Dissertation

THE ROLE OF PROBIOTICS IN COLORECTAL CANCER LIVER METASTASES TREATMENT

submitted by

Matas JAKUBAUSKAS

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Department of Surgery, Division of Transplant Surgery

under the supervision of

Assoz. Prof. Priv.-Doz. Dr.med.univ. Philipp STIEGLER

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DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice “.

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Co-authors who contributed to these publications:

Lina Jakubauskiene[1,2], Bettina Leber[1], Angela Horvath[3], Kestutis Strupas[2], Philipp Stiegler[1] and Peter Schemmer[1]

[1] General, Visceral and Transplant Surgery, Department of Surgery, Medical University of Graz, Graz, Austria.
[2] Faculty of Medicine, Vilnius University, Vilnius, Lithuania.
[3] Division of Gastroenterology and Hepatology, Medical University of Graz, Graz, Austria.

I confirm that all co-authors have agreed to include their published data in my dissertation. The manuscript “Probiotic Supplementation Suppresses Tumor Growth in an Experimental Colorectal Cancer Liver Metastasis Model” was published in peer-reviewed journal “International Journal of Molecular Sciences” by MDPI AG, Basel, Switzerland under Creative Commons Attribution (CC BY 4.0) “Open Access” license, which allows for unlimited distribution and reuse as long as appropriate credit is given to the original source and any changes made compared to the original are indicated. The parts of published work are being reproduced identically in this dissertation.

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ABBREVIATIONS

5-FU	5-fluoruracil
ABC	Adenosine triphosphate binding cassette
ALT	Alanine transaminase
ANOVA	Analysis of Variance
APC	Adenomatous polyposis coli
AST	Aspartate transaminase
CALI	Chemotherapy associated liver injury
CAPIRI	Capecitabine, irinotecan
CAPOX	Capecitabine, oxaliplatin
CD31	Cluster of differentiation 31
CRC	Colorectal cancer
CRCLM	Colorectal cancer liver metastasis
CT	Computer tomography
CTx	Chemotherapy
DAB	Diaminobenzidine
EGFR	Epidermal growth factor receptor
FOLFIRI	Leucovorin, 5-fluorouracil, irinotecan
FOLFOX	Leucovorin, 5-fluorouracil, oxaliplatin
FOLFOXIRI	Leucovorin, 5-fluorouracil, oxaliplatin, irinotecan
GLP	Glucagon-like peptide
HRP	Horseradish peroxidase
IL	Interleukin
IM	Intestinal mucositis
LV	Left ventricle
LVEF	Left ventricle ejection fraction
MAPK	Mitogen activated protein kinase
MMR	Mismatch repair
MPO	Myeloperoxidase
MVD	Microvascular density
PLAX	Parasternal long-axis
PSAX	Parasternal short-axis
RPMI	Roswell Park Memorial Institute
SOS	Sinusoidal obstruction syndrome

TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
US	Ultrasound

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ZUSAMMENFASSUNG

Etwa 15-25% der Patienten, bei denen Darmkrebs (CRC) neu diagnostiziert wird, haben bereits Metastasierung, was als hauptsächliche Todesursache angesehen wird. Der am häufigsten anzutreffenden Ort von Metastasen von CRC ist die Leber (CRCLM). Chirurgische Entfernung von CRCLM gilt als die einzige Option zur Heilung, wobei aber nur etwa 20% der Patienten zum Zeitpunkt der Diagnose Kandidaten für diese radikale Behandlung sind. Für die anderen 80% der Patienten bleibt die hauptsächliche Behandlungsoption der systemischen Chemotherapie (CTx). Während der CTx Behandlung können eine Reihe unerwünschter Nebenwirkungen auftreten, bis hin zu lebensgefährlichen Zuständen. Darüber hinaus tendiert CRCLM dazu, eine Resistenz gegen CTx zu entwickeln. In solchen Fällen sind andere, zusätzliche Behandlungen notwendig, um die Krankheit zu kontrollieren und um die Lebensqualität des Patienten zu verbessern. Gesteigertes Forschungsinteresse zur Gesundheit des Verdauungstrakts und zu Probiotika haben zu vielversprechenden Erkenntnissen für die Behandlung verschiedener klinischer Zustände geführt, darunter auch Daten zur Anwendung von Probiotika bei CRC. Allerdings sind derzeit keine Studien bekannt, welche die Auswirkungen von Probiotika auf CRCLM direkt untersuchen. Diese Doktorarbeit soll die Rolle probiotischer Ergänzungsstoffe bei der Behandlung von CRCLM bewerten.

Sechs Wochen alte männliche Wistar-Ratten erhielten entweder ein Multi-Spezies-Probiotikum ($1{,}2 \times 10^9$ CFU/t) oder ein Placebo-Mix. Am 14. Tag des Experiments wurden den Ratten CRC-Zellen (CC531) unter der Leberkapsel implantiert. Am 28. Tag des Experiments erhielten die Ratten FOLFOX CTx. Die Veränderungen des Tumorvolumens wurden per Mikro-CT-Scans gemessen. Proben des Stuhls wurden für nachfolgende Analysen des Mikrobioms gesammelt. Zusätzlich erfolgte immunhistochemisches Anfärben von Proben von Darm, Leber und Herz.

Die Analyse der Tumorvolumens zeigte, dass probiotische Ergänzungen das Tumorwachstum reduzierten. Zwischen den probiotischen Ergänzungen und FOLFOX CTx konnten keine synergetischen Effekte beobachtet werden. Verlangsamtes Tumorwachstum wurde erreicht, indem die Angiogenese unterdrückt wurde, da die mikrovaskuläre Dichte des Tumors bei den Ratten, die probiotische Ergänzungen erhielten, deutlich geringer war. Zusätzlich linderten die Probiotika die Schwere und Dauer der durch CTx induzierten Diarrhoe. Weiterhin verringerten die Probiotika den durch FOLFOX verursachten Gewichtsverlust und den Verlust von Albumin im Blut deutlich. Bei genauerer Untersuchung von Blutproben und Lebergewebe wurde keine Verletzungen von Herz und Leber festgestellt, die durch CTx verursacht wurden.

Zusammenfassend kann also gesagt werden, dass unsere Studie gezeigt hat, dass Gaben von Multi-Spezies-Probiotika die Angiogenese bei CRCLM merklich behindert und damit das Tumorwachstum verlangsamt wird. Zusätzlich konnten wir zeigen, dass probiotische Ergänzungen die Symptome intestinaler Mukositis, die durch FOLFOX induziert wurden, lindern können und so das Körpergewicht und die Albuminspiegel im Blut konstant halten.

ABSTRACT

Around 15-25% of patients, newly diagnosed with colorectal cancer (CRC), already have metastatic disease and it is considered to be the major cause of death. The most common site for CRC metastasis is the liver (CRCLM). Surgical CRCLM removal, is considered to be the only curative option, but only around 20% of all patients are candidates for radical treatment at the time of diagnosis. For the other 80% of these patients, the main treatment option remains systemic chemotherapy (CTx). During the administration of CTx, numerous adverse effects can occur, which can cause life-threatening conditions. Furthermore, CRCLM eventually tends to develop resistance to CTx. In such cases, novel treatment additives are necessary to better control the disease and improve patient quality of life. The increased research interest in gut health and probiotics lead to promising findings for the treatment of various clinical conditions, including data on probiotics as a tool in CRC management. Currently, there are no studies directly examining probiotic effects on CRCLM. This dissertation thesis aimed to assess the role of probiotic supplementation in CRCLM treatment.

Six-week-old male Wistar rats received either a multispecies probiotic (1.2×10^9 CFU/day) or a placebo mixture. On day 14 of the experiment, rat CRC cells (CC531) were implanted under the liver capsule. On the 28^{th} experiment day rats received FOLFOX CTx. Change in tumor volume was measured by performing micro-CT scanning. Stool samples were collected for further microbiome analysis. Additionally, immunohistochemical stainings of gut, liver and heart samples were performed.

Tumor volume analysis revealed that probiotic supplementation reduces tumor growth. No synergistic effects between probiotic supplementation and FOLFOX CTx was observed. Diminished tumor growth was achieved by suppressing angiogenesis, as tumor microvascular density was significantly lower in rats receiving probiotic supplementation. Additionally, probiotics alleviated the severity and length of CTx-induced diarrhea. Furthermore, probiotics significantly reduced FOLFOX induced weight and blood albumin loss. No CTx-induced liver and heart injuries were noted upon closer blood and tissue sample inspection.

In conclusion, our study was able to demonstrate that multispecies probiotic supplementation significantly inhibits CRCLM angiogenesis and decreases tumor growth. Additionally, we showed that probiotic supplementation could alleviate FOLFOX-induced intestinal mucositis symptoms and preserve both weight and blood albumin levels.

INTRODUCTION

Epidemiology

According to the International Agency for Research on Cancer annual report on global cancer statistics, an excess of 1.9 million new cancer cases and around 930,000 deaths were attributed to colorectal cancer (CRC) in 2020 (2). These aforementioned numbers represent about one-tenth of all new cases and cancer-related deaths, ranking CRC second in mortality and third in incidence malignancy worldwide (2). Incidence rates vary greatly between different world regions, with the highest being in Australia, Europe, and North America and the lowest being in South Central Asia and Africa (2,3). Specifically in Europe, the highest incidence rates for CRC were seen in Slovakia, Hungary, and Slovenia and the lowest in Albania, mortality rates mainly follow the incidence (3).

The incidence trends are closely related to countries' socioeconomic development. With the rise of countries' human development index, society also face an increase in both incidence and mortality of CRC (4,5). This shift is mostly explained by the changes in dietary patterns and lifestyle. People adopt a more Western-style diet with an increased intake of sugar, fat and animal-source foods. Additionally, the pandemic of obesity is further fueled by the decrease in physical activity and an increase in sedimentary lifestyle (6,7). Moreover, the increase of CRC in the countries overgoing the transition phase could also be explained by the increase in alcohol consumption and smoking (8,9). Different patterns are observed in high human development index countries, as the incidence throughout the years is either decreasing (USA, Japan), remaining stable (UK), or rising (Italy, Denmark) (4,10). These differences in high human development index countries could be attributed to the change in risk factor prevalence and different implementation of CRC screening programs (11–13).

Various types of screening programs are mainly responsible for the decline in CRC incidence and mortality in developed countries. Several studies suggest that screening can reduce CRC incidence and mortality by around 50% (14,15). Most of the established screening programs are aimed at patients over 50 years old, however, in recent years, a dramatic rise in CRC was seen in the younger (<50 years) individual cohort (2,16). The exact underlying reasons are unknown, but some authors provide evidence that obesity, dietary changes, and physical inactivity play a crucial role (16,17). This trend is quite concerning, as outcomes for the early onset CRC are often worse due to delayed diagnosis and more aggressive cancer

differentiation (18). Taking this into account the American Cancer Society updated its recommendations and reduced the CRC screening start to 45 years of age (19).

Pathogenesis

Adenoma-Carcinoma sequence

The first suspicion that CRC is very closely related to colorectal adenomas became apparent in the 1970s. Morson systematically presented evidence that cancerous foci are found in benign adenomas, residual benign tissue can be found in carcinomas and several published cases described carcinoma development from previously non-cancerous polyps (20). After this initial report, several other, more comprehensive studies followed. Stryker et al. showed the natural course of untreated colonic polyps and reported a 24% cumulative risk to develop cancer at the polyp site (21). Winawer et al. demonstrated that endoscopic polypectomies significantly reduce the risk of subsequent CRC, thus further adding evidence that adenomas gradually develop into adenocarcinomas (22). The works of Fearon and Vogelstein were fundamental for the understanding of the Adenoma-Carcinoma pathway (23). They proposed a genetic colorectal tumorigenesis model stating that the mutational inactivation and activation of the tumor suppressor and oncogenes respectively, lead to the development of CRC (24).

Some of the comprehensive sequence-based studies gave further clarification in identifying the genes that are commonly mutated in CRC (25,26). A study by Wood et al. identified over 80 somatic mutations that are found in CRC, however further statistical analysis revealed that less than 15 mutations are necessary for the initiation, progression, and maintenance of the tumor (25). The most frequent somatically mutated genes, such as the adenomatous polyposis coli (APC), KRAS, and p53 genes, play crucial roles in the development of CRC (25,26).

One of the first mutations in the adenoma-carcinoma sequence occurs in the APC gene that is located on chromosome 5q21 (27–29). Germline mutations of this gene result in a disease named familial adenomatous polyposis which is characterized by the development of a large number of adenomas in the large bowel that eventually transform into CRC if left untreated (30). This tumor-suppressor gene codes a 312-kDa protein that has a crucial role in suppressing the Wnt signaling pathway, which regulates cell proliferation and differentiation in the intestine (31). As Kinzler and Vogelstein point out that APC is a “gatekeeper” and it is important in sustaining a constant cell number in regenerating cell colonies (32). Additionally, this gene controls cell adhesion, migration, and chromosomal segregation (33). Somatic APC gene mutations are present in 40 to 80% of colorectal adenomas and CRC (23,29,34,35).

Moreover, even microscopic adenomas (<0.5cm) present with this mutation, this finding further supports the idea that the APC mutation occurs very early in the adenoma-carcinoma sequence (29).

Another frequent and important CRC pathogenetic event is the somatic mutation of the KRAS gene. Humans have three RAS genes (KRAS, NRAS and HRAS) which respectively encode small G-proteins KRAS4A, KRAS4B, NRAS and HRAS. These proteins are crucial components of the mitogen-activated protein kinase (MAPK) pathway that is essential for cell growth and differentiation (36). The frequency of KRAS mutations increases with the growth of colorectal adenomas. In smaller adenomas (size <2cm) the prevalence of KRAS mutations is around 10-14%, however, this number drastically increases with the growth of adenomas and can reach up to 50% (23,37). A similar prevalence of KRAS mutations is found for CRC as for large adenomas, indicating that this oncogene participates in the early stages of adenoma-carcinoma sequence, however, it is probably not the initiating factor.

Mutations in the p53 gene are considered to occur most frequently in many human tumors (38). This gene encodes a transcription factor that regulates the production of proteins that have tumor suppressor properties by being involved in DNA repair, apoptosis, and angiogenesis (39,40). The disruption of the p53 protein is thought to be very important for the colorectal adenoma transition to CRC. This idea is strongly supported by the increasing frequency of p53 alterations throughout the adenoma-carcinoma sequence, as only around 5% of adenomas have it when compared to the 50% for adenomas with invasive properties and to the 50-75% p53 alteration frequency in CRC (23,37,41,42).

There are several other gene mutations, such as SMAD2, SMAD4, or PIK3CA, that can also be involved in the adenoma-carcinoma sequence (36,43). However, their mutation prevalence is much lower, when compared to the frequencies of APC, KRAS, or p53 mutations, indicating that they have less important roles in the development of CRC.

Serrated neoplasia pathway

In 1990 the term serrated adenomas was first proposed and it was used to define polyps that had serrated architecture of the crypt epithelium (44). During the last 20 years, the increase of evidence indicates that around 15% of CRC develop through the serrated neoplasia pathway (45–47). This pathway has some resemblance to the aforementioned adenoma-carcinoma sequence, however, they are considered to function independently (48). This pathway is thought to be initiated with the mutation of the BRAF oncogene, which, as the mutated KRAS

gene, activated the MAPK pathway leading to uncontrolled cell proliferation (49,50). However, the BRAF mutation alone is not enough to develop a serrated adenoma, as the cells eventually enter senescence (51). This is overcome by the hypermethylation of CpG islands on the tumor suppressor genes, resulting in their silencing. Most commonly genes such as p16INK4a, IGFBP7, and MLH1 are affected by the hypermethylation process (52). The silencing of the MLH1 gene results in the development of microsatellite unstable CRC, sometimes this process is called the sporadic microsatellite instability pathway (48,53).

Microsatellite instability

Microsatellites are short repeating sequences consisting of 1-6 nucleotides and are usually located near the coding region (54). Microsatellites are very susceptible to errors during the DNA replication process, however, these errors are usually repaired by the mismatch repair (MMR) proteins (55). In cases when the MMR is nonfunctioning, errors in the microsatellites start to accumulate. Additionally, not only the microsatellites are affected by the MMR system dysfunction, but also other nucleotide sequences, enhancing the mutation possibility in oncogenes and tumor suppressor genes, this mutator phenotype idea was first suggested by Loeb et al in 1974 (56,57). In contrast to the classic adenoma-carcinoma pathway, it is characteristic that microsatellite instability-associated tumors have a large number of point mutations in various genes. However, it is mostly unclear which of them are important in the subsequent development of CRC. As mentioned in the previous paragraph, errors in the MMR system can be caused by sporadic MLH1 gene silencing due to hypermethylation, moreover, they may be caused due to germline mutations (48,53). Lynch syndrome, previously termed hereditary nonpolyposis CRC, is the most common hereditary CRC condition, which is caused by germline mutations in the MMR genes MLH1, MSH2, MSH6, and PMS2 (56). This syndrome is mostly associated with a higher lifetime risk of CRC, however, the risk for other adenocarcinomas, such as endometrial or gastric, is also increased (58).

