioavailability of the micronutrients iron, zinc, iodine and vitamin A, thereby causing

deficiencies (Platel & Srinivasan, 2016). However, a study performed by Kebebe *et al.* (2023) on nutritional impacts of excluding red meats from the diet, showed that excluding red meat from the diet significantly reduced the intake of proteins, saturated fatty acids, octadecanoic acid, cholesterol, vitamin D, riboflavin, niacin and sodium (Kebebe *et al.,* 2023). The same study also highlighted that both carnivores and herbivores were deficient in dietary fiber, vitamins A and D, calcium, magnesium, and potassium. The study sums up that herbivores had an increased risk of inadequately meeting the requirements of energy, calcium, potassium and vitamin D, but carnivores had a risk of deficiency of dietary fiber, vitamin A and magnesium. Interestingly, excluding red meat also significantly reduced the intake of caffein and alcohol. Excess intake of alcohol is also a well-known cancer risk factor. We question whether there are interactions between meat and alcohol, resulting in a higher risk of cancer when meat is consumed together with alcohol than without (ibid.).

5.0 Conclusion

This study is a part of an exploratory pilot study. The overall aim was to investigate the carcinogenic potential of experimental diets prepared from commercially available processed meat- and plant-based meat alternatives in A/J Min/+ mice. To our knowledge, this is the first study to show initiating effects of a commercially available hot dog on small intestinal carcinogenesis in the Min/+ mouse model. Furthermore, the reference diet exhibited a promoting effect on the SI and colon, as well as an initiating potential on the colon. However, the reference diet exhibited a promoting effect on the SI and colon, as well as an initiating effect on the colonic carcinogenesis. Interestingly, a promoting potential on the colonic carcinogenesis was also found for the vegan diet. These findings were unexpected and our hypothesis that processed meat products have a higher carcinogenic potential than plant-based meat alternatives could not be confirmed. The hamburger without nitrite did not show a significantly different carcinogenic potential than the hot dog. The hot dog represented a processed meat product with nitrite, however other components were also different between the hot dog and the hamburger. Therefore, this study cannot rule out whether a higher processing degree of meat play an important role in the carcinogenic potential of such products.

Indeed, we found gender-differences in carcinogenic potential of the diets in colon. The hot dog diet and the reference diet exhibited initiating potential in males and females, respectively. Moreover, the vegan diet showed a promoting potential in males. This was somewhat surprising, as we hypothesised that females would be more sensitive to the processed meat products than males. This study indicates that different diets have different carcinogenic potential depending on the gender, and we highlight the importance of studying both genders in future experimental studies. The biometrical parameters were not affected significantly by diets dominated by processed meat products. The length of the intestines was affected by the vegan diet, however the relevance of this finding in a carcinogenesis perspective is unclear but should be further investigated.

Altogether, we have revealed a carcinogenic potential of not only commercially available processed meat products, but also a processed vegan product. Clearly, the protein source and the processing method may be important contributing factors to the colorectal carcinogenesis. As CRC is related to a complex interplay between dietary, genetic and lifestyle factors, and the

underlying mechanisms and causality are not completely understood, the present study highlights the need of further research.

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Appendix A: List of additives

(appendix) Table A1. Description of the different E-numbers in the food products used in the experimental diets.

E-number	Name	Type	Description
E250	Sodium nitrite	Preservative	Stops bacterial growth of e.g. *Clostridium Botulinum*, gives colour
E261	Potassium acetate	Preservative, acidity regulator	Potassium salt of E260 (Acetic acid)
E315	Erythorbic acid	Antioxidant	Isomer of E300 (Ascorbic acid – Vitamin C)
E326	Potassium iactate	Antioxidant, acidity regulator	Extends shelf life (antimicrobial – inhibits spoilage and pathogenic bacteria)
E451	Triphosphate	Emulsifier, stabilizer	Salt of phosphoric acid. 2 types: Pentapotassium triphosohate and Pentasodium triphosphate
E150	Caramel	Colorant	To give the product an appealing and "correct" colour
E461	Methyl cellulose	Gelling agent, stabilizer, covering agent, thickening agent	Methyl cellulose gives liquids a chewy consistency and forms strong gels when heated.
E162	Beetroot colour	Colorant	Beetroot concentrate.

Appendix B: Mean/Median and range (min-max)

(appendix) Table B1. Mean/median values and minimum-maximum range of lesions in the SI (small/large) and in the colon (fACF/tumor), stratified by gender.

	Vegan	**Hot dog**	**Hamburger**	**Reference**
SI	Mean/Median [Min-Max]	Mean/Median [Min-Max]	Mean/Median [Min-Max]	Mean/Median [Min-Max]
Small number male	3.00/3.66[0.00-13.00]	6.00/6.44 [1.00-14.00]	4.00/4.6 [2.00-8.00]	2.00/2.66 [0.00-6.00]
Small number female	3.00/3.63 [2.00-8.00]	6.00/6.36 [0.00-14.00]	4.00/4.78 [1.00-11.00]	2.00/3.53 [0.00-14.00]
Small load male	0.22/0.32 [0.00-1.09]	0.54/0.63 [0.07-1.56]	0.26/0.32 [0.14-0.52]	0.20/0.23 [0.00-0.58]
Small load female	0.25/0.31 [0.11-0.87]	0.59/0.66 [0.00-1.58]	0.34/0.43 [0.04-1.19]	0.15/0.32 [0.00-1.20]
Small size male	0.08/0.07 [0.0 – 0.14]	0.09/0.09 [0.0– 0.15]	0.07/0.07 [0.02– 0.15]	0.09/0.08 [0.0 – 0.15]
Small size female	0.08/0.08 [0.04-0.12]	0.10/0.09 [0.00-0.11]	0.08/0.08 [0.02-0.15]	0.08/0.08 [0.00-0.15]
Large number male	19.00/17.33 [7.00– 25.00]	44.00/42.78 [4.00– 86.00]	15.00/15.2 [7.00– 22.00]	21.50/23.00 [10.00–42.00]
Large number female	16.00/22.82 [4.00– 75.00]	26.00/34.55 [12.00-79.00]	26.00/33.43 [3.00-80.00]	24.00/29.69 [8.00– 89.00]
Large load male	11.30/11.79 [8.03-19.00]	29.06/32.52 [3.40-74.46]	11.88/9.58 [3.39– 14.14]	19.52/19.65 [6.27– 36.54]
Large load female	11.44/17.04 [2.04-68.02]	13.43/23.74 [7.81-53.37]	17.33/25.41 [1.28-72.20]	18.17/29.51 [5.24-103.36]
Large size male	0.68/0.74 [0.46-1.14]	0.68/0.72 [0.49-0.86]	0.62/0.62 [0.48-0.79]	0.78/0.82 [0.62-1.09]
Large size female	0.60/0.69 [0.48-0.98]	0.69/0.66 [0.45-0.86]	0.67/0.66 [0.38-0.91]	0.82/0.84 [0.47-1.49]
Colon				
fACF number males	12.00/7.00 [2.00-30.00]	30.10/32.00 [3.00-67.00]	5.40/3.00 [1.00-15.00]	24.20/20.00 [15.00-41.00]
fACF number females	6.20/4.00 [0.00-24.00]	9.60/5.50 [1.00-35.00]	11.57/4.50 [1.00-44.00]	40.14/10.50 [3.00-237.00]
fACF size males	0.03/0.02 [0.01-0.07]	0.02/0.02 [0.01-0.03]	0.01/0.01 [0.01-0.03]	0.02/0.02 [0.01-0.02]
fACF size females	0.02/0.02 [0.00-0.03]	0.02/0.01 [0.00-0.03]	0.01/0.01 [0.00-0.02]	0.02/0.02 [0.01-0.03]
fACF load males	0.26/0.21 [0.05-0.62]	0.49/0.50 [0.02-1.16]	0.07/0.07 [0.01-0.18]	0.46/0.43 [0.25-0.87]
fACF load females	0.13/0.07 [0.00-0.46]	0.15/0.12 [0.01-0.44]	0.17/0.04 [0.01-0.54]	0.73/0.26 [0.08-4.32]
Tumor number males	1.20/1.00 [0.00-4.00]	1.80/1.50 [0.00-5.00]	1.00/1.00 [0.00-2.00]	1.00/1.00 [0.00-3.00]
Tumor number females	0.40/0.00 [0.00-1.00]	1.00/0.00 [0.00-4.00]	0.43/0.00 [0.00-2.00]	1.14/1.00 [0.00-4.00]
Tumor size males	0.33/0.27 [0.00-1.13]	1.48/1.16 [0.00-4.46]	1.11/1.13 [0.00-1.78]	1.10/1.13 [0.00-2.69]
Tumor size females	0.15/0.00 [0.00-0.74]	0.36/0.00 [0.00-1.57]	0.51/0.00 [0.00-2.64]	0.65/0.23 [0.00-2.83]
Tumor load males	0.64/0.40 [0.00-2.60]	4.93/1.76 [0.00-14.29]	1.47/1.13 [0.00-3.56]	1.77/1.13 [0.00-5.02]
Tumor load females	0.15/0.00 [0.00-0.75]	0.98/0.00 [0.00-4.84]	0.84/0.00 [0.00-5.28]	1.29/0.23 [0.00-5.65]