Development of CRC metastasis

Around 15-25% of patients already have metastatic disease at the time of primary CRC diagnosis and it is considered to be the major cause of death (59,60). The most common site for CRC metastasis is the liver (CRCLM), accounting for around 70% of cases, followed by the lungs, with around 30% of cases, the incidence for other site metastasis such as bones or nervous system are much rarer (61). The two main metastatic spread theories “anatomical” and “seed and soil” are quite universal for all cancers and are also applicable to CRC (62,63). The “anatomical” theory explains that the metastasis spread according to the blood-draining

site of the primary tumor, explaining the high number of metastases in the liver as the blood from the colon and rectum is mainly drained through the portal system to the liver (63). The other hypothesis explains the metastatic spread on a molecular level by showing that metastasis can develop from a single cell and that the target organ microenvironment is crucial for the metastasis to evolve (62). Specifically for CRC, several studies analyzed genetic mutation differences between primary CRC and its metastasis. These studies concluded that the main driving genetic mutations, such as KRAS, BRAF, TP53, and APC, are usually maintained during the metastatic spreading of CRC (64). The genetic mutation analysis of CRC metastasis is crucial for developing novel treatment options, for example, target therapy in the presence of a BRAF mutation showed promising results in a clinical trial (65,66).

Treatment

Treatment of primary tumor

During the last several decades, endoscopic treatment options for early local CRC have made considerable progress. Currently, only early CRC lesions, specifically T1 cancer with no additional tumor risk factors, can be treated safely using endoscopic resection techniques (67,68). As the en-bloc resection is crucial for further adequate tumor pathological evaluation, technically challenging approaches, such as endoscopic submucosal dissection or full-thickness resection are needed (69). Although these procedures are technically demanding, they yield great functional outcomes and adequate oncological outcomes (70).

As endoscopy has a very narrow field in CRC treatment, tumor surgical resection remains the key element of curative treatment. Colon and rectal surgeries are quite different in the technical difficulties of the procedures and further postoperative outcomes. Fortunately, a lot of surgical research efforts result in ever-improving surgical and technical advances which correspond to improved patient survival (71).

Treatment of liver metastasis

It is considered that up to 50% of patients develop CRCLM throughout the course of the disease, making their treatment almost as important as the primary tumor (72). Only a few decades ago patients with metastatic CRC had limited treatment options resulting in a 5-year survival of just 3% (73). With the introduction of more aggressive local and systemic treatment tactics, this number gradually increased and reached 20% for all patients with metastatic CRC and around 50% for patients with CRCLM (65,74).

Surgical CRCLM removal, as for the primary CRC tumor, is considered the only curative option. Resection of the CRCLM is the most often performed procedure and it has several main aspects that have to be respected to achieve sufficient oncological results (75). Nowadays, the importance of R0 resection, when the resection margin is at least 1mm is debated, with some studies showing that a tumor-free margin with less than 1mm is not associated with worse patient survival (76,77). The other important consideration is the expected future liver remnant after the surgery. A resection is considered safe if the remaining liver remnant is around 25% of the total non-cirrhotic liver volume (78). If the liver is damaged by cirrhosis or chemotherapy (CTx) induced injury the remaining remnant has to be considerably larger, reaching 40% of the initial liver volume (79). Several techniques such as portal vein embolization, ligation, or the association of liver partition with portal vein ligation may be used to induce liver growth and increase future liver volume (80–82). Additionally, in some cases CRCLM may be located in anatomically unfavorable places, such as the hilum of the liver, rendering the resection technically unachievable. Taking all these potential surgery obstacles into account, only about 20% of patients remain suitable candidates for surgical treatment upon the diagnosis of CRCLM (65).

For patients with unresectable CRCLM liver transplantation may be optional (83). However, strict patient selection is necessary to achieve great long-term survival rates (84). Moreover, this treatment option remains controversial due to the high tumor recurrence rates, that are associated with the use of immunosuppression after transplantation (85).

The use of ablation techniques for CRCLM is quite popular, especially for patients when resection is not possible. Two of the most frequently used ablation methods include radiofrequency ablation and microwave ablation. Both techniques use alternating electric current to generate heat in the target tissues, but the main difference is the frequency that they use (86). Microwave ablation systems use higher frequency and this allows for larger ablation area and more homogenous tissue necrosis (87). Best results, even comparable to survival after resection, are achieved when ablation is used on lesions that do not exceed 3cm in diameter (86). In some occasions, ablation techniques are combined with surgical resection, this combination allows to remove part of the liver with the most metastatic lesions and ablate smaller lesion, preserving the liver volume (88).

Chemotherapy

Unfortunately, only around 20% of all patients diagnosed with CRCLM are candidates for radical treatment options at the time of diagnosis (1). For the other 80% of these patients, the

main treatment option remains systemic CTx. The selected CTx regimens depend on the patient functional status, tumor molecular profiling, and the target result (65).

The fluoropyrimidine-based CTx has been the cornerstone treatment option for metastatic CRC for over half a century (89). 5-fluorouracil (5-FU) is the most often used agent from the fluoropyrimidines group and it is usually administered with leucovorin to increase the CTx effectiveness (90). Capecitabine is also a fluoropyrimidine class agent, which is converted to 5-FU inside the body, however, its main advantage is the ease of use as it is administered orally. Monotherapy of these agents is usually used to treat older and comorbid patients as it is quite well tolerated (65). However, for the more general population fluoropyrimidines are administered as a cytotoxic combination with either oxaliplatin (FOLFOX or CAPOX) or irinotecan (FOLFIRI or CAPIRI).

Patients with initially unresectable CRCLM may undergo intensive first-line neoadjuvant CTx to achieve a significant response that would enable further resection. Although this provides a lot of hope to the patient only around 12-33% of these patients achieve the target and undergo the subsequent liver resection (91). Currently, several studies indicate that the most effective regimen to achieve resectability is FOLFOXIRI (leucovorin, 5-FU, oxaliplatin, and irinotecan) with the addition of a vascular endothelial growth factor inhibitor (VEGF) bevacizumab (92,93). However, patients receiving FOLFOXIRI should be non-frail as this CTx is associated with a higher complication rate such as neutropenia and diarrhea (93,94).

Several biologic therapy agents are being used for the treatment of CRCLM (65). Bevacizumab is a VEGF inhibitor and it is the most frequently used target therapy agent. It acts by inhibiting tumor angiogenesis and it is administered as an additive to the irinotecan or oxaliplatin-based CTx regimen (95). The further use of target therapy agents is limited to the molecular profile of the CRCLM. Patients that present with KRAS/NRAS wild-type CRCLM may benefit from epidermal growth factor receptor (EGFR) inhibitors Cetuximab and Panitumumab. Similarly to bevacizumab, these agents can be administered as an additive to the first line CTx (96). In the presence of CRCLM with microsatellite instability or MMR deficiency target therapy agents such as Pembrolizumab or Nivolumab should be considered, as they showed promising results in increasing progression-free survival period (97,98). In difficult, CTx refractory cases novel agents such as the multi-targeted tyrosine kinase inhibitor Regorafenib or oral chemotherapeutical agent TAS-102 could further prolong patient survival (99,100).

Resistance to chemotherapy drugs

Patients receiving CTx for CRCLM usually have to transition from one treatment scheme to the other as the tumor eventually develops resistance to the administered CTx drug and starts to progress. There are several known mechanisms of how drug resistance emerges (101).

CTx resistance can develop if changes to drug metabolism occur. For instance, 5-FU undergoes several biochemical reactions to achieve its active form, mainly the decreased enzymatic activity of thymine phosphorylase or any other disturbances in these pathways that lead to resistance (102). On the other hand, most of the 5-FU is catabolized by hepatic dihydropyridine dehydrogenase and the increased enzyme activity can lead to CTx resistance (103). Several novel substances are being investigated to decrease the activity of the aforementioned enzyme to improve 5-FU efficacy (104–106).

The increased CTx drug efflux through various membrane transporter pumps results in decreased drug concentration in cancer cells. ATP-binding cassette (ABC) transporters are mainly involved in this resistance pathway and are responsible for the development of multi-drug resistance (107). As multi-drug resistance is a considerable obstacle to achieving sufficient CTx effect, a lot of research effort is focused on various agents that could modulate the activity of these transporters (108,109).

Changes in cancer cell death mechanisms are associated with subsequent resistance to CTx. The balance between apoptosis-regulating proteins becomes disturbed in cancer cells by the upregulation of anti-apoptotic proteins, mainly the Bcl-2 family, and the downregulation of anti-apoptotic proteins, such as p53 and Bax (110). These aberrations increase tumor resistance to CTx by inhibiting apoptosis, therefore, inhibiting the Bcl-2 proteins or increasing p53 activity, may result in increased tumor susceptibility to CTx (111,112). Contrary to apoptosis, the increased activity of cancer cell autophagy allows for better energy and protein distribution within the tumor, which is crucial for survival during anti-cancer treatment (113). Additionally, it is believed that autophagy plays a role in cancer immune escape further amplifying tumor resistance to treatment (114).

Cancer cells can recruit additional cells, such as cancer-associated fibroblasts, inflammatory and immune cells to create what is called a tumor microenvironment, which regulates tumor progression (115,116). Depending on the administered treatment different cell types can be recruited to increase tumor resistance to CTx agents (117). Cancer-associated fibroblasts play

a major role as they comprise most of the tumor stroma. They produce various pro-inflammatory substances that increase cancer cell survival against CTx substances (101).

Chemotherapy-induced side effects

During the administration of CTx, numerous adverse effects can occur, potentially involving every organ system and causing life-threatening conditions (118). Here I will discuss several CTx-induced adverse effects, that may not be the most prevalent ones, but their occurrence could significantly limit further cancer treatment options (119).

Intestinal mucositis

One of the most common CTx side effects is intestinal mucositis (IM). The prevalence of this condition varies from 40 to 100% of all cancer patients depending on the CTx regime used (120,121). For instance, the incidence of IM is notably higher when regimens that include irinotecan are used for treatment (122). IM may clinically present with very different severity starting with mild symptoms such as bloating, nausea, or diarrhea that may further develop into severe, life-threatening conditions (121,123). Moreover, severe cases of IM may lead to suboptimal cancer treatment as CTx doses may need to be reduced or delayed altogether (122). Lastly, as IM prolongs patient hospitalization, it significantly increases both resource and financial burden to the healthcare system (122).

For a long time, mucositis was thought to develop due to direct drug-induced injury to the epithelium, however, the last few decades of research effort gave us a better understanding of the underlying pathogenetic mechanisms. Usually, intestinal damage occurs at the tip of the villi from mechanical or chemical factors. After damaged epithelial cells undergo death, they are replaced by migrating neighboring cells (124). The persistent cell renewal is driven by the proliferation of stem cells that are located in the intestinal crypts (125). The use of CTx agents disturbs this process by inducing apoptosis in the continuously proliferating stem cell pool, thus the epithelial cell shedding in the villi is no longer backed up by sufficient renewal. This balance is additionally disturbed as CTx drugs may have a direct cytotoxic effect also to the villi cells, thus further increasing cell loss (126). Due to around 10 times higher spontaneous apoptosis rates in the small intestine compared to the colon, the small intestine is more prone to damage caused by CTx (127).

Although the development of IM is a dynamic process, to better understand it Sonis et al. have proposed a revolutionary five-stage oral mucositis pathogenesis model, that is also applicable

to CTx-induced IM (128,129). During the first, initiation phase, CTx drugs cause DNA strand breaks and additionally reactive oxygen species are generated. These changes occur not only in the epithelium but also in the submucosa, which may be more impactful to the development of injury (130). As soon as the DNA damage occurs, the second, primary damage, phase starts. This phase is defined by the activation of various signaling pathways such as Bcl-2, p53, caspase-1/3, and NF-κB (131). The activation of the NF-κB transcription factor plays a major role in the development of IM as it regulates various cytokines (IL-6; IL-1β, TNF-α), adhesion molecules, and apoptosis (129,131,132). The inflammatory response is further exacerbated during the third, signal amplification, phase. Some of the cytokines, that were generated during the previous phase, not only cause damage to the intestinal tissue but also amplify the inflammation by sustaining a positive-feedback loop (128). For example, TNF-α can augment the primary signal by activating NF-κB. The initial three phases are usually subclinical to the patient as the integrity of the epithelium is not yet compromised, however this changes during the fourth, ulceration, phase. During this phase mucosal tight junctions and therefore integrity deteriorates, allowing for bacterial translocation from the intestinal lumen to the submucosa and further activation of inflammation, which eventually results in tissue damage and intestine ulcerations (128,129). The fifth, healing, phase starts spontaneously after the initiating damaging factor ceases to act. Signals from the extracellular matrix promote epithelial proliferation and regeneration (122). Usually, IM symptoms peak 3 days after and normalize 14-16 days after the administration of CTx drugs (123).

Symptomatic treatments such as rehydration and adequate electrolyte replacement therapy remains essential for IM management as currently, there are no approved specific treatment options for IM (121,128). Fortunately, the ever-increasing understanding of the underlying pathogenetic IM mechanisms helps researchers to adapt already existing drugs or to create completely new therapeutical agents. It was shown that the development of intestinal dysbiosis is closely related to the clinical manifestation of IM (133). Several studies showed that the use of various options to alter the intestinal microbiota, such as antibiotics, fecal transplantation, or probiotics, helps to alleviate IM (131,134,135). Probiotics seem to be the most promising of them all, as they exert numerous benefits to the intestine while being easy to administer and safe (136). However, more research is needed to determine the most beneficial bacterial strains and to better understand the interaction between microbiota and mucosa in the presence of IM (131). Some indirect evidence suggests that Melatonin may be beneficial in reducing CTx-induced IM as it has anti-oxidative and mucosal barrier-regulating properties in the intestine (137,138). Additionally, anti-inflammatory agents, namely selective COX-2 inhibitors reduced intestinal damage in animal studies, however, their clinical results are

inconclusive (139,140). Other substances, such as anti-apoptotic agents (IL-1 antagonists; β arrestins) and especially incretins (GLP-1; GLP-2), may be also beneficial in managing IM, however, they are in the early research stages and their effects are still tested in animal models (141–143).

CTx-associated liver injury

The ever-increasing CTx use promoted the recognition of more subtle side effects. One of them is CTx-associated liver injury (CALI), which is especially relevant in the management of CRCLM. There is a wide variety of possible hepatotoxicity that systemic CTx causes, however specifically steatosis, steatohepatitis, and sinusoidal obstruction syndrome are the most common ones when CTx agents are used for the treatment of CRCLM (144). Steatosis develops due to impaired metabolism of free fatty acids and it is histologically characterized by the deposition of lipids within the cytoplasm of the hepatocytes, causing impaired cellular function (145,146). A further step in hepatic injury is chemotherapy-associated steatohepatitis, which features hepatocyte ballooning and inflammation upon microscopic evaluation (145). It is believed to develop due to increased oxidative stress from CTx agents in already steatosis-affected hepatocytes (147). Another severe liver toxicity is sinusoidal obstruction syndrome (SOS). Different from the previous two described injuries it is a result of CTx toxic effects not to the hepatocytes themselves but to the sinusoidal endothelial cells (148). Upon macroscopic examination, the liver appears bluish-red due to venous blood congestion and the microscopy reveals perisinusoidal fibrosis and erythrocyte congestion within the subendothelial space (148).

Interestingly, results from several studies indicate that the type of liver injury is closely associated with the CTx regimen used. All of the main CTx agents (5-FU, irinotecan, and oxaliplatin) used for CRCLM treatment are known to induce steatosis, irinotecan based therapies increase the development of steatohepatitis and the use of oxaliplatin greatly increases the rate of SOS (149–152). Additionally, CTx-induced liver injury develops gradually and prolonged exposure to CTx is usually needed (153).

CALI is extremely relevant when treating CRCLM as quite often these patients undergo surgical treatment and the presence of CALI is known to negatively impact postoperative outcomes (154). Few studies report that the presence of steatosis at the time of liver resection increases infective complication and overall morbidity rates (155,156). Interestingly, there are reports that the presence of steatosis may have a protective effect (157). The impact of steatohepatitis is less well-studied. A study by Vauthey et al. found that the presence of

steatohepatitis significantly increased the postoperative mortality rate, furthermore, such patients more often develop postoperative liver failure (158). Similarly, to steatohepatitis, SOS also increases complication rates and decreases long-term patient survival (157,159).

Currently, there are no therapeutic modalities approved for the treatment of CALI. In experimental settings, several agents showed liver protective effects and reduced the incidence of CTx-induced injury. One of them is bevacizumab, primarily used to increase the efficacy of CTx for CRCLM, however, it seems to also prevent the development of SOS (160,161). Another promising liver protector in this scenario is S-adenosyl-methionine. It acts by increasing the concentration of glutathione, which scavenges oxygen free radicals and thus decrease inflammation in the liver (162). Usually, SOS tends to resolve after the cessation of CTx but the time period, as Vigano et al. point out, is around 9 months. CTx-induced liver steatosis or steatohepatitis tends to persist for an even longer time period (163).

CTx-induced cardiotoxicity

As mentioned previously, FOLFOX or FOLFIRI are the main CTx regimens used for the treatment of CRCLM. The individual drugs and the combination of them are known to cause cardiotoxicity and even up to 39% of patients receiving systemic treatment for metastatic CRC experience cardiovascular adverse events (164,165). Most often cardiotoxicity is caused by 5-FU or its pro-drug capecitabine. Additionally, cardiac adverse events were reported with the use of monoclonal antibodies, most commonly bevacizumab (166–168).

Usually, 5-FU induced cardiotoxicity becomes apparent during the first CTx cycle and the symptoms develop within 72 hours of drug administration (169). Clinically cardiotoxicity can present with a number of symptoms, with the most common being angina and myocardial infarction followed by arrhythmias, pericarditis, myocarditis, and pulmonary embolism (169,170). The exact molecular mechanisms are not fully understood, however, the main underlying pathway is thought to be coronary spasm and increased thrombosis due to 5-FU damage to the coronary artery endothelial cells (171). Additionally, it was shown that 5-FU can directly cause damage to the cardiomyocytes by impairing the energy production in the mitochondria and by inducing apoptosis (172).

The problem with CTx-induced cardiotoxicity is not only the life-threatening adverse event itself but also the problematic further systemic treatment. Currently, there is no high-quality clinical evidence providing an effective substitute for 5-FU when treating CRCLM (167). Rechallenge

with 5-FU is life-threatening, with a reported mortality rate of up to 13%, and should be attempted only in a monitored setting after a thorough multidisciplinary discussion (167,169).

Usually, the treatment of toxicity consists of immediate 5-FU discontinuation, followed by diagnostics and symptomatic treatment of cardiac ischemia. Uridine triacetate is an antidote developed to treat fluoropyrimidine overdose and it may be used in cases of suspected severe toxicities (173). Several drugs, novel ones such as an iron-chelator dexrazoxane and long-known ones such as β-Blockers, renin-angiotensin-aldosterone system inhibitors, or statins, may be used for CTx-induced cardiotoxicity prevention (174).

Gut microbiota, probiotics and CRC

Gut microbiota, CRC and CRCLM

As described previously, the development of cancer is a very complex mechanism, highly influenced by genetic and immunological conditions. As research on gut microbiota indicates, an organisms' immunological status is strongly affected by the microorganisms that colonize the digestive tract (175). Back in 1980 Goldin and Gorbach were among the first to show that changes in the gut microbiota may have an influence on the development of CRC (176). Since then several other pieces of research linked gut dysbiosis with the development of various, including non-gastrointestinal, tumors (177,178).

Currently, most of the research done in this field investigates the link and underlying mechanisms between gut dysbiosis and the development of CRC (179,180). Years of research identified several bacteria strains such as *Escherichia coli, Bacteroides fragilis, Fusobacterium nucleatum,* and *Salmonella enterica* that can potentiate the development of CRC (180,181). A strong microbiome influence was proven by Wong et al. (182). Their preclinical study showed that gavaging mice with stool from CRC patients increases dysplasia and the number of polyps in the colon. The exact mechanistic data, on how gut dysbiosis promotes CRC, is lacking (180). It is hypothesized that the disruption of the gut barrier leads to foreign antigen influx and chronic inflammation, which allows for escalated tumor development (183). Additionally, some bacterial strains, such as *B. fragilis*, may directly cause chronic inflammation and promote carcinogenesis by producing toxic metabolites (184). Furthermore, *F. nucleatum* can increase cell proliferation by initiating the β-catenin–Wnt oncogenic pathway (185).

The liver is exposed to bacterial components and metabolites through the portal venous system. It is quite well studied and reported that gut microbiome plays a role in the

development of non-alcoholic steatohepatitis, liver cirrhosis, and hepatocellular carcinoma (186). However, not much is known about the gut microbiome's influence on the development and progression of CRCLM. Several bacterial strains, especially *E. coli*, have been linked with the CRC metastatic spread, mainly to the liver (181). A study by Ma et al. showed that the development of primary liver tumors and CRCLM may be related to the microbiota-modified bile acids and their interaction with the hepatic natural killer T cells (187).

Probiotics

Changes in the gut microbiota can be influenced by using different methods and substances, one of which being probiotics (188). By a widely accepted definition probiotics are "non-pathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (189).

Probiotics act in the intestine by several complex and intertwined mechanisms leading not only to intestine-specific but also to systemic changes (190). One of the action mechanisms is immune system modulation by regulating inflammation-related gene expression and this may lead to both local and systemic inflammation reduction (191). Probiotics may improve the intestinal barrier by simply competing with pathogenic microbes, such as *E. coli O157:H7*, and not allowing them to colonize the intestine (192). Additionally, probiotics help to stabilize the intestinal barrier by upregulating tight-junction protein (claudin-1, occluding, and zonulin) production, leading to a more hostile environment for pathogenic bacteria (193). Interestingly, research indicates that some of the probiotic strains can produce systemically acting molecules such as oxytocin, serotonin, noradrenaline, gamma-aminobutyric acid, or dopamine, through which they can impact mental health (194).

The increased research interest in gut health and probiotics for the last few decades lead to promising findings for the treatment of various clinical conditions, including data on probiotics as a tool in CRC and CRCLM management (191,195).

Probiotics and CRC treatment

There are two main ways in how the gut microbiome and its modulation with probiotics can influence CRC and CRCLM treatment. One is via direct bacteria and their metabolite interaction with the tumor and the second is the microbiome impact on CTx (183,196). Different probiotics may have a lot of distinct tumor-inhibiting characteristics, however, immunomodulation seems to be among the most important (197).

Several bacterial strains and their combinations have been tested for suppressing CRC. Most of the studies were done in animal models and the used probiotic bacteria are lactic acid producing bacteria, usually belonging to the *Bifidobacterium* and *Lactobacillus* genera (175,198). Probably the most popular commercially available probiotic mixture VSL#3 managed to reduce colitis-associated adenocarcinoma in a mice model (199). Moreover, promising data was seen in a clinical trial by Rafter et al., where the intervention consisting of synbiotics with *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb12* strains altered several CRC biomarkers (200).

Currently, there are no studies directly examining probiotic effects on CRCLM, however, some data indicate that the gut microbiome could influence the disease course. A study by Ma et al. showed that changes in the gut microbiome, especially the decreased abundance of *Clostridium* species, can regulate bile acid metabolism (187). These changes subsequently result in the accumulation of natural killer T cells in the liver and inhibit both primary and metastatic liver cancer growth. A study by Li et al. found that oral multispecies probiotic (*Lactobacillus rhamnosus* GG, viable *Escherichia coli* Nissle 1917, and VSL#3) supplementation can inhibit systemic inflammatory response by decreasing the Th17 cell count, which constrains hepatocellular cancer microvasculature formation (201).

Several bacteria strains have been identified to play a role in CTx agent chemoresistance and overall patient outcomes (202). A study by Yu et al. determined that *F. nucleatum* plays a role in the development of resistance to oxaliplatin and 5-FU in CRC patients (203). Additionally, scientists showed a link between bacteria from the genus *Sutterella* and species *Veillonella dispar* in CRC patients and the development of resistance to oxaliplatin and 5-FU (204). This research indicates that microbiome manipulation could have an impact on the effectiveness of CTx. This research topic is not very widely studied and only one animal study indicates a positive probiotic supplementation effect in increasing 5-FU effectiveness (205).

Probiotics for treating CTx-induced side effects

As previously mentioned, CTx-induced side effects have a great clinical impact on the success of cancer treatment, thus researchers are experimenting with novel treatment options to alleviate them, one of them being probiotics (195). Summarized data with currently available studies is presented in Table 1.

The role of probiotics in managing gastrointestinal mucositis has been studied both in animal and human studies. A meta-analysis by Feng et al. and a systematic review by Garczyk et al.

that included only clinical studies indicated that the oral administration of various probiotic strains reduced the incidence and length of CTx-induced diarrhea (206,207). Unfortunately, the heterogeneity of the included studies limits any firm conclusion-making. Although there are quite a few clinical studies done in this field, animal studies are still important, as they provide more in-depth knowledge on the microbiome shift during the development of mucositis (131). Similarly, to clinical studies, most of the published animal studies report a decrease in diarrhea severity and length in the probiotic intervention group. Moreover, histological, immunological, and biochemical analysis revealed that probiotic supplementation inhibits apoptosis, increases crypt proliferation and decreases proinflammatory cytokine and oxidative stress marker levels (208).

Data regarding the probiotic supplementation effects on other CTx-induced side effects, such as CALI or CTx-induced cardiotoxicity are lacking. Currently, no studies directly examine probiotic supplementation effects on CALI but some data indicate that probiotics may inhibit similar liver changes as develop from CTx agents. Several animal studies showed that probiotic supplementation reduces fatty acid accumulation and liver steatosis (209–211). Furthermore, probiotic supplementation can protect the liver from steatohepatitis development (212). Moreover, a study by Dewanjee et al. shows that probiotic supplementation may have protective effects not only in a chronic setting but also in an acute one (213). To date, only one study examines probiotic effects on CTx-induced cardiotoxicity. Zhao et al. determined that oral supplementation with *Lactobacillus gasseri* reduces cisplatin-caused body weight loss and cardiotoxicity. Although, their findings were inconclusive, their data suggests that the cardiac protection was a result of probiotic's systemic anti-inflammatory properties (214).

Aims

The aim of this dissertation thesis was to assess the role of probiotic supplementation in CRCLM treatment. The specific aims of the thesis were: 1) to assess the effects of probiotic supplementation on the effectiveness of CTx for CRCLM in an experimental rat model. 2) To assess if probiotics reduce CTx-induced gastrointestinal symptoms. 3) To assess the hepatoprotective properties of probiotic supplementation during CTx for CRCLM. 4) To examine a possible negative influence of FOLFOX CTx on heart function and the potential protective effects of probiotics

Table 1. Summary of studies examining the effects of probiotics for treating CTx-induced side effects.

Reference	Cancer type	Chemotherapy	Probiotic strains	Clinical outcomes
INTESTINAL MUCOSITIS/ DIARRHEA				
Animal studies				
Yeung et al. (215)	N/A	5-FU	*L.s casei* variety *rhamnosus; L. acidophilus; B. bifidum*	Decreased weight loss and diarrhea scores
Bowen et al. (216)	N/A	Irinotecan	VSL#3	Decreased weight loss and diarrhea scores
Bastos et al. (217)	N/A	Irinotecan	Viable and heat killed *S. cerevisiae*	Decreased weight loss
Sezer et al. (218)	N/A	Irinotecan	*S. boulardii*	Decreased weight loss and diarrhea scores
Chang et al. (219)	N/A	5-FU + Oxaliplatin	*L. casei* variety *rhamnosus*	Decreased diarrhea scores
Huang et al. (220)	N/A	5-FU	*L. casei* variety *rhamnosus; L. acidophilus; B. bifidum*	Decreased weight loss and diarrhea scores
Kato et al. (221)	N/A	5-FU	*B. bifidum* G9-1	Decreased weight loss
Mi et al. (222)	Colorectal	5-FU + Oxaliplatin	*B. infantis*	Decreased weight loss and diarrhea scores
Wang et al. (223)	N/A	Doxorubicin	*S.thermophilus* TH-4	Decreased weight loss
Whitford et al. (224)	N/A	5-FU	*S. thermophilus* TH-4	No beneficial clinical effects
Yuan et al. (225)	N/A	5-FU	*B. infantis*	Decreased weight loss and diarrhea scores
Tang et al. (226)	N/A	5-FU	DM#1 (*L.casei; L. acidophilus; S. thermophilus; B. breve)*	Decreased weight loss
Justino et al. (227)	N/A	5-FU	*L. acidophilus*	No beneficial clinical effects

Smith et al. (228)	N/A	5-FU	*L. fermentum* BR11	No beneficial clinical effects
Clinical studies				
Tian et al. (229)	Lung	Platinum-based	*C. butyricum*	Reduced incidence of diarrhea
Motoori et al. (230)	Esophageal	Docetaxel, Cisplatin and 5-FU	*B. breve* strain Yakult; living *L. casei* strain Shirota	Reduced severity of diarrhea and lymphopenia
Mego et al. (231)	Colorectal	Irinotecan and cetuximab	10 lyophilized various *Bifidobacterium* and *Lactobacillus* strains	Decreased incidence of grade 3 or 4 diarrhea
Osterlund et al. (232)	Colorectal	Mayo or de Gramont regimens	*L. rhamnosus GG*	Decreased incidence of grade 3 or 4 diarrhea
Chemotherapy associated liver injury				
Currently, no clinical or animal studies directly examine probiotic supplementation effects on chemotherapy associated liver injury				
Chemotherapy induced cardiotoxicity				
Animal studies				
Zhao et al. (214)	N/A	Cisplatin	*Lactobacillus gasseri*	Increased body weight and preserved cardiac function
Clinical studies				
Currently, no clinical studies directly examine probiotic supplementation effects on chemotherapy induced cardiotoxicity				

MATERIAL AND METHODS

The description of some parts of Material and Methods section may be similar to those published in Jakubauskas M et al (233,234).

Animals

Six-week-old male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) weighing approximately 200–280 g at the start of the experiment were used. Animals were housed at the Institute for Biomedical Research (Medical University of Graz, Austria). Two to four rats were housed per cage with unrestricted access to pelleted chow and tap water. Rats were kept under controlled conditions of temperature (22-23°C) and humidity (45-50%) in a 12 h light/dark cycle. The Austrian Committee for Animal Trials approved all procedures (Approval number: BMWF-66.010/0158-V/3b/2019).

Experimental groups and design

All rats were randomly assigned to the desired experimental groups (Table 2). The detailed scheme of the experimental design is presented in Figure 1.

Table 2. Animal groups in the study.

	SHAM Tumor (-) /Chemotherapy (-)		Non FOLFOX Tumor (+) /Chemotherapy (-)		FOLFOX Tumor (+) /Chemotherapy (+)	
Gavage	Placebo	Probiotics	Placebo	Probiotics	Placebo	Probiotics
Started (n)	10	10	15	15	20	20
Finished (n)	10	10	15	15	20	19*

*One death on the last protocol day due to CTx toxicity

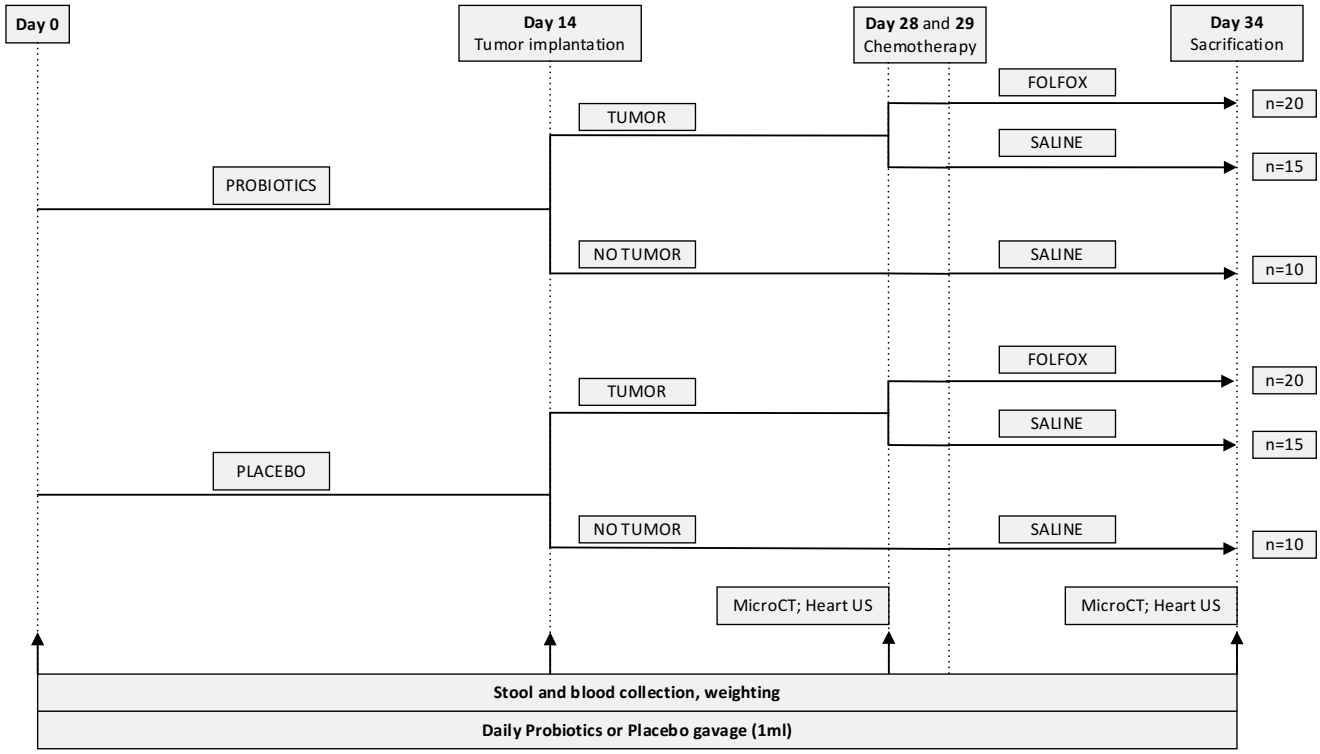

Figure 1. Experimental design of the study. Figure reproduced from reference (233).

Probiotics

In this study, we used a commercially available multispecies probiotic mixture (provided by Institut Allergosan, Graz, Austria) composed of 8 different bacterial strains: *Lactobacillus casei W56; Lactobacillus acidophilus W37; Lactobacillus brevis W63; Lactococcus lactis W58; Bifidobacterium lactis W52; Lactococcus lactis W19; Lactobacillus salivarius W24* and *Bifidobacterium bifidum W23.* Bacteria were mixed with 1g of matrix (maize starch, maltodextrins, vegetable protein, potassium chloride, magnesium sulphate, amylases, and manganese sulphate). Matrix without bacteria was used as a placebo. The probiotic/placebo powder was dissolved using tap water every morning approximately 15 minutes before gavaging. Each rat received 1 ml of suspension containing either 1.2 x 10^9 CFU/ml of the probiotic or placebo mixture.