Appendix C: protocols for termination (Norwegian)

Protokoll for organuttak PILOT-forsøk 2022-2023

Dato: 03.09.22

Forberedelser – senest 1 dag før uttak:

- Finne frem og markere cryorør med mus ID, dato og vevstype.
- Passe på at det finnes flytende nitrogen og finne frem liten termos.
- Klippe til filterpapir til fiksering av tarmer.
- Klargjøre PBS i 1L flasker og sette i kjøleskap (tabletter med PBS blandes med RO vann/MilliQ vann).
- Skrive ut ark til terminering for å notere vekter.

Forberedelser – samme dag som uttak:

Ta med ned på muselab:

- Markerte cryorør.
- Flytende nitrogen i termos, bruk briller og hansker ved overføring til termos. Ikke skru lokket på termosen helt på, det skal alltid ligge løst på toppen slik at gassen slippes ut!
- Boks til fiksering av tarmer – fyll med formalin (OBS! må stå i avtrekksskap hele tiden! Hvis boks med formalin flyttes utenfor avtrekksskap så må den være inni en lukket og tett pose. Bruk i tillegg en transportkasse).

Finne frem på muselab:

- Vekt (2 desimaler).
- Boks til veiing av mus og veieskip til veiing av organer.
- Gassanestesiapparat (Univentor 400 Anasthesia Unit) med Isofluran. Maske og kammer til mus.
- Isoporplate og pinsett til å sjekke dybde av anestesi.
- 1,5 mL Eppendrof-rør til blodprøve før sentrifugering – må markeres med mus ID.
- 1 mL sprøyter med blå kanyler (23 G ca 20 mm).
- EDTA til skylling av sprøyte og kanyle – hell opp litt i et lite begerglass.
- Isoporplate og pose til døde mus.
- 1 liten skarp saks, 1 skarp/tynn pinsett, 1 butt liten pinsett og skalpellblad til uttak av tarmer.
- Etanol til desinfeksjon av utstyr + overflatedesinfeksjon til desinfeksjon av overflater**. OBS! Pass på at etanol har fordampet fra alt utstyr før de er i kontakt med organer/vev!**
- Sentrifuge (Hermle Z 160M, Hermle labortechnik, Hersteller Spintron inc., radius = 7,3 cm).
- 200 µL pipette og tilhørende spisser til å føre over plasma.
- 2 stk isoporbokser med is.
- 3-4 begerglass til PBS, plasseres på is.
- 1 stk 20 mL sprøyte med rosa myk buttanert kanyle.
- 1 skarp/tynn pinsett, 1 rett butt pinsett og 1 skalpell + blad til uttak av lever.
- Filterpapir til tarmer – husk å markere med mus ID på begge sider!
- Stiftemaskin og stifter.

- Boks med formalin til fiksering av tarmer (OBS! må stå i avtrekksskap hele tiden! Ta av posen inni avtrekk i tilfelle noe formalin har lekket ut av boksen).
- Tørkepapir

Uttak av organer:

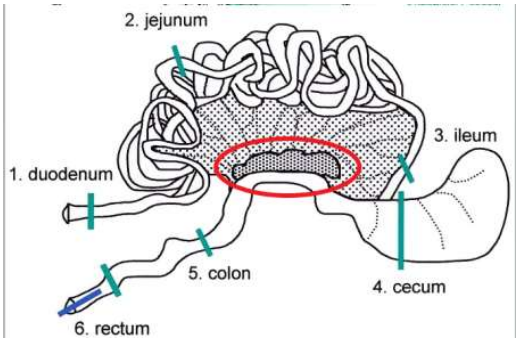
Før avliving:

1. Finn frem riktig mus.
2. Registrer vekten av musen.
3. Ta prøver av fersk feces. Dette legges i cryorør og fryses på flytende nitrogen.

Avliving og organuttak:

1. Legg musen i anestesi:
    a. Ved start av apparatet, ha luftstrøm mot kammer og skru flow til ca 600 mL/min + 4,0 % isofluran.
    b. Ha første mus i kammeret.
    c. Skru ned til ca 300 mL/min + 3,0 %.
    d. Vent til musen har sovnet. Sjekk ved å tippe musen over på ryggen: hvis den blir liggende på ryggen så sover den.
    e. Flytt luftstrømmen over til masken. Deretter flyttes musen raskt over til masken.
    f. Test reflekser på alle føtter og la musen ligge litt lengre i masken hvis ikke anestesien er dyp nok (ved reflekser).
2. Blodprøve ved hjertestikk:
    a. Skyll sprøyte og kanyle godt med EDTA.
    b. Stikk nålen 90 grader i midtlinjen rett under de to øverste brystvortene og samle 0,5-1 ml blod fra venstre eller høyre ventrikkel.
    c. Ta blod over i 1,5 mL Eppendorfrør merket med mus ID.
    d. Sett blod på is frem til sentrifugering.
    e. Sentrifuger blodet: 1,5 mL rør skal spinnes på 2000 g (=4953 rpm) i ca 10 min.
    f. Pipetter plasma over i et cryorør. Vær forsiktig med å ikke få med buffy coaten eller de røde blodlegemene.
    g. Frys på flytende nitrogen.
3. Skru av anestesiapparat, ta musen ut av masken og ta nakkestrekk.
4. Bløt pelsen på buken med PBS for å ikke få hår på organene.
5. Ta ut tarmer:
    a. Åpne buken og lokaliser colon.
    b. Fukt fingrene med PBS.
    c. Klipp av colon så nærme anus som mulig.

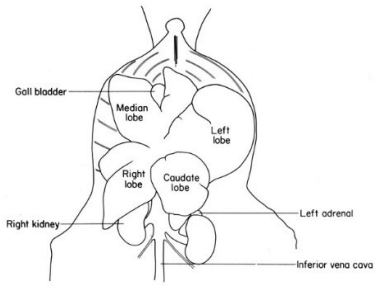
d. Trekk forsiktig i tarmen, slik at hele colon blir fri fra krøset.



Figur C1: illustrasjon av tarmer med kuttsteder.

e. Klipp av colon ved cecum og legg i et begerglass med iskaldt PBS på is. **Det er viktig at tarmen holdes kjølig og fuktig hele tiden for å unngå degenerasjon (inaktivering av enzymer).**
f. Klipp av cecum og legg på et veieskip.
    i. Vei cecum og skriv ned vekt.
    ii. Klem ut innholdet av cecum over i et cryorør.
    iii. Legg cecumvevet i et annet cryorør. Det er ikke nødvendig å skylle vevet.
    iv. Frys på flytende nitrogen.
g. Trekk videre i tarmen slik at tynntarmen også blir fri fra krøset.
h. Klipp av tynntarmen slik at en liten del av magesekken blir med.
i. Legg tynntarmen i iskald PBS. **Det er viktig at tarmen holdes kjølig og fuktig hele tiden.**
j. Bruk sprøyta med buttanert kanyle til å skylle tynn- og tykktarmen grundig med iskald PBS.
k. Klipp opp tarmen på langs.
l. Skyll tarmen i et nytt glass med iskald PBS.
m. Legg tynntarmen på bordet i en dam av iskald PBS. Orienter tarmen med magesekken øverst til venstre og cecum nederst til høyre (i en z-form).
n. Klipp tynntarmen i 3 like lange deler.
o. Flat ut tynntarmen forsiktig med fingrene.
p. Bruk skalpellbladet og merk tynntarms-del nr.2 med 1 snitt med skallpellbladet og tynntarm-del nr.3 med 2 snitt. **Delene skal snittes distalt**.
q. Legg colon nedenfor den distale delen av tynntarmen og flat forsiktig ut med fingrene.
r. Legg et filterpapir oppå alle tarm-delene. Løft det forsiktig opp igjen slik at tarm-delene sitter fast på papiret.
s. Legg det andre filterpapiret forsiktig inntil arket med tarmen og fukt med litt PBS fra sprøyta.
t. Stift arkene sammen og legg i boksen med formalin.