Tumor implantation

The rat colorectal cancer cell line (CC531) (Cell Lines Service, Eppelheim, Germany) was cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Global Life Sciences Solutions, Marlborough, MA, USA), 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 1% L-glutamine

(Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 25mM HEPES (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in cell culture flasks (Corning, Corning, NY, USA) at 37°C in a humidified atmosphere containing 5% CO_2. On the morning of tumor implantation, CC531 cells were harvested and suspended at a density of 5 x 10^6 cells in 100µl of sterile PBS. The tumor inoculation procedure was performed under general anesthesia (induction with 4-5% isoflurane and maintenance with 2% isoflurane) with addition of intramuscular injection of fentanyl (8µg/kg). After induction of anesthesia, the animal was placed in a supine position on an automatically regulated heating pad to maintain a body temperature of 37°C throughout the procedure. The right subcostal area was shaved and disinfected. Afterward, a 1cm subcostal incision approximately 0.5cm below the ribcage was made. After reaching the peritoneum the median liver lobe was gently exposed using the opposite end of microsurgical forceps (Figure 2A). Using a 25-Gauge 25mm length needle (B. Braun Melsungen AG, Melsungen, Germany) 100µl of the prepared CC531 cell suspension was slowly injected under the liver capsule. An appearance of a whitish protrusion indicated successful tumor implantation (Figure 2B). With a short compression of the injection site using a small sterile gauze hemostasis, to prevent tumor cell leakage, was achieved. A two-layer interrupted suture (Vicryl 4-0, Ethicon, Somerville, NJ, USA) was performed to close the abdominal wound (Figure 2C), additionally, the skin was adapted and glued with topical skin adhesive (LiquiBand Optima, Advanced Medical Solutions, Devon, UK) (Figure 2D). At the end of the operation, all animals received 4.5mg/kg carprofen subcutaneously. To further reduce pain drinking water was supplemented with ibuprofen (0.4 mg/ml) for the first 5 postoperative days.

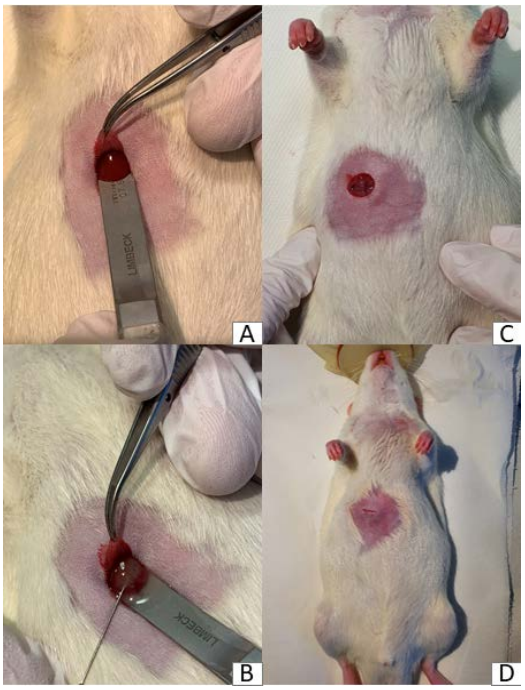

Figure 2. Surgical procedure of intrahepatic implantation of colorectal adenocarcinoma cells. **A**-exposure of the median liver lobe. **B**- Tumor inoculation. **C**- Wound closure using a double layer interrupted suture. **D**- Skin adapted using topical adhesive. Figure reproduced from reference (233).

Micro-CT scanning

24 hours before the first Micro-CT scans a single dose of ExiTron nano 12000 (Viscover™, nanoPET Pharma GmbH, Berlin, Germany) contrast agent was injected via the tail vein using a 25-Gauge 25mm length needle (B. Braun Melsungen AG, Melsungen, Germany). The contrast agent was injected under 2% isoflurane anesthesia. Micro-CT scans were performed on experimental day 28 (two weeks after tumor implantation) and on the last experiment day 34, just before scarification. Due to its long-lasting properties, a single application of the contrast agent was sufficient for good tumor visibility in both micro-CT scans. In this study, we used the SkyScan 1276 (Bruker, Karlsruhe, Germany) micro-CT scanner using the following scanning parameters: 1mm filter, 85kV, 200 µA, exposure time 132ms, detector binning 4×4 and resolution of 40.5µm. Anesthesia induction was performed using 5% isoflurane, afterwards, the rat was transferred to the rat cassette. Anesthesia was maintained using 3% isoflurane with continuous heart and respiratory rate monitoring. Scanning length varied from 13 to 19 minutes depending on the chosen scanning field.

Micro-CT image analysis

The micro-CT image analysis was done using CTAn software (Bruker, Karlsruhe, Germany) by a single examiner in a blinded fashion. At first, the reconstructed images were filtered using the following parameters: Filter: anisotropic diffusion filter; Type: Privilege wide regions; 15 iterations and a gradient threshold of 15. Liver volume was calculated by running a prespecified task list. Threshold values were corrected for each case individually (Figure 3). The tumor volume was measured by first defining a custom region of interest based on the tumor shape and size and then running a pre-specified task list (Figure 4). 3D reconstructions were created using the CTVox software (Bruker, Karlsruhe, Germany) (Figure 5). Change in tumor volume was expressed as tumor volume at day 28 (mm^3) x 100% / tumor volume at day 34 (mm^3).

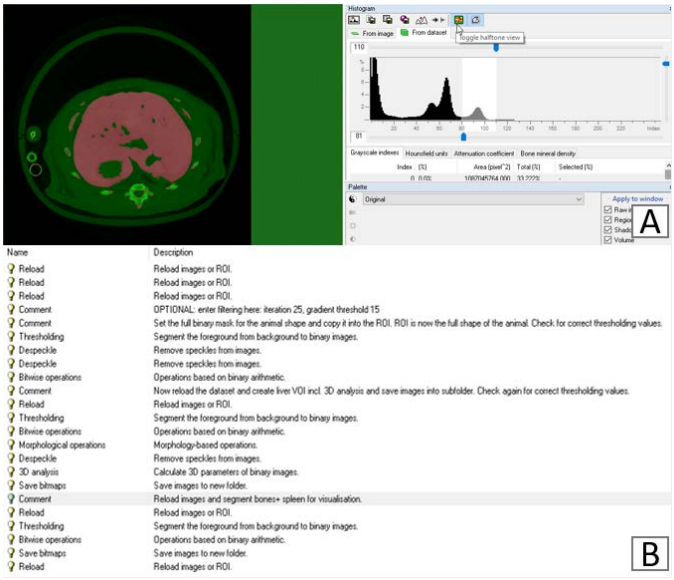

Figure 3. Liver micro-CT analysis. **A**- Threshold value determination. **B**- Pre-specified task list used for liver volume calculations.

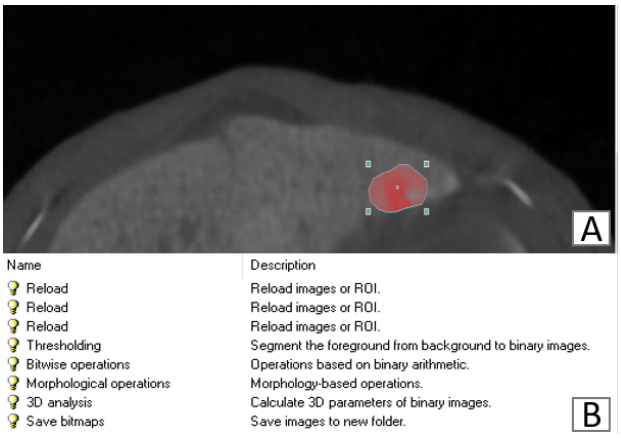

Figure 4. Tumor micro-CT analysis. **A**- Defining a custom region of interest based on the tumor shape and size. **B**- Pre-specified task list used for tumor volume calculations.

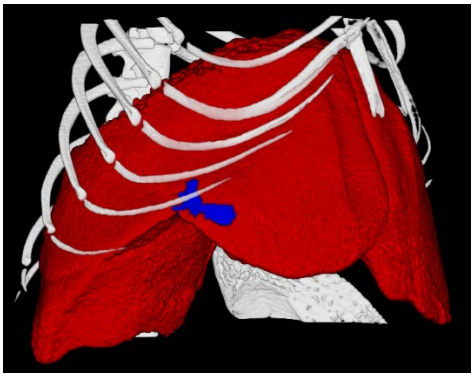

Figure 5. Micro-CT 3D image reconstruction. Red color represents liver; blue- tumor; white – skeleton and spleen.

Heart ultrasound scanning

On experimental days 28 and 34 heart ultrasound (US) was performed using a Vevo 3100 preclinical imaging system (FUJIFILM, VisualSonics, Toronto, Canada) equipped with an MX250 (frequency range: 15-30 MHz, axial resolution: 75 µm) ultrahigh-frequency linear array transducer. Immediately after the Micro-CT scan was finished, rats were transferred and secured in a supine position on a 37°C heat pad (Vevo Imaging Station, FUJIFILM VisualSonics, Toronto, Canada). According to the electrocardiogram, anesthesia was adapted using 1-2% isoflurane in order to obtain constant and comparable heart rates. Chest hair were removed using a shaver and depilatory cream, afterwards preheated ultrasound gel was applied. The left ventricle (LV) was visualized using both B- and M-modes in the parasternal long-axis (PLAX) and short-axis (PSAX) views. B- and M-Mode images of LV in PLAX view were acquired in its maximum dimension from apex to base. In the PSAX view, B-Mode images were recorded at the level of the mitral valve (base), papillary muscles, and apex, while M-Mode images were only at the level of papillary muscles.

Heart ultrasound image analysis

Heart US image analysis was performed in a blinded fashion by a single investigator. LV ejection fraction (LVEF) was evaluated using the VevoLab software version 3.2.6 (FUJIFILM VisualSonics, Toronto, Canada). The LVEF was calculated from the PSAX M-mode images using the LV trace function from at least 3 consecutive cardiac cycles. Change in LVEF was expressed as LVEF(%) after CTx at day 34 x 100%/LVEF(%) before CTx at day 28.

Chemotherapy

The FOLFOX CTx regimen was chosen in this experiment due to its proven potency in treating colorectal cancer liver metastasis (235,236). This regimen was also used in another similar study by our research group with the desired tumor suppressive effect (237). All CTx agents were administered via intraperitoneal injections using a 25-Gauge 16mm length needle (Terumo AGANI, Shanghai International Holding Corp. GmbH, Hamburg, Germany) under short general 2% isoflurane anesthesia. The detailed CTx protocol is presented in Figure 6.

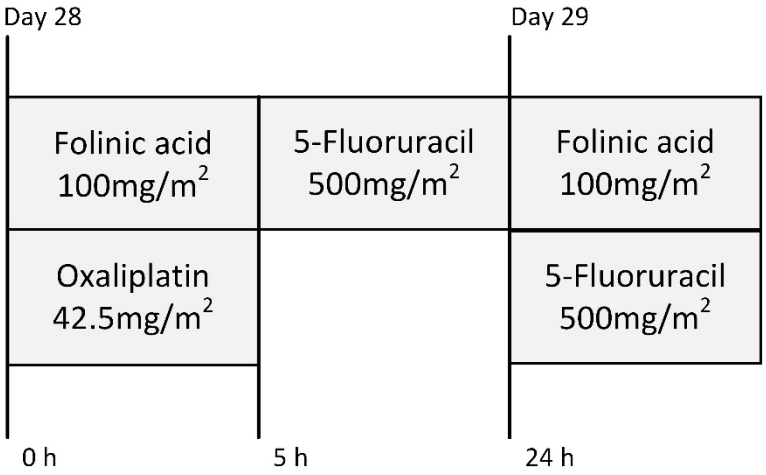

Figure 6. FOLFOX CTx dosage scheme. Figure reproduced from reference (234).

Diarrhea assessment

All animals were examined two times daily following the first injection of the chemotherapeutical agents. Diarrhea was graded using a published scale: grade 0, no diarrhea; grade 1, mild diarrhea (staining of the anus); grade 2, moderate diarrhea (staining of the lower abdomen) and; grade 3, severe diarrhea (staining over legs and higher abdomen or continual oozing) (Figure 7) (238).

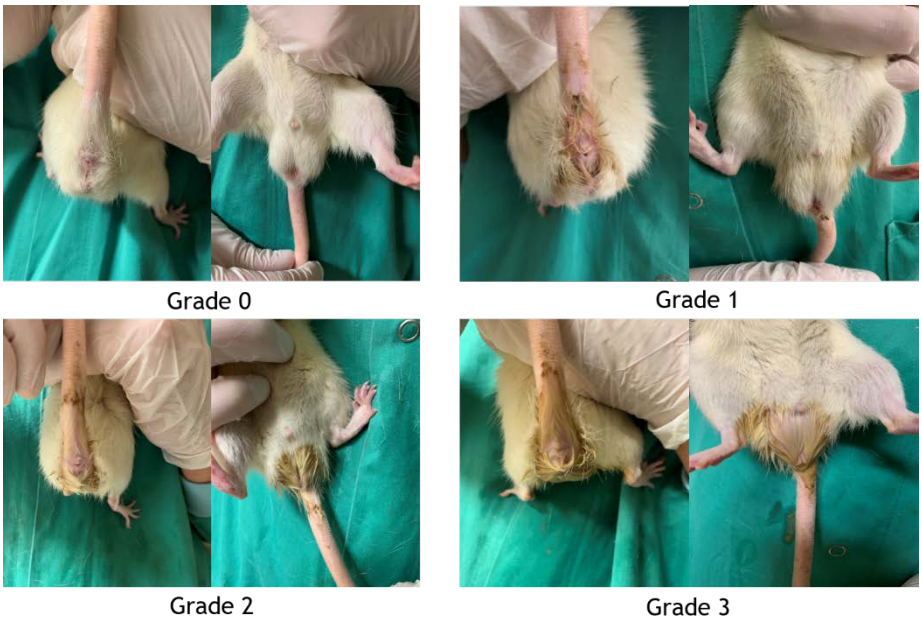

Figure 7. Diarrhea assessment. Grade 0 - no diarrhea. Grade 1 - mild diarrhea (staining of the anus). Grade 2 - moderate diarrhea (staining of the lower abdomen). Grade 3 - severe diarrhea (staining over legs and higher abdomen or continual oozing). Figure reproduced from reference (234).

Blood sample analysis

On experimental days 0, 14, and 28 venous blood samples were collected from the subclavian vein under 2% isoflurane anesthesia. On the last experimental day, blood was collected from the inferior vena cava. Complete blood count was measured using a V-Sight hematology analyzer (A. Menarini Pharma GmbH, Vienna, Austria). Biochemical blood measurements were performed with a Spotchem EZ (A. Menarini Pharma GmbH, Vienna, Austria) analyzer.

Histology

Harvested tissue samples were fixed in a 4% buffered formaldehyde solution, washed with distilled water and dehydrated with ascending ethanol series. Following incubation at 60 °C, tissues were embedded in paraffin. 2 µm thick tissue sections were cut using a rotary microtome (HM 340E, Thermo Fisher Scientific, Waltham, MA, USA) and mounted on superfrost plus slides (Thermo Fisher Scientific, Waltham, MA, USA). All tissue sections were stained with hematoxylin and eosin using a standard protocol.

Immunohistochemical staining was performed using the following primary antibodies: Anti-MPO (Dako, Via Real Carpinteria, CA, USA, A0398, dilution 1:800), Anti-Ki67 (Abcam, Cambridge, UK; ab16777, dilution 1:200), and Anti-CD31 (Abcam, Cambridge, UK; ab182981, dilution 1:2000). The UltraVision LP Detection System: HRP Polymer (Thermo Fisher Scientific, Waltham, MA, USA) and DAB Chromogen (Dako, Via Real Carpinteria, CA, USA) were used to visualize the target antigen. Sections were counterstained with hematoxylin.

Liver apoptotic cells were assessed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) technique using TUNEL Assay Kit - HRP-DAB (Abcam, Cambridge, UK ab206386). The method was applied according to the manufacturer’s manual.

All stained sections were scanned and positive cells were counted from all the area of the sample using the open-source QuPath software (239). The tumor microvasculature quantification and analysis were performed additionally using the ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Crypt and villi length in both the ileum and colon were measured according to a publication by Adelman et al. (240). Five random crypt and villi lengths of the ileum were measured and a villi/crypt length ratio was calculated for further data analysis. The median value of five random crypt lengths of the colon for each rat was used.

Microbiome analysis

DNA isolation from fecal samples was performed using the Magna Pure LC DNA III Isolation Kit (Bacteria, Fungi) (Roche, Mannheim, Germany) according to published protocols (241,242). A stool pellet was mixed with 500µl phosphate buffered saline (PBS) and 250µl bacterial lysis buffer. Afterward, the sample was homogenized using the MagNA Lyser instrument (Roche Life Science. Mannheim, Germany) at 6500 rpm for two 30s cycles. Enzymatic lysis was done using 25µl lysozyme (100ng/ml, 37°C for 30 min) and 43.4 µl proteinase K (20 mg/mL, 65°C for 1 h). After the samples were heat inactivated at 95°C for 10 min, DNA was extracted using a MagnaPure LC instrument (Roche, Mannheim, Germany) according to the manufacturer's instructions. Extracted DNA was eluted in 100µl elution buffer and stored at −20°C until analysis. 2µl of total DNA was used in a 25µl PCR reaction in triplicates using a FastStart High Fidelity PCR system (Sigma, Darmstadt, Germany) according to the manufacturer's instructions and the target specific primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′GGACTACNVGGGTWTCTAAT-3′) for 30 cycles. Triplicates were pooled, normalized, indexed and purified according to a published protocol (241). The final pool was sequenced on an Illumina MiSeq desktop sequencer at 9 pM and v 3 600 cycles chemistry. FASTQ raw reads were processed using QIIME2 tools implemented on a local galaxy instance (https://galaxy.medunigraz.at). Taxonomic assignment was done using a Naïve Bayesian Classifier trained on the SILVA V132 database. Features were summarized on genus level for further analysis. Using the web-based analysis platform "Calypso", group specific general linear models identified genera that significantly changed during the course of the study. For the selected genera, differences between day 28 and day 34 were calculated and entered into a Spearman correlation analysis with diarrhea characteristics, albumin and weight changes to assess the associations between microbiome changes and the effects of the probiotics. P-values were adjusted with Benjamini-Hochberg correction and visualized using the R package "corrplot" (Version 0.92).