6. Uttak av lever:
    a. Lokaliser leveren og galleblæra.
    b. Fjern galleblære uten å søle noe av innholdet på leveren. Hvis dette likevel skjer, må leveren skylles i PBS.
    c. Klipp leveren løs fra skrotten.
    d. Legg leveren i et veieskip på vekten med litt iskald PBS. Veieskipet med PBS skal allerede være tarert (nullet ut) før leveren legges oppi.
    e. Vei hele leveren og skriv ned vekten.
    f. Ta veieskipet av vekten.
    g. Kutt bort hele **'Left lobe'** og legg i cryorøret merket med **Lever A**.
    h. Legg resten av leveren i cryorøret merket med **Lever B**.
    i. Frys på flytende nitrogen.



Alle cryorør overføres til -80 fryser.

Formalinboksen transporteres **i en lukket pose** til avtrekkskapet på lab for skåring.

Dagen etter uttak – etterarbeid med tarmer:

1. Tarmen ligger på formalin (10%) i boks i avtrekkskap ved romtemperatur i ca 24 timer før farging:
    a. Ta tarmen ut av filterpapiret og legg i et glass med Metylenblått (0,1% metylenblått i 10% formalin) i 20-25 sekunder. Rør rundt med en pinsett.
    b. Skyll tarmen i 3 glass med formalin (10%).
    c. Legg tarmen i et 50-mL rør med 70% etanol. Husk å markere røret med musens ID, forsøksnavn og dato.
    d. (Det er mulig å gjenta fargingen hvis for mye av fargen forsvinner under skylling eller lagring).
2. Vent minimum 24 timer før tarmene kan skåres for lesjoner.

Appendix D: Protocol genotyping (Norwegian)

Dag 1

Forberedelses til DNA ekstraksjon:

- Ta en ørebrusk-prøve av en mus og legg den i et 1,5 mL eppendorfrør med sikringslokk. Skriv ID-nummeret til musen tydelig på røret.
- Hent is i isopor-boks.
- Slå på varmeblokk (95-96°C) og vannbadet (55°C).
- Ta ut TE-rør (det er nok TE-buffer til 15 prøver per rør, inneholder totalt 995 µl) og proteinase K-rør (inneholder 250 µl, konsentrasjon 10 mg/ml) fra fryseren og tin dem i romtemperatur.
- Tint TE-rør (med 995 µl) tilsettes 5 µl 10% SDS løsning (SDS står i romtemperatur). Dette gir en konsentrasjon på 0,05% SDS. Må lages på nytt for hver runde genotyping.

Gjennomføring av DNA ekstraksjon:

1. **Tilsett 60 µl TE.buffer med 0,05% SDS til hver prøve** – øret må være under væskeoverflaten. Lukk lokket på eppendorfrørene godt.
2. **Kok prøvene i 10 minutter ved 95-96°C i varmeblokk.**
3. **Sett prøvene på is i ca 2 minutter.**
4. **Sett prøvene i sentrifugen, trykk start, når hastigheten har nådd 13,5 trykk på stopp.** Etter sentrifugering kan prøvene stå i romtemperatur.
5. **Tilsett 6 µL Proteinase K til hver prøve** (dette tilsvarer 1/10 av volumet i røret). Lukk lokket på rørene godt. (Proteinase K kan oppbevares i kjøleskapet etter tining, men bør brukes innen én uke).
6. **Whirle prøvene og sett dem i gult flytestativ** – øret må være under væskeoverflaten.
7. **Inkuber prøvene ved 55°C i vannbad over natten (eventuelt i minimum 2 timer: hvis denne metoden velges må man whirle prøvene ofte).** Sett gjerne prøvene i rekkefølge som sikkerhet i tilfelle merket på rør blir borte.

Dag 2:

Forberedelse til PCR:

- Regn ut volum av komponentene i MasterMixen (i Excel-ark). Lag nok til x antall prøver + 3 standarder + 2 ekstra. Husk at minste volum av Gotaq som kan pipetteres er 0,5 – 1 µl.
- Slå på varmeblokk (95-96°C).
- Ta ut komponentene til MasterMixen fra fryseren (bortsett fra GoTaq som må stå i fryser helt til den skal brukes) og tin dem på is (dette tar lang tid! Kan redusere tiden ved å whirle rørene i korte perioder, obs ikke lenger enn 10-20 sek av gangen)
- Ta ut MQ (autoklavert vann) og standardene (MIN, WT og Blank) fra fryseren og tin i romtemperatur.
- Forbered fortynningene: Lag ett 0,6 ml eppendorfrør per prøve og tilsett 196 µl MQ (autoklavert vann). MIN og WT standarder må også fortynnes (OBS! noen av MIN og WT standardene er allerede fortynnet!) Kan lages på Dag 1 og oppbevares i kjøleskap til Dag 2.
- Slå på BioRad PCR-maskinen. Velg «Saved programs», «Min» og «Run».

Gjennomføring av PCR og gel elektroforese:

8. **Whirle prøvene** (alle står i gult flytestativ, tørk av litt vann fra utsiden av rørene før whirlingen).
9. **Kok prøvene i 10 minutter ved 95-96°C i varmeblokk.** (Dette steget inaktiverer Proteinase K).
10. **Sett prøvene på is i ca 2 minutter.**
11. **Sett prøvene i sentrifugen, trykk start, når hastigheten har nådd 13,5 trykk på stopp.** Vær forsiktig når du tar prøvene ut av sentrifugen. Etter sentrifugering kan prøvene stå i romtemperatur. Du har nå ekstrahert DNA fra ørebrusk-prøvene.
12. **Fortynn prøvene: Merk 0,6 ml eppendorfrørene med 196 ul MQ med ID-numrene til musene. Tilsett 4 µl DNA fra hver prøve til hvert 0,5 ml rør med sammen ID-nummer.** (DNA trekkes opp fra omtrent midt i væsken i det store røret som har vært kokt og sentrifugert). **Fortynn også standardene MIN, WT og Blank (hvis disse ikke er fortynnet fra før). Whirle prøvene enkeltvis fortløpende etter at DNA er tilsatt.** (DNA er nå fortynnet 1:50). OBS! Ta en ny spiss til hver prøve! Prøvene er nå klare for PCR, eller kan fryses på -20°C.
13. **Lag MasterMixen: Sett et tomt 1,5 ml eppendorfrør på is. Tilsett alle komponentene til røret** (Husk å blande hver enkelt komponent med pipettespissen før oppsuging og følg gjerne rekkefølgen i Tablelen under). **Tilsett GoTaq helt til slutt.** GoTaq er flytende ved -20°C og må stå på blå isblokk eller is så lenge den er ute av fryseren. Sett GoTaq tilbake i fryseren så fort du har tilsatt beregnet volum til mixen. **Bland MasterMixen godt ved å pipettere opp og ned i røret omtrent 4 ganger med halvparten av totalvolumet i mixen.** OBS! Ta ny spiss til hver komponent!

Tablel D1: Blandingsforhold Mastermix.

	Pr prøve
10X PCR Buffer II	1.00
2 mM dNTP mix	1.00
25 mM MgCl2	1.00
M*APC*-9 (0,8 µM)	0.25
M*APC*-MT (32,2 µM)	0.25
M*APC*-15 (16,0 µM)	0.25
MQ (fra fryseren)	1.22
GoTaq (5u/µL)	0.033
Tot vol MM	5.00

14. **Sett riktig antall PCR-rør på den blå isblokken. Merk rørene godt slik at du vet hvilken prøve som tilhører hvert rør. Tilsett 5 µl av MasterMixen i hvert PCR-rør.** Legg dråpen inntil innsiden av røret slik at den fester seg der. **Påse at alle rør har fått MasterMix og dunk dråpene forsiktig ned i bunnen av rørene.** Pass på at rørene står på den blå isblokken hele tiden.
15. **Tilsett 5 µl fra hver DNA-fortynning i hvert PCR-rør.** Legg dråpen med DNA langs innsiden av røret slik at den fester seg der. OBS! Ta ny spiss til hver prøve! **Påse at alle rør har fått DNA-fortynning og dunk dråpene forsiktig ned i bunnen av rørene.** Trykk lokkene på PCR-rørene godt på. (Totalvolum per prøver er 10 µl).