Statistical analysis

Analysis was performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Distribution of the variables was investigated using the Shapiro–Wilk test. Normally distributed data were analyzed using t-test and one-way ANOVA with Tukey's posthoc test or Mann-Whitney U and Kruskal-Wallis with Dunn's posthoc test was used for not normally distributed data. A p value ≤0.05 was considered significant. Data is reported as median and quartiles (Q1; Q3).

RESULTS

Some parts of the results are reported in Jakubauskas M et al (233,234).

General data and weight change throughout the study

During the experiment, one premature death due to CTx-induced toxicity occurred in the Probiotics + FOLFOX Group (Table 2). No other unexpected serious adverse events were noted.

At the start of the study, rat weight increased evenly in all groups (Figure 8). This tendency continued for rats that did not receive CTx. However, rats that received FOLFOX significantly lost weight (Figure 9A). Probiotic supplementation managed to significantly limit weight loss caused by CTx (83.97% (79.65; 86.61) vs. 86.76% (84.29; 88.46); p=0.016) (Figure 9B).

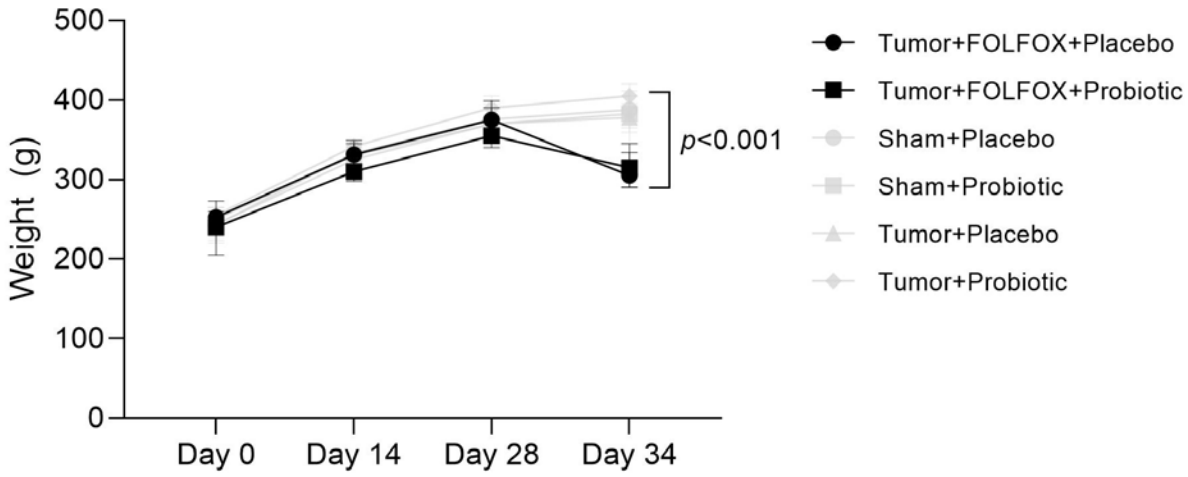

Figure 8. Weight change throughout the study.

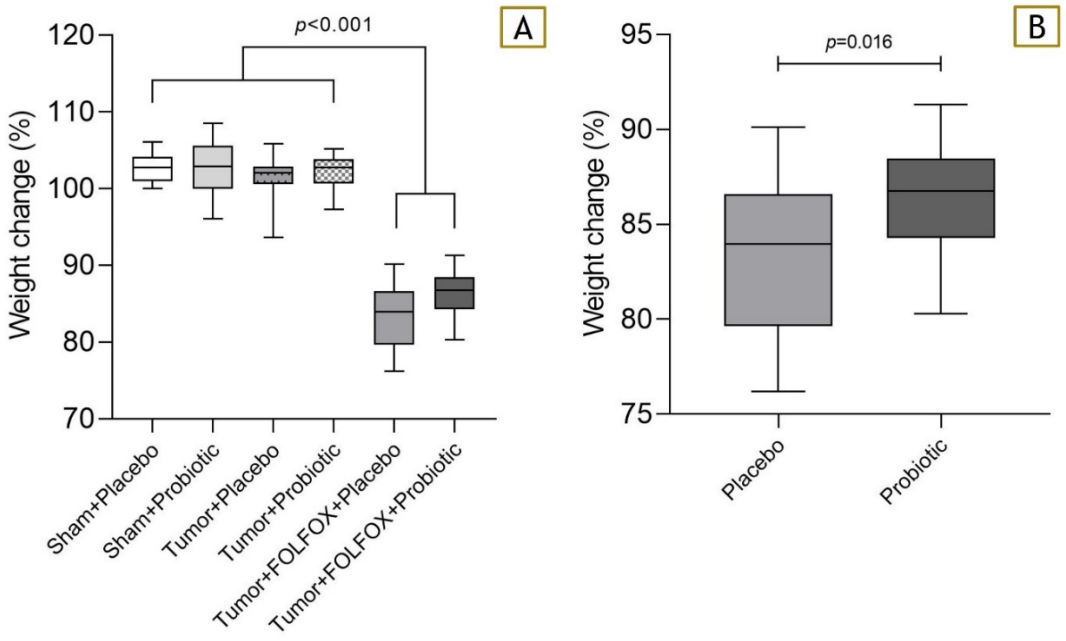

Figure 9. **A**- weight change between rats that received FOLFOX CTx and did not. **B**- weight change after FOLFOX CTx between probiotic and placebo groups. Weight change calculated as weight day 28 (g) x 100% / weight at day 34 (g). Figure reproduced from reference (234).

Blood analysis results

WBC

FOLFOX CTx induced severe myelosuppression as white blood cell (WBC) levels were significantly reduced in both CTx-receiving rat groups when compared to rats that did not receive CTx (Figure 10). Probiotic supplementation did not effect the WBC count during the study and did not influence the severity of leukopenia.

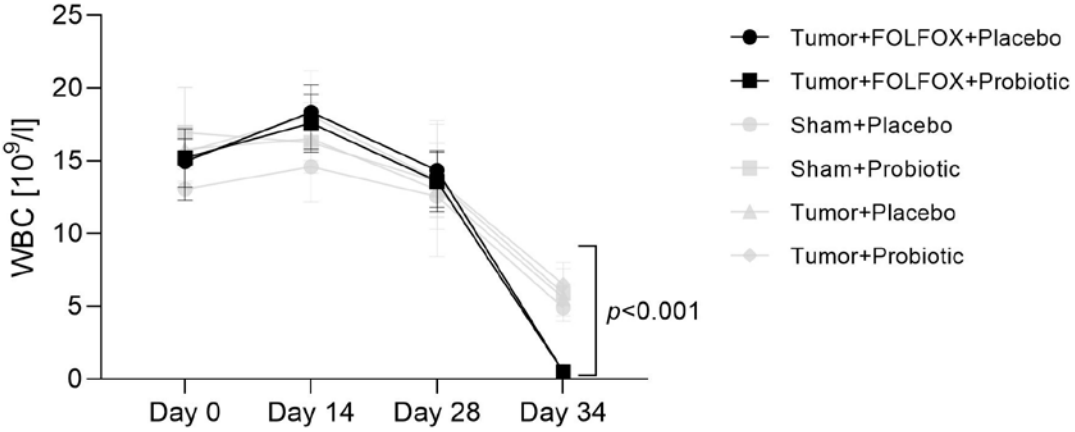

Figure 10. White blood cell count change throughout the study. Figure reproduced from reference (233).

Blood albumin levels

Rat blood albumin levels were consistent across all groups at the start of the study (Figure 11A). FOLFOX CTx significantly reduced blood albumin levels in both placebo and probiotic groups. We further analyzed blood albumin level changes between protocol days 28 and 34. Figure 11B shows that probiotic supplementation managed to significantly reduce CTx-induced blood albumin loss (80.35% (68.03; 84.88) vs. 83.60% (80.28; 89.48); p=0.021).

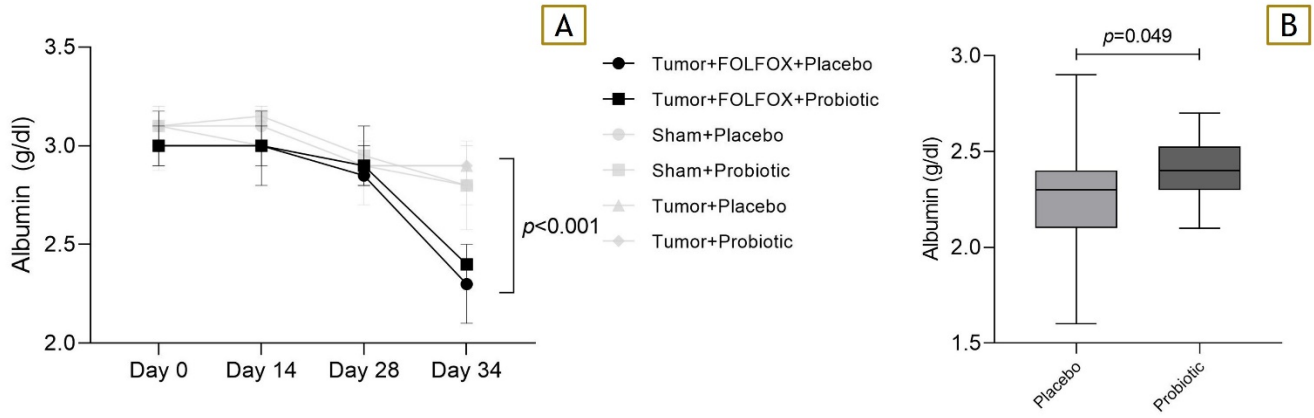

Figure 11. A- Albumin concentration change throughout the study. **B** - Blood albumin level change in percent between days 28 and 34 after FOLFOX CTx. Figure reproduced from reference (234).

Tumor analysis

Tumor volume change

Micro-CT data analysis shows that probiotic supplementation alone, without CTx, significantly impairs tumor growth in rats compared to the placebo group (109.20% (91.27; 152.70) vs. 57.67% (49.06; 69.76); p = 0.044) (Figure 12). However, there was no difference in tumor growth between intervention groups in rats receiving FOLFOX CTx (35.74% (25.87; 45.65) vs. 34.90% (27.68; 43.15); p = 1.000).

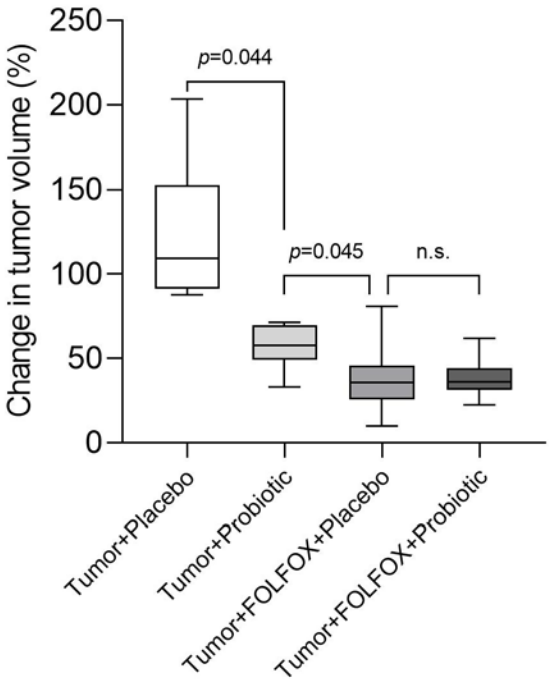

Figure 12. Change in tumor volume at the end of the study. Figure reproduced from reference (233).

Tumor immunohistochemical analysis

The highest percentage of MPO-positive cells was observed in the tumor+placebo group. The tumor+probiotics group had a lower percentage of MPO-positive cells, however, the difference was not significant (Figure 13). Both CTx groups had a significantly lower tumor infiltration with MPO-positive cells when compared to both groups that did not receive CTx.

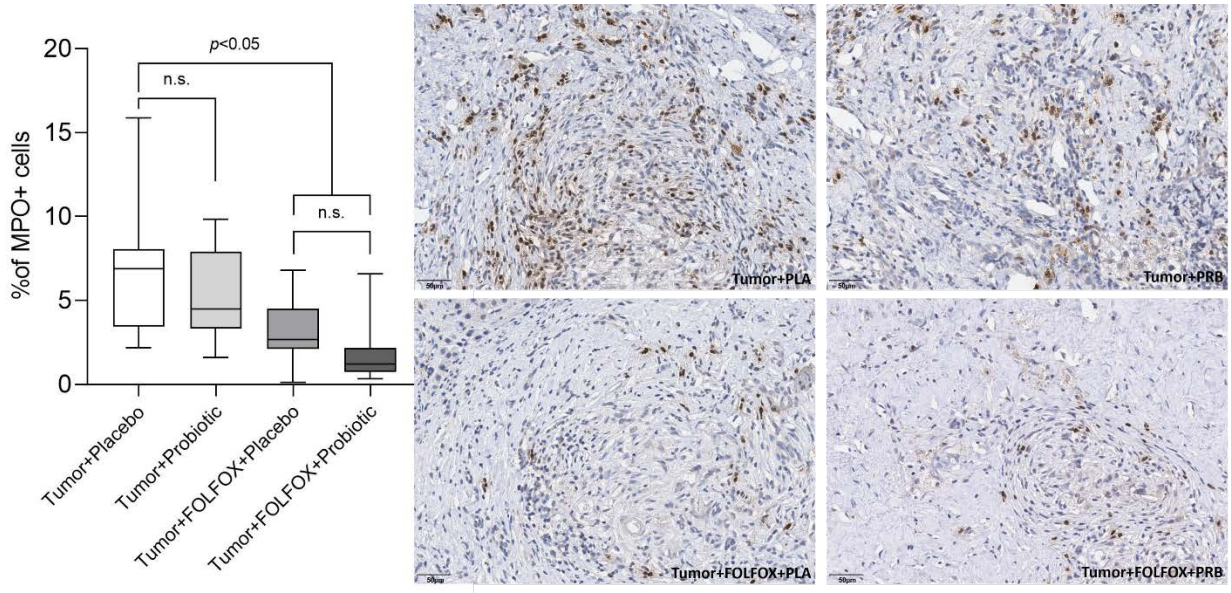

Figure 13. Percentage of MPO+ cells in the tumor. Figure reproduced from reference (233).

A similar tendency was observed with the tumor proliferation index (% of Ki67 positive cells). The highest tumor proliferation index was detected in the tumor+placebo group (Figure 14). Both CTx groups had a significantly lower tumor proliferation index when compared with both groups that did not receive CTx.

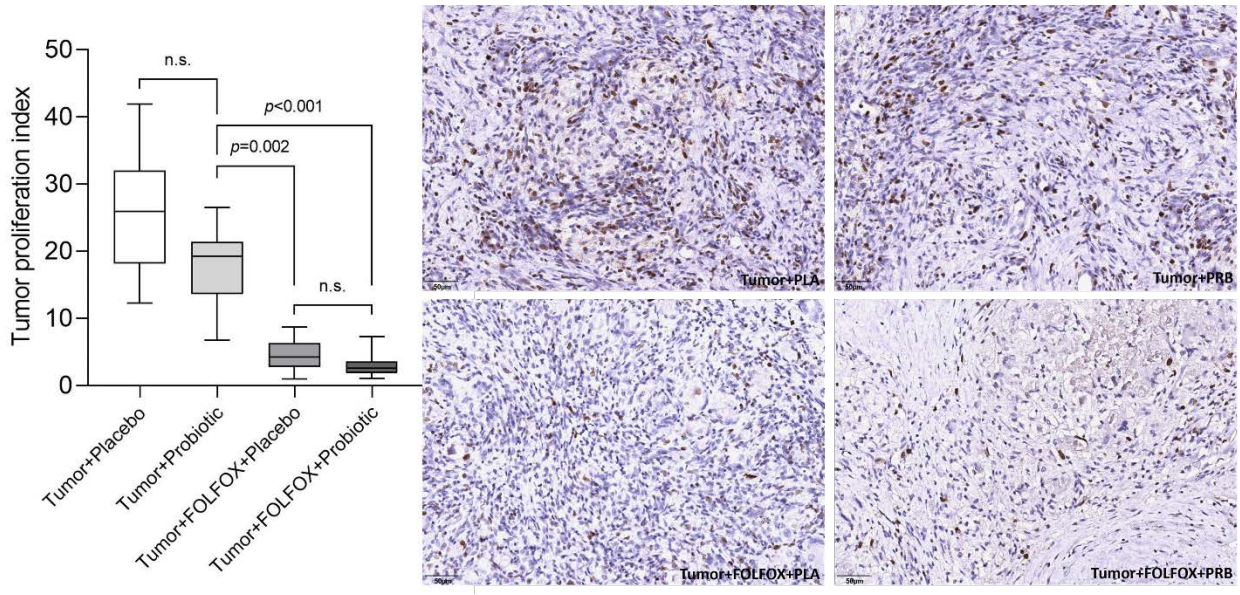

Figure 14. Tumor proliferation index (% of positive Ki67+ cells in the tumor). Figure reproduced from reference (233).

A significantly higher tumor apoptosis index (% of TUNEL positive cells) was seen in samples from rats that received CTx (Figure 15). We did not observe any tumor apoptosis index differences between rat groups that did not receive CTx.

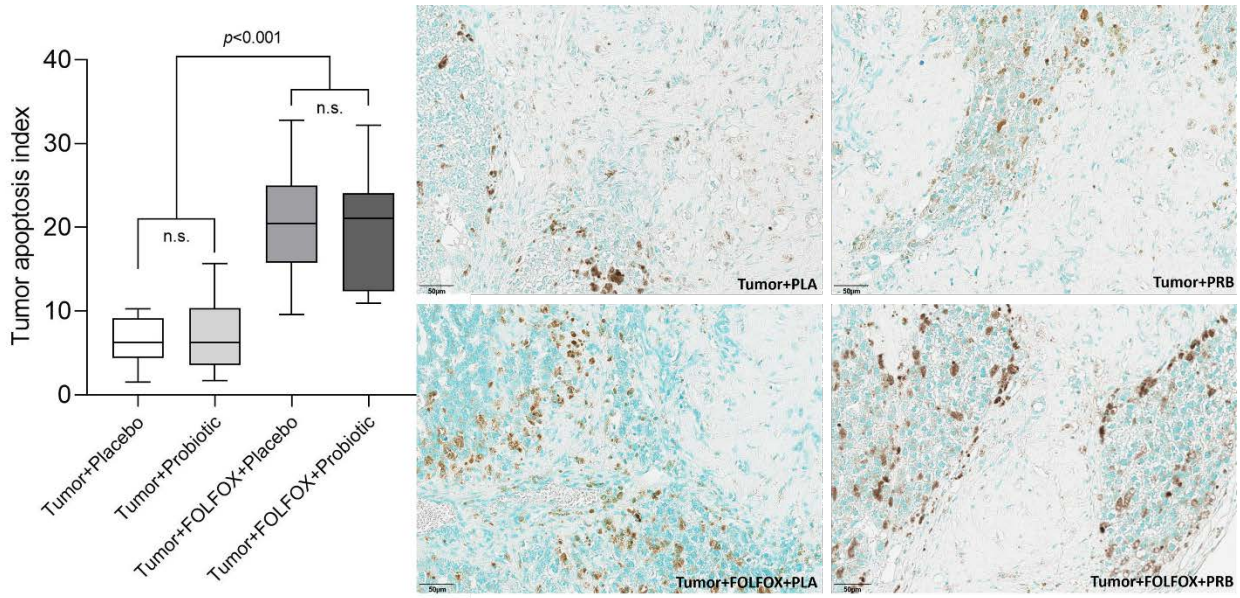

Figure 15. Tumor apoptosis index (% of TUNEL+ cells in the tumor). Figure reproduced from reference (233).

Probiotic supplementation alone, without FOLFOX therapy, significantly reduced Tumor microvascular density (MVD) (Figure 16). MVD differences were significant between both rat groups that did not receive CTx. Furthermore, there was no significant difference between the tumor+probiotic group and both rat groups that received FOLFOX CTx.

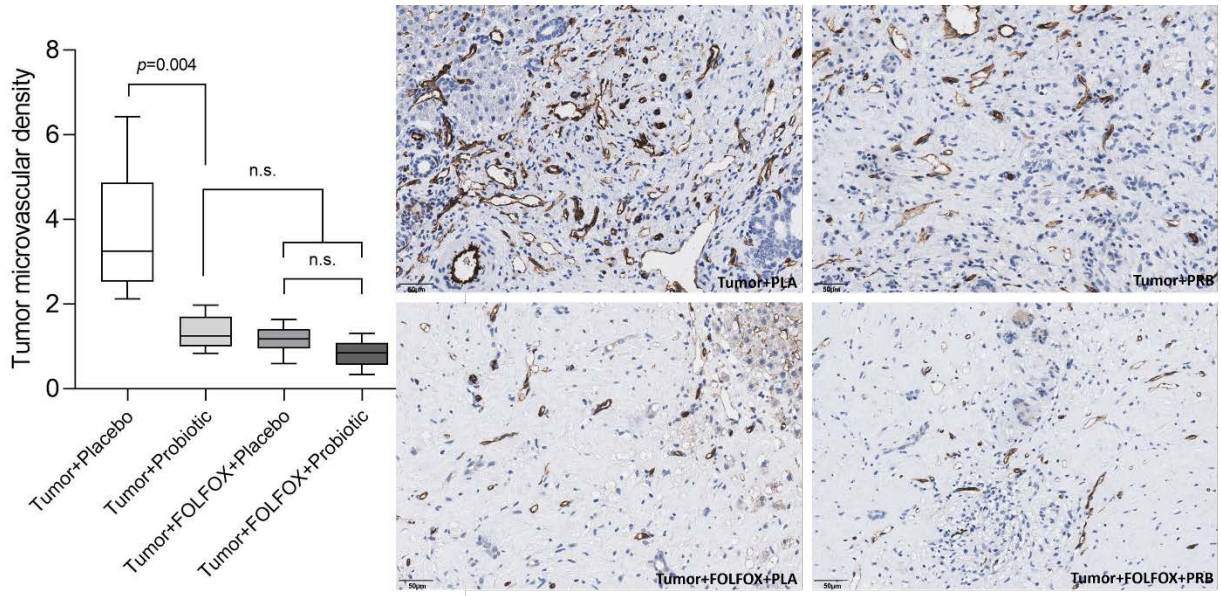

Figure 16. Tumor microvascular density (number of vessels sprouts per 1000μm2). Figure reproduced from reference (233).

CTx-associated liver injury

Blood sample analysis of liver injury markers (AST, ALT and Total bilirubin) did not indicate any CTx-induced liver damage (Figure 17).

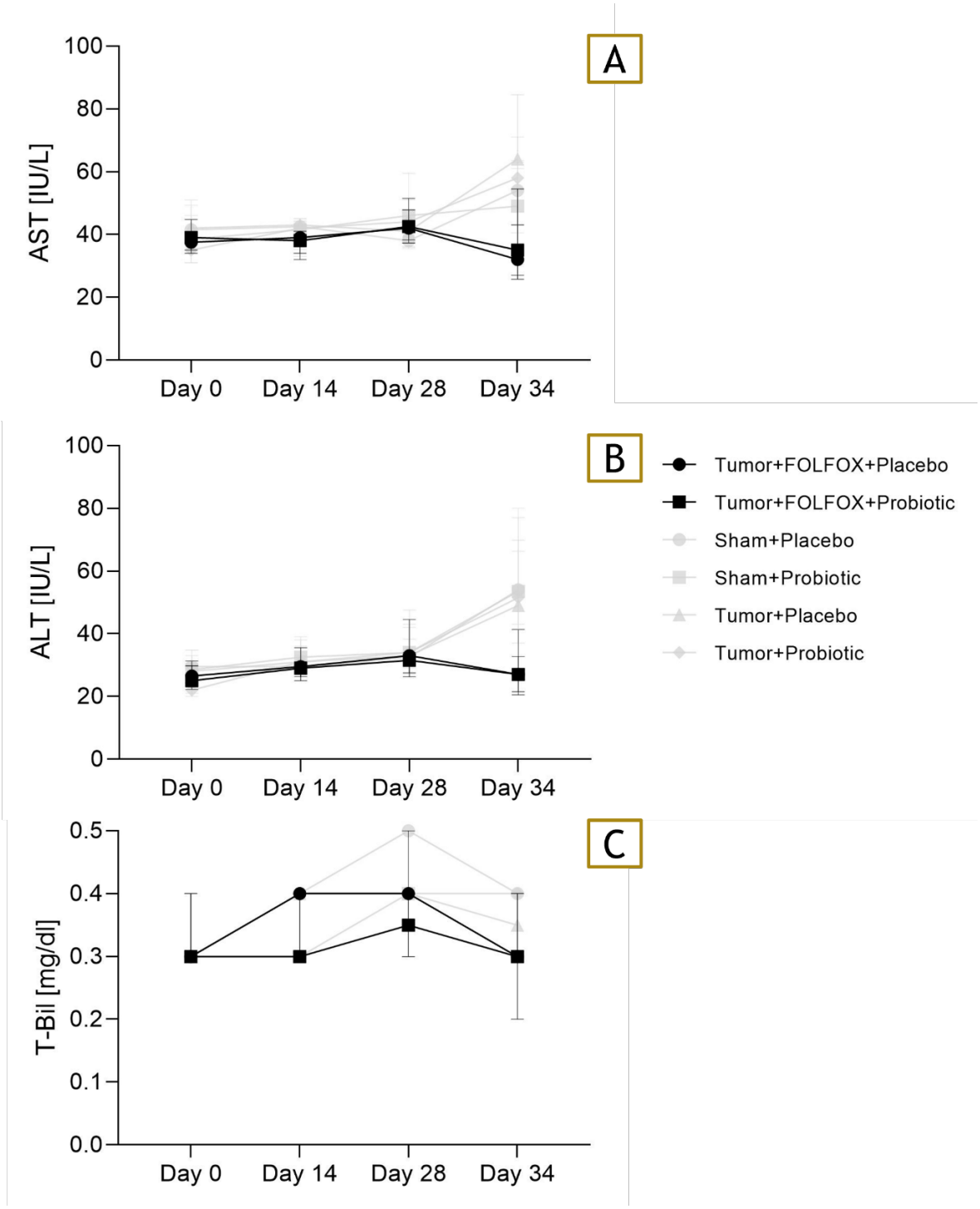

Figure 17. Blood liver injury markers. **A** - AST levels throughout the study. **B** - ALT levels throughout the study. **C** - Total bilirubin levels throughout the study.

Furthermore, the initial histological evaluation of liver samples did not show any cellular alterations, thus a full-extent analysis was not performed.

Heart analysis

Change in LVEF

Baseline values of the LVEF measurement varied from 74.43% to 87.01% and there were no significant differences when compared between groups. We did not observe any decrease in LVEF between days 34 and 28 in groups that did not receive CTx, but there was a decrease in LVEF in both CTx groups and this difference was statistically significant (p<0.001) (Figure 18A). Furthermore, rats receiving probiotic supplementation experienced a lower decrease of LVEF after FOLFOX CTx (94.81% (91.87; 95.57) vs. 97.15% (95.63; 99.37); p<0.001) (Figure 18B).

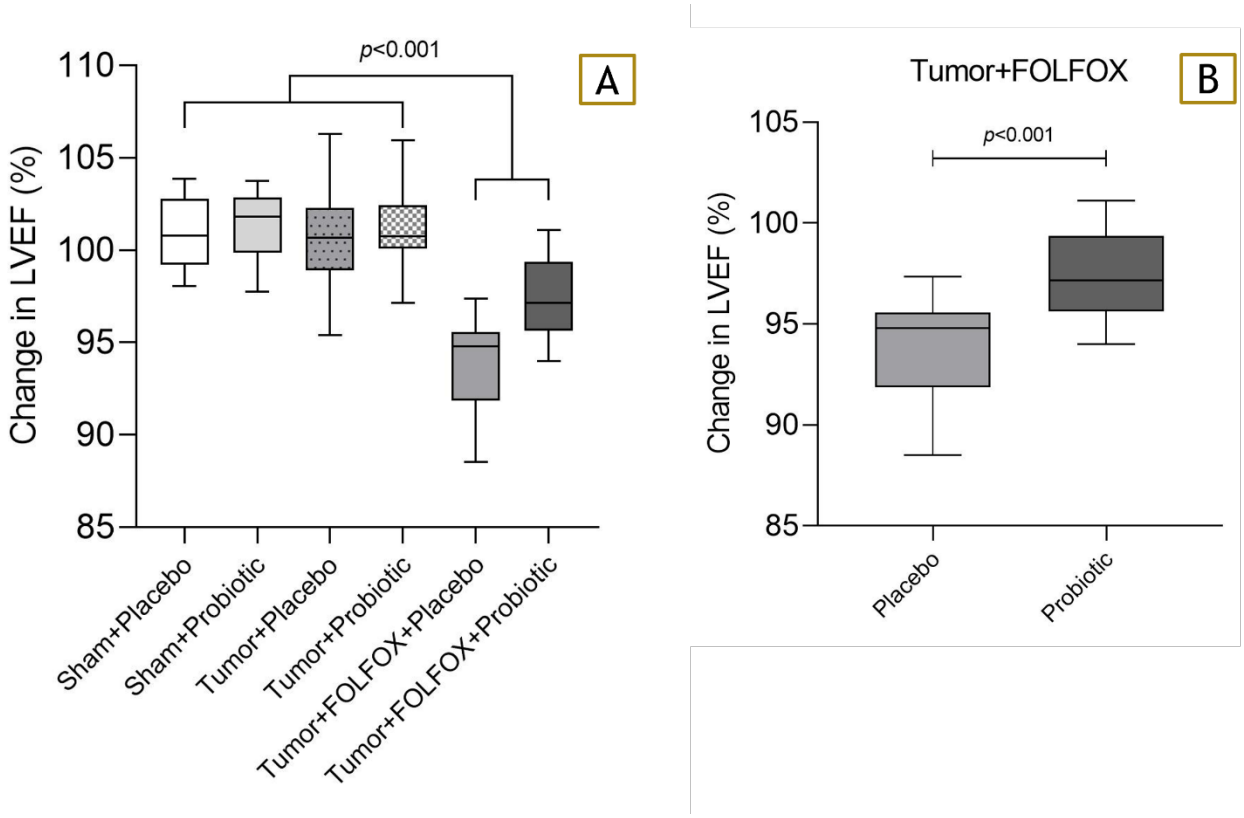

Figure 18. Change in LVEF after FOLFOX CTx administration. **A**- LVEF change between rats receiving and not receiving FOLFOX CTx. **B**- LVEF change after FOLFOX CTx between probiotic and placebo groups.

Heart immunohistochemical analysis

The analysis of MPO and Caspase 3 positive cells did not indicate any beneficial effects of probiotic supplementation on heart tissue. Moreover, we did not observe an increase of apoptotic cells in both CTx groups when compared to the non-CTx groups (Figure 19 A, B).

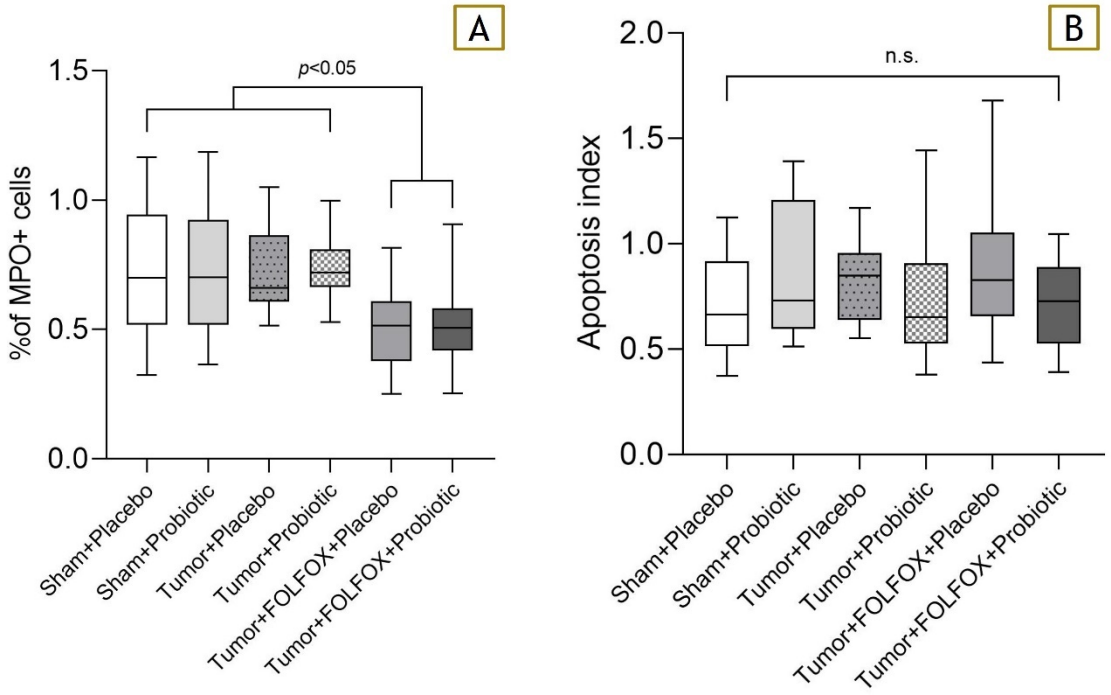

Figure 19. Heart tissue immunohistochemical analysis results. **A** - percentage of MPO+ cells in the heart. **B** – Heart apoptosis index (percentage of Caspase3+ cells).

Intestinal mucositis evaluation

Diarrhea assessment

A total of 15 animals (75%) receiving a placebo and 19 animals (100%) receiving probiotics developed some degree of diarrhea (Figure 20). The peak incidence of diarrhea was observed 96 hours after the first CTx injection in both groups. At the peak incidence, 7 animals (35%) receiving placebo and 4 (21%) receiving probiotics developed severe diarrhea. Furthermore, severe diarrhea tended to resolve quicker for animals receiving probiotic supplementation (24h (12.0; 30.0) vs. 12h (12.0;12.0); p=0.026).