16. **Sett PCR-rørene i PCR-maskinen. Velg «Skip step» og «Yes».** Du har nå startet PCR. Programmet tar ca.1 time og 14 minutter.

Tablel D2: PCR-Program: MIN-MUS

1	96°C	∞	
2	94°C	3 min	36 sykluser
3	94°C	15 sek	
4	56.5°C	15 sek	
5	72°C	20 sek	
6	72°C	7 min	
7	4°C	∞	

17. **Ta PCR-rørene ut av PCR-maskinen og sett dem på is** (i det rosa stativet som settes oppå en håndfull is som er lagt på isoporlokket).
18. **Tilsett 2 µl blå loadingbuffer til hver prøve** (totalvolum per prøve blir nå 12 µl). OBS! Ta ny spiss til hver prøve! Loadingbuffer lagres i romtemperatur og det er enklere å pipettere fra dette røret hvis du først pipetter en større mengde (f.eks. 60 µl) over i lokket.
19. **Skyll Lonzagelen med MQ og tørk forsiktig av overflødig væske.** Pass på at alle brønnene får vann i seg og tørk av med kleenex som ikke gir fra seg lo. Ikke tørk bort vannet som ligger nedi brønnene.
20. **Tilsett prøvene til brønnene i gelen: Den første brønnen på venstre side skal inneholde 2 µl DNA ladder** (dette er en DNA-standard som lagres i kjøleskapet). **Tilsett så 5 µL fra hver prøve til hver brønn.** (Hold pipettespissen skrått ned i brønnen når du skyver ut prøven). OBS! Ta ny spiss til hver prøve! **Husk også å tilsette 5 µl av standardene MIN, WT og Blank** (MIN = positiv standard, WT = negativ standard og Blank = nullprøve).
21. **Fyll inn rekkefølgen på prøvene i arbeidsarket slik at du har god oversikt over alle prøvene og hvilken brønn de er tilsatt i**.
22. **Kjør elektroforese: Koble til og slå på elektroforese-maskinen, koble stativet til maskinen ved å plugge rød ledning inn i rød inngang og svart ledning inn i svart inngang. Sett gelen inn i stativet.** Spenningen skal stå på 250V. Følg med på gelen! (Det tar ca 5 min og det er enklere å se resultatene hvis rommet er mørkt). **1 bånd = WT. 2 bånd = MIN. Registrer resultatene i arbeidsarket.**
23. **Kast PCR-rørene, gelen og fortynningene. Øremerkene (1,5 ml eppendorfrør med ekstrahert DNA fra ørebrusk-prøvene) spares på -20°C. Sett alle komponentene til MasterMixen, standardene og MQ tilbake i fryseren. Slå av alle maskiner. Kast isen i vasken. Vask benken med sprit.**

Appendix E: Health monitoring score sheet

Experiment
Project manager
Veterinarian
Daily monitoring by

ID/genotype
From 8 weeks of age

	Daily			Weekly		Comment
Date	Rectal bleeding	Rectal prolapse	Appearance and behavior	Weight	Weight diff	

Humane endpoints			
Parameter	Score	Category	General approach
Weight difference (7 days)	1	No loss / gain	ok
	2	0-15% loss / gain	Notification on cage card and keep under observation
	3	>15% loss / gain	Euthanize
Rectal bleeding	1	Ok	ok
	2	Blood in cage	Notification on cage card and keep under observation
	3	Blood around anus	Euthanize
Rectal prolapse	1	Ok	ok
	2	A bulging of the distal colon out of the rectum during defecation	Notification on cage card and keep under observation
	3	Complete bulging of distal colon out of rectum	Euthanize
Appearance and behavior	1	Well- conditioned, active, alert, normal movement	ok
	2	Under- conditioned, sluggish, aggitated, fuzzy fur	Notification on cage card and keep under observation
	3	Emaciated, non-responsive, hunched, eyes squinted, eyes sunken, ears pulled back, rough and dirty fur	Euthanize

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