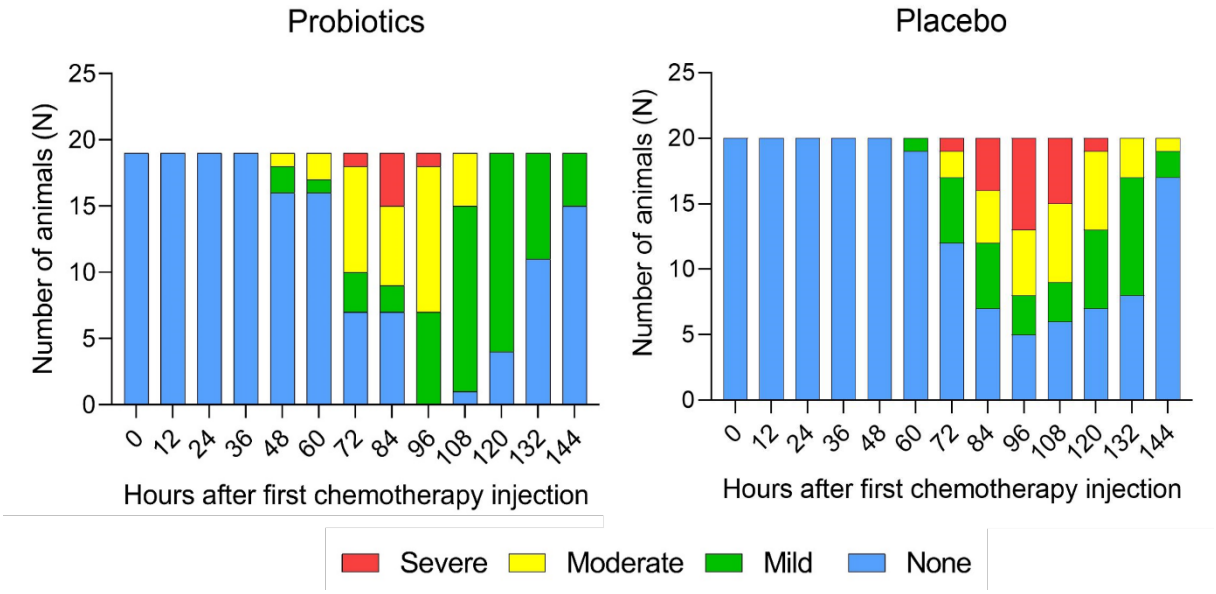

Figure 20. Diarrhea assessment results. Figure reproduced from reference (234).

Intestine immunohistochemical analysis

Analysis of rat terminal ileum villi length and crypt depth ratio showed that probiotics helped to alleviate CTx-induced intestinal damage (1.59 (1.44; 1.76) vs. 1.93 (1.73; 2.09); p<0.001)) (Figure 21A). A similar tendency was seen with the rat colon crypt depth analysis (366.00µm (331.70; 402.20) vs. 309.80µm (286.20; 345.10; p<0.001)) (Figure 21B).

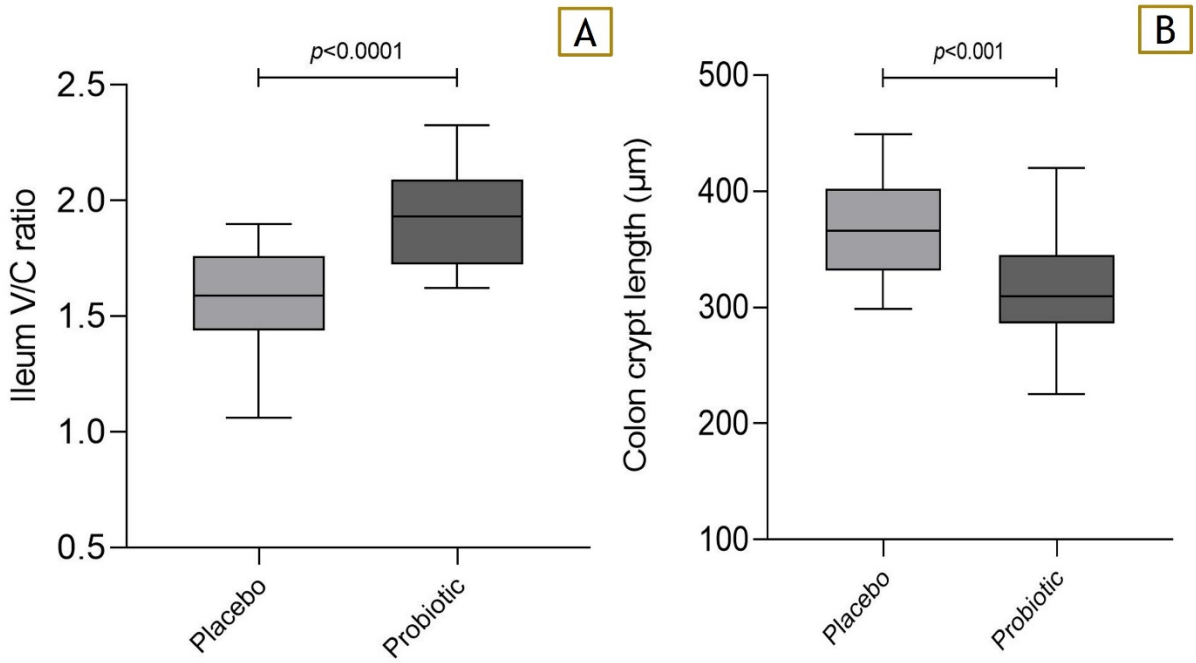

Figure 21. A- Terminal ileum villi/crypt length ratio after FOLFOX CTx **B**- Colon crypt length after FOLFOX CTx. Figure reproduced from reference (234).

The percentage of MPO-positive cells was significantly lower in both CTx-receiving groups when compared to rats that did not receive CTx, and this was observed both in colon and ileum tissues. There were no differences between both CTx groups (Figure 22A, B).

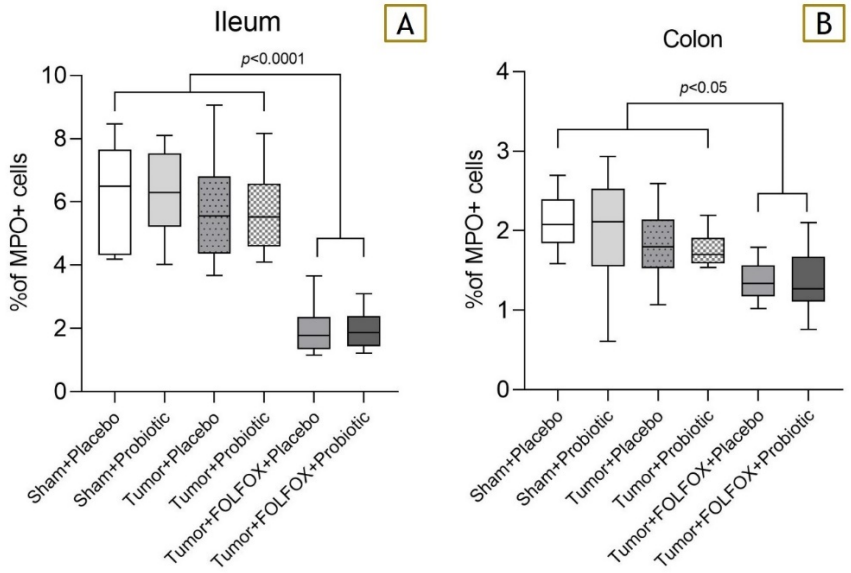

Figure 22. Intestine infiltration with MPO+ cells. **A**- percentage of MPO+ cells in the terminal ileum **B**- percentage of MPO+ cells in the colon. Figure reproduced from reference (234).

Probiotic supplementation greatly increased intestinal cell proliferation and the differences between both CTx groups were statistically significant (Figure 23A, B).

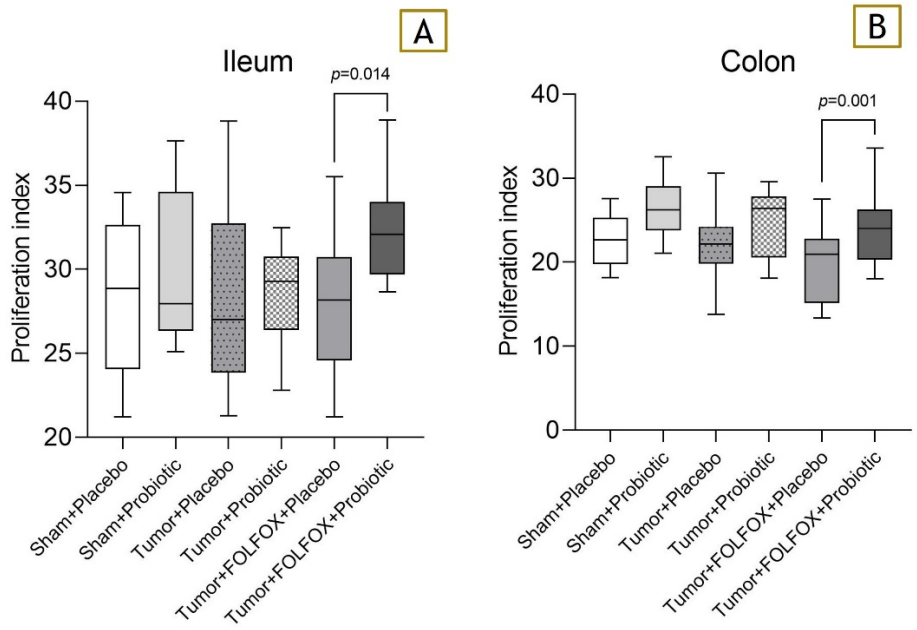

Figure 23. Intestine proliferation index. **A**- percentage of Ki67+ cells in the terminal ileum **B**- percentage of Ki67+ cells in the colon. Figure reproduced from reference (234).

Furthermore, it seems that probiotic supplementation also managed to have a protective effect from CTx-induced apoptosis on ileum cells (9.57% (7.98; 11.07) vs. 7.58% (6.50; 9.30); p=0.001) (Figure 24A). However, the apoptosis index in the colon samples was similar across all experiment groups (Figure 24B). Figure reproduced from reference (234).

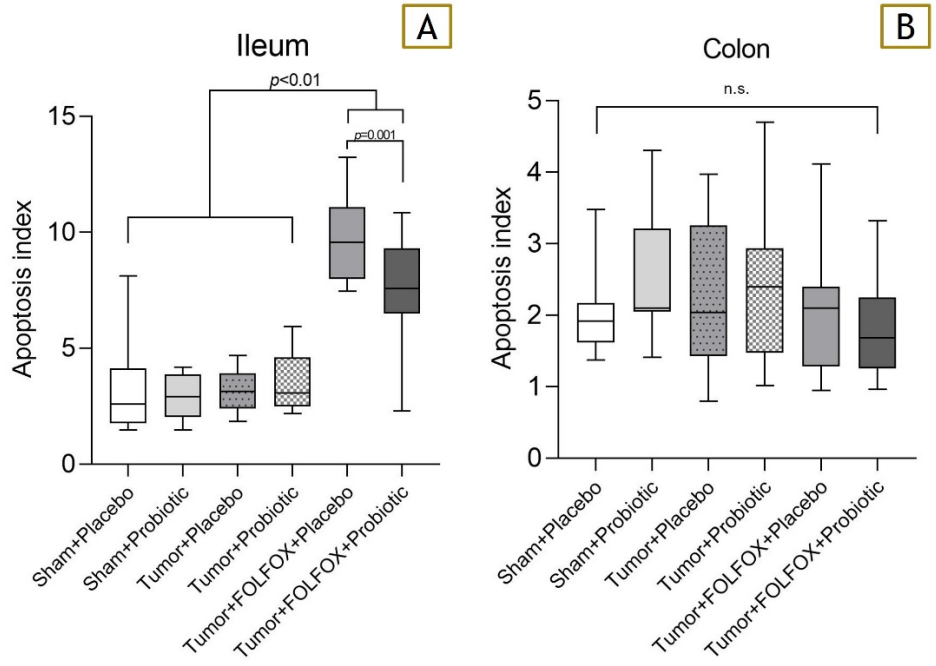

Figure 24. Intestine apoptosis index. **A**- percentage of Caspase3+ cells in the terminal ileum **B**- percentage of Caspase3+ cells in the colon. Figure reproduced from reference (234).

Microbiome analysis

Associations between CTx adverse effects and the microbiome

Correlation analysis results are summarized and presented in Figure 25. The length of diarrhea shows a strong correlation with an increase in *Bacteroides* in stool samples during CTx (r_s=0.76; p_{adj}=0.002). Additionally, our correlation analysis shows that the higher abundance of *Ruminococcaceae NK4A214* group bacteria may further promote albumin loss during CTx (r_s=-0.68; p_{adj}=0.015).

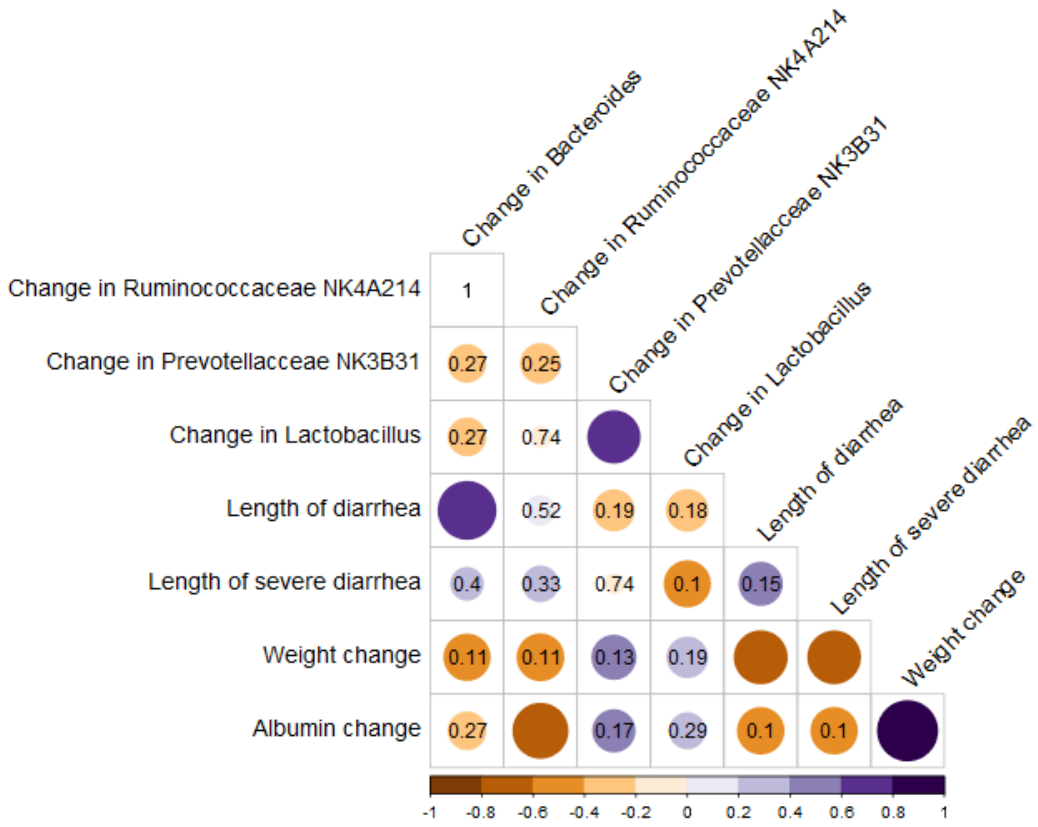

Figure 25. Correlation plot of changes in abundance of bacteria genera and clinical outcomes. Figure reproduced from reference (234).

DISCUSSION

Some results were discussed in a similar manner in Jakubauskas M et al (233,234).

According to global statistics, CRC ranks second in mortality and third in incidence malignancy worldwide (2). Around 15-25% of patients already have metastatic disease at the time of primary diagnosis and it is considered to be the major cause of death (59,60). The most common site for CRC metastasis is the liver (CRCLM), accounting for around 70% of cases (61). Surgical CRCLM removal, as for the primary CRC tumor, is considered to be the only curative option (75). Unfortunately, only around 20% of all patients diagnosed with CRCLM are candidates for radical treatment options at the time of diagnosis (1). For the other 80% of these patients, the main treatment option remains systemic CTx. During the administration of CTx, numerous adverse effects can occur, potentially involving every organ system and causing life-threatening conditions (118). Furthermore, CRCLM tends to eventually develop resistance to the administered CTx drug. In such cases, novel treatment additives are necessary to better control the disease and improve patient quality of life. The increased research interest in gut health and probiotics during the last few decades lead to promising findings for the treatment of various clinical conditions, including data on probiotics as a tool in CRC management. Currently, there are no studies directly examining probiotic effects on CRCLM, however, some indirect data indicates that the gut microbiome could influence the disease course. This dissertation thesis aimed to comprehensively assess the role of probiotic supplementation in CRCLM treatment.

Our study group developed this orthotopic CRCLM rat model and used it to experiment with different substance supplementation and CRCLM growth (237,243). We further improved this model and to our knowledge, it was the first ever published study investigating the effects of probiotics on CRCLM growth in an orthotopic CRCLM rat model.

In the past 20 years, probiotic use gained a lot of interest in the oncology field. One of the main research focus areas is cancer prevention and tumor growth inhibition. A lot of the currently published studies evaluate probiotics' effects in influencing local, intestinal CRC growth and only a few studies investigate the systemic anti-tumorigenic properties (195).

Tumor growth suppression

One of the dissertation research topics was to assess the effects of probiotic supplementation on the effectiveness of CTx for CRCLM. Results from the experimental animal study showed

that multispecies probiotic supplementation had a significant tumor-suppressing effect. The comprehensive histological tumor evaluation suggests that the tumor volume reduction was mostly attributed to the decreased tumor microvasculature.

Angiogenesis is crucial in providing CRCLM with nutrients and oxygen to sustain adequate growth (244). The tumor microenvironment which is comprised of different immune cells plays an important role in angiogenesis by modulating the inflammation caused by the invading tumor (245,246). Additionally, gut dysbiosis leads to increased gut permeability, which results in the activation of the immune system and chronic inflammation (247). The pro-inflammatory environment results in increased angiogenesis and escalated tumor growth. Probiotics may be beneficial in reducing tumor-induced inflammation in the liver as their use already showed promising results in controlling chronic inflammatory liver diseases (248).

Currently, only a few experimental studies reveal antiangiogenic probiotic properties. A study by Li et al. evaluates the effects of probiotic supplementation on tumor growth and tumor microvasculature (201). The study was conducted on mice and they used a unique probiotic mixture consisting of *Lactobacillus rhamnosus* GG, viable *E. coli* Nissle 1917, and VSL#3. Although this was not an orthotopic tumor model, their initial results indicated that probiotic supplementation inhibited subcutaneous hepatocellular carcinoma neoangiogenesis, thus decreasing volume. An extensive immunologic analysis showed that probiotics had a systemic anti-inflammatory effect, resulting in the downregulation of the proangiogenic IL-17 expression by the decrease of Th17 cell count. Additionally, the shotgun-metagenome sequencing identified several gut bacteria that produce anti-inflammatory metabolites, which affect the differentiation of T-cells, resulting in decreased Th17 cell counts. Similar findings were reported from a cell culture study by Liotti et al. indicating that *Lactobacillus rhamnosus* GG helps to retain epithelial homeostasis in the gut and inhibits angiogenesis in CRC cells by activating the formyl peptide receptor 1 (249).

Another possible liver tumor-inhibiting pathway was suggested by Ma et al. (187). They showed that changes in the gut microbiome, especially the decreased abundance of *Clostridium* species, impair the intestine bile acid metabolism resulting in increased primary bile acid concentrations. As the researchers determined, primary bile acids increase the accumulation and activation of natural killer T cells in the liver leading to inhibited primary and metastatic liver cancer growth.

The tumor immunohistochemical analysis in our study indicates that the probiotic supplementation primarily exerted tumor-growth suppressing but not cytotoxic effects. Tumor

apoptosis index was comparable between both groups not receiving CTx, however, the tumor proliferation index was lower in the probiotics group, although the difference, when compared to placebo, did not reach statistical significance. These results imply that the probiotic combination with CTx theoretically could act synergistically, as one acts by inhibiting angiogenesis and possibly tumor growth and the other by exerting cytotoxic properties. Unfortunately, we could not prove this hypothesis in our current study, but further research evaluating this theory is of interest.

CTx associated liver injury

The use of CTx agents is of paramount importance for the treatment of CRCLM. Unfortunately, CTx administration is associated with a variety of side effects. One of them is CALI, which is especially relevant in the management of CRCLM. There is a wide variety of possible hepatotoxicity that systemic CTx causes, however specifically steatosis, steatohepatitis, and sinusoidal obstruction syndrome are the most common ones when CTx agents for the treatment of CRCLM are used. One of the aims of this dissertation was to assess the hepatoprotective properties of probiotic supplementation during CTx for CRCLM.

Unfortunately, we could not provide sufficient results on this topic, as we did not observe any CTx-induced liver damage in our used CRCLM animal model. First of all, we did not observe any relevant liver injury marker (AST, ALT, and T-Bilirubin) changes, additionally, the initial histological evaluation of liver samples did not show any cellular alterations. After closely evaluating other, similar studies, it seems that chronic CTx agent exposure is needed to develop liver injury (250,251).

In an animal study, Robinson et al. managed to induce SOS by repetitively administering CTx agents (250). Their model relied on weekly administration of FOLFOX CTx drugs for 5 consecutive weeks. Distinct liver histological changes and elevated AST and ALT levels were seen in all FOLFOX receiving mice. Interestingly, they observed that a chow diet, which we also used in our study, seems to protect the liver from CTx-induced injury. Their study did not analyze how a chow diet protects the liver, however, they hypothesized that high concentrations of phytoestrogens in the chow diet were responsible for the hepatoprotection (252). Similarly, Keizman et al. established an animal model for CTx-associated steatohepatitis (251). After a pilot study using both oxaliplatin and irinotecan in mice, they determined that a 6 mg/kg weekly oxaliplatin dose for 4 weeks was most consistently associated with CTx associated steatohepatitis. In our study we used even higher CTx doses as in the two

aforementioned studies, however, no liver damage was observed, suggesting that the total toxic dose needs to be reached over time to induce CTx-associated liver injury.

CTx induced cardiotoxicity

The CTx regimens that are used to treat CRCLM are known to cause cardiotoxicity. Even up to 39% of patients receiving systemic treatment for metastatic CRC experience cardiovascular adverse events (164,165). Thus, one of the aims of this dissertation was to examine a possible negative influence of FOLFOX CTx on heart function and the potential protective effects of probiotics.

We did see a significant decrease of LVEF in both CTx receiving rat groups, indicating that cardiotoxicity caused by FOLFOX CTx may have occurred. Additionally, in our study we determined that rats pretreated with probiotics had a significantly lower decrease in their LVEF, suggesting that probiotics may have had cardioprotective properties. However, previous animal studies showed these findings can be confounded by other important variables. As Stohr et al report in their study, dehydration leads to a decrease in left ventricle filling, eventually lowering ventricle ejection volumes (253). Similar findings were observed by Watanabe et al (254). They showed that dehydration caused a decrease in venous return and ventricle filling and not the left ventricular function leading to reduced heart stroke volumes. It is very likely that the difference of LVEF in our study was also confounded by the aforementioned variables, as CTx receiving rats experienced severe diarrhea and dehydration. This was supported by heart immunohistochemical analysis that did not show any changes in the heart apoptosis index in rats that received CTx. Furthermore, there were no differences between rats that received placebo or probiotic gavage throughout the study. Additionally, the LVEF did not fall below 50%, which is considered a concerning threshold to cross.

Although it is reported that up to 39% of patients receiving systemic treatment for metastatic CRC experience cardiovascular adverse events the exact pathogenetic mechanisms and provoking factors are not fully understood (167). In our study, we did not see any heart structural or relevant functional changes, unfortunately, the reason for such results remains unclear. Maneikyte et al. used a very similar animal model to analyze the potential cardiotoxic effects of FOLFOX CTx (255). They reported that a single cycle of FOLFOX CTx can induce both functional and structural heart changes. As in our study, FOLFOX significantly reduced LVEF, reaching the median value of 72%, which is still high and hardly relevant. However, they also saw increased myocardial apoptotic activity after administering CTx. Interestingly, the

apoptotic index reached around 10%, which is high considering the robustness of heart tissue to CTx-induced apoptosis (256). These different and interesting findings, when compared to our study, could be partly explained by the different CTx agent doses used. We used half the dose of FOLFOX as Maneikyte et al. used, but as other authors argue, the single high dose of the CTx agent does not significantly increase the cardiotoxicity risk, thus we, could only hypothesize that other, unknown variables could be the cause of such between-study variability (167).

CTx associated intestinal mucositis

One of the most prevalent CTx side effects is IM. Its occurrence can range from 40 to 100% depending on the CTx drug and its administration (120,121). More severe cases of IM may lead to suboptimal cancer treatment as CTx doses may need to be reduced or delayed altogether (122). Additionally, as IM prolongs patient hospitalization, it significantly increases both resource and financial burden to the healthcare system (122).

The pathophysiology of IM is quite intricate and mainly involves five, in the introduction section detailed, phases. Although, this generalization helps to better understand the overall pathophysiology of IM, but different CTx regimens affect the gut barrier differently. Currently, only a few studies examine the impact of FOLFOX CTx on the development of IM (219,222,257). Therefore, one of the aims of this dissertation was to assess if probiotic supplementation reduces FOLFOX CTx-induced gastrointestinal symptoms.

The results of our animal study indicate a successfully established IM model. Additionally, the administered multispecies probiotic mixture managed to alleviate the severity and length of FOLFOX-induced diarrhea. These findings were further supported by the gut histological analysis, which showed initial CTx-induced gut damage and the subsequently increased cell regeneration in the probiotics group.

Usually, IM symptoms peak 3 days after, which coincides with the ulceration phase, and normalize 14-16 days after the administration of CTx drugs (123). The peak diarrhea incidence in this study was 96 hours and was in line with the commonly reported IM development timeframes. Chang et al. also analyzed the probiotic impact on FOLFOX-induced IM (219). In their study mice received various doses of *Lactobacillus casei* variety *rhamnosus* bacteria and the authors noted lower diarrhea severity scores in these mice, with the peak being reached 6 days after the first CTx agent injection. Different FOLFOX CTx injection timing and dosage could probably explain the delayed diarrhea peak in their study.

Weight loss is a common occurrence during CTx administration. It is very closely related to the presence of IM and is often associated with poor patient outcomes (258). Similar other studies also support our findings and report that probiotics, especially those containing *Lactobacillus spp.* and *Bifidobacterium spp.*, help to mitigate CTx-caused weight loss (221,227). Moreover, Bowen et al. demonstrated that the commercially available multispecies probiotic mixture VSL#3 can alleviate irinotecan-induced weight loss (216). As this study determined, probiotics retain body weight by preserving the intestinal barrier and reducing CTx-induced diarrhea. Additionally, weight loss, especially in acute cases, is often accompanied by hypoalbuminemia, which results in increased mortality and CTx failure rates (259,260). Our study was the first to report that multispecies probiotic supplementation could help to preserve albumin levels in a cancer model.

Histological evaluation of the ileum and colon tissue gave us information on the possible probiotic action mechanisms. FOLFOX CTx caused significant damage to the intestinal crypts and villi. Fortunately, it seems that the probiotic supplementation mitigated this injury and this is reflected by the retained ileum villi/crypt length ratio and lower colon crypt depth. These results are in line with the findings published by Chang et al. that were observed also in a CRC model (219). Additionally, Justino et al. and Yeung et al. saw almost identical results when administering 5-FU and various probiotic strains to mice (215,227).

A more in-depth immunohistochemical evaluation revealed the underlying processes that occur during intestinal injury. Analysis of samples stained with anti-MPO antibodies brought some unexpected results. The percentage of anti-MPO positive cells was significantly lower in both CTx groups and also for both the ileum and colon. This result seems contradictory, as various inflammatory cells play an important role in the initiation and development of IM (131). This result could be mainly affected by the fact that tissue samples were obtained 6 days after the first CTx dose, when IM shifts towards the regeneration phase, especially in rats (129). Moreover, CTx caused severe leukopenia theoretically leaving fewer neutrophils for tissue infiltration. A crucial event for IM development is the loss of epithelial cells, namely by apoptosis, which we objectified by using the Caspase-3 staining (129). The analysis data indicate that the probiotics supplementation decreased the apoptosis rate in the ileum, but no effects were seen in the colon. Due to around 10 times higher spontaneous apoptosis rates in the small intestine compared to the colon, the small intestine is more prone to damage caused by CTx (127). Additionally, we showed that probiotic administration significantly increased ileum and colon epithelial cell regeneration. As other researchers report, several different

probiotic strains have also shown intestine healing effects in CTx-associated IM models (216,219).

CTx drugs drastically transform the gut microbiome (261). These changes usually consist of a decrease in microbiome diversity and a relative abundance surge of *proteobacteria* (262–264). We did a microbiome correlation analysis, which showed that the increased relative abundance of *Bacteroides* was closely correlated with prolonged diarrhea. Data on this finding in other articles is very inconsistent as some authors report an increase and others a decrease of *Bacteroides* group bacteria abundance after CTx administration (262,263). It is known that *Ruminococcaceae NK4A214* group bacteria produce short-chain fatty acids, thus causing an anti-inflammatory effect in the bowel (265–267). However, findings from our study were contradictive showing an opposite effect that the higher abundance of *Ruminococcaceae NK4A214* group bacteria did correlate with a bigger loss of albumin during CTx administration. We did not find any other articles that would support this result, therefore it should be critically reinvestigated in a future study.

Safety

The use of probiotics for the general population is usually considered safe for most of the strains and this is acknowledged by regulatory agencies (268). However, concerns regarding probiotic use in certain populations such as neonates, intensive care unit, or immunocompromised patients are still debatable (190). The main concern is that probiotic supplementation can promote bacteriemia and fungemia, which could lead to septic complications, due to increased gut permeability in the aforementioned health conditions. As a high-volume systematic review concluded, this issue is hard to address scientifically, as the safety data is usually very poorly reported (269). Gathered data by Nieuwboer et al. indicate that adverse events do not occur more frequently in immunocompromised patients, however, the author also raised concerns that probiotic safety lacks appropriate regulations and standard reporting guidelines (270). Although this study was not designed to detect bacterial translocation, but during the execution of our study we did not witness any severe complications or deaths that could be related to probiotics use. Additionally, there are concerns that probiotic use in the presence of gut dysbiosis could lead to persistent and long-lasting gut colonization with probiotic bacterial strains (271). This is important as such persistent probiotic-induced dysbiosis leads to an elevated risk of infectious diseases and even possibly non-infectious diseases such as diabetes, asthma, obesity, allergies, or inflammatory bowel disease (272–275). Further research on this topic could be very impactful as currently, we do

not have enough data to explain the possible long-term probiotic effects, especially in vulnerable populations.

Strengths and weaknesses of the study

The main strength of this study was the employed tumor model. It was the first-ever published study investigating the effects of probiotics on CRCLM growth in an orthotopic CRCLM rat model. This allows to establish a more realistic tumor model, as the proper tumor microenvironment can develop. For example, Li et al. performed a very detailed mechanistic study evaluating the effects of probiotics on hepatocellular cancer growth in a subcutaneous tumor model (201). In the discussion section of the study, they acknowledged this drawback admitting that the subcutaneous model does provide a realistic tumor model. They planned to further extend their study using an orthotopic liver cancer model, however, no follow-up study has been published to date.

This study also had several limitations that should be addressed. First of all, this whole project failed to generate enough sufficient data to adequately address all the research questions that were raised during the conception of the study. However, after the data analysis, it seems that all of them are hardly achievable within a single animal study due to the different pathogenesis of all these CTx-associated complications that were discussed previously.

Moreover, we were unable to perform the full in-depth gut microbiome analysis that was initially planned. The main limiting factor was the observed differences in microbiome composition between the groups upon baseline evaluation and the small sample size did not allow to correct for them. Due to all the technical issues that we encountered; we could not achieve any high-quality definitive results from the microbiome analysis. Fortunately, we were able to adapt our workflow by normalizing the relative abundance data and performing correlation analysis. Although this limits the interpretation of the findings, however, we were able to determine a few bacterial groups that could be attributed to prolonged diarrhea and blood albumin concentration changes. We did go through all the possible variables that could influence such microbiome deviations upon baseline, however, we failed to identify a single one of them that would at least partly explain such deviations at the start of the study. In future studies, we are planning to prolong the animal incubation period before the start of the study to even out their baseline microbiome.

Lastly, this study relied only on an animal model, and the results were not further tested in other species. As Lazaris et al. published in their study, animal models do not fully represent

the diversity of liver metastasis histopathological growth patterns that are known to be present in humans (276). Additionally, the innate and adaptive immune systems differ between humans and rats, thus contrasting interactions could occur between the administered probiotics and the hosts gut microbiome and immune systems (277). But it should be noted that rat models are superior to the more popular mouse models as rats have a more similar immune system to humans than mice do (277). Furthermore, exposure to environmental factors and dietary habits, which are very important for microbiome changes, differ drastically between rats and humans. Nevertheless, as the mucositis study group points out, animal studies are still relevant in initially evaluating the general microbiome change trends after intervention and determining the beneficial effects of certain overlapping features (131). Both successful and unsuccessful probiotic effect translation from rodents to humans have been published, further supporting the use of animal models for probiotic research, but results should be interpreted critically (278,279).

Future perspectives and dilemmas

Probiotic and gut microbiome research lately became very popular, however, few controversies are still not answered and new research directions have been proposed.

Current literature knowledge indicates that gut microbiome manipulations can be beneficial for cancer treatment, especially immunotherapy, effectiveness. This is especially relevant for CRC with MMR gene mutations as extremely promising data has being published with the use of anti-CTLA-4 and anti-PD-1 therapies (280). However, the changes in the microbiome structure should be done very cautiously, as it could result in both increased and decreased tumor response to immune checkpoint inhibitors (281,282).

Such findings, further promote research to identify the ideal microbiome structure to achieve the best CTx response. Several clinical trials, with promising initial results, are exploring the effectiveness of fecal microbial transplantation to modulate the unfavorable (low diversity, unfavorable species) microbiome to achieve better immunotherapy response (283). Whole microbiome transplantation is quite an aggressive approach to the problem with several shortcomings, such as ideal donor identification, and transplantation regimen determination (284). To overcome these issues new generation targeted microbial interventions are being developed.

More and more effort is put into the development of personalized probiotics, that would go beyond the commonly used *Bifidobacterium spp. and Lactobacillus spp.* Several bacterial such

as *Clostridium butyricum, Akkermansia muciniphila, Enterococcus gallinarum,* or *Escherichia coli* Nissle 1917, showed beneficial effects in cancer therapeutics, but these results should be further proven in future cancer trials. On the other hand, targeting some bacterial strains, such as *F. Nucleatum*, that are associated with increased CRC risk could also yield beneficial health effects (185).

To further generate valuable data in probiotic research more attention should be also given to proper study planning and reporting. There is a need for more standardized studies (sample collection, sequencing and analysis protocols) that would allow us to better determine the causal effect of certain bacterial strains on cancer treatment. Furthermore, most of the probiotics studies focus on fecal microbiome analysis, which mostly accounts for the microbiota in the terminal large bowel, however, the primary interaction between the gut microbiota and the host is expected to occur at the small intestine's ileal mucosa, where the majority of the local immune system resides (285). Moreover, the potential role of microorganisms other than bacteria, such as fungi, viruses, and protozoa, is understated and should be better accounted for (286).

Conclusions

In conclusion, our study was able to demonstrate that multispecies probiotic supplementation significantly inhibits CRCLM angiogenesis and decreases tumor growth in an experimental animal model. Additionally, our research shows that probiotic supplementation can alleviate FOLFOX-induced IM symptoms and preserve both weight and blood albumin levels. These findings should be further evaluated in the upcoming studies, to determine in-depth molecular action mechanisms and better determine underlying microbiome changes. Although, we aimed to examine the effects of probiotic supplementation on CTx-induced liver and heart injuries, but our study was unable to generate sufficient data to reach these goals.

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