UNIVERSITA' DEGLI STUDI DI CATANIA
DEPARTMENT OF BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES
International Ph.D. in Basic and Applied Biomedical Sciences
XXXIV Cycle

Ph.D. Thesis

ANGELO AMICO

Maintenance of normal gastro-intestinal function with dietary supplement containing *Lactobacillus rhamnosus* GG in patients treated with abdominal or pelvic radiotherapy e/o chemotherapy: Clinical trials

Ph.D. Tutor:
CHIAR.MO PROF. ANTONIO DI CATALDO

Ph.D. Coordinator:
CHIAR.MA PROF. STEFANIA STEFANI

ACADEMIC YEAR 2020-2021

TABLE OF CONTENTS

1. INTRODUCTION

"We are what we eat" is a commonly used phrase, which summarizes the complex interactions between the outside world and the inside of our body in a minimalist form; interactions that often result in benefits for our body, but sometimes represent the basis of the onset of our diseases, including neoplasms (1). The exogenous contribution of the surrounding environment related to the onset of neoplasms is enclosed in the term exposure which can be defined as the cumulative measurement of entire exposures such as dietary factors, drugs, exposure to infectious agents, UV radiation or environmental toxins / pollutants, behavioural and socio-economic factors and their associated biological responses throughout the individual lifespan (2). The cumulative risk depends on: (1) dose, time and duration of exposures; (2) the specific combination of individual exposures with each other; (3) the interaction of such exposures with the individual genetic and epigenetic background (3). In recent years, a number of studies have shown that microorganisms that colonize exposed body surfaces are key determinants of host health maintenance, as well as causative agents of many diseases, including malignant tumours (4). Such colonizing microorganisms or microbiota can be considered part of our internal exposome (5). Among the microbial populations, the gastrointestinal microbiota is the most studied, given its great influence on host homeostasis (6). The intestinal microbiota includes a large population of microorganisms that inhabit the gastrointestinal tract, in particular the large intestine. They are mainly prokaryotes, but also viruses and fungi. Overall, the gut microbiota can be considered as a factor that we are exposed to in high doses throughout life (7).

The intestine represents the interface between the intestinal microbiota and the human body. The gut microbiota performs a number of vital functions (8):

• Production or transformation of molecules modulating host metabolism.

• Maintaining the integrity of the intestinal barrier.

• The metabolism of xenobiotics.

• Protection against gastrointestinal pathogens.

• Modulation of the host's immune system.

Notably, some commensal bacteria produce essential micronutrients, including vitamin K and vitamin B. Additionally, a number of intestinal commensals can transform amino acids into signalling molecules, such as glutamate into gamma-amino butyric acid (GABA) or histidine to histamine. It should be emphasized that intestinal commensals can secrete the so-called short-chain fatty acids (SCFA), derived from the bacterial fermentation of dietary fibres. Once released in the intestine, SCFAs are absorbed and transported to the liver where they are used as an energy source. Furthermore, SCFAs play a role in controlling glucose and lipid metabolism (9). A stable intestinal microbial balance plays a key role in the proper fulfilment of all these fundamental metabolic functions. Any imbalance in this delicate balance can lead to an altered microbiota, a condition called dysbiosis, linked to several human diseases, including cancer (10). The gut microbiota population genome represents the gut microbiome, encoding 100 times more genes than the human genome. In the last decade, the advent of metagenomics, which combines next-generation sequencing (NGS) with

computational analysis of 16S rRNA, has allowed the characterization of both diversity and abundance, typical of the gut microbiome (11). These functional studies are now helping to elucidate the true impact of microbiome architecture on human health (12). Recent experimental work has established for the first time a complete collection of the human gut microbiome, consisting of over 200,000 non-redundant genomes from 4,644 gut prokaryotes, which allow for future use as a reference in metagenomics studies (13). Given the close interconnection between the intestinal microbiome and the human host, it is of fundamental importance to analyse host-related variables such as physiology, lifestyle habits and diet, in order to increase both the robustness and the reproducibility of the metadata analysis (14). This will help identify members of the gut microbiome that are directly associated with human disease, including carcinogenesis and host response to anticancer therapy (15). Links have been found both with local gastrointestinal tumours and with other tumours in other sites (7). Recently, the number of studies and projects, such as the ONCOBIOME project (16) that shows correlations between the gut microbiome and disease has enormously increased. From this, derives an interesting and wide potential for use and modulation of the intestinal microbiome in the health sector (17).

1.1 Microbiome-cancer interactions

In the light of these evidences, intestinal bacteria could favour the onset of tumours in two different ways: a first mechanism involves the activation of the TLR signalling pathway, which leads to chronic inflammation of the gastric mucosa, in turn, linked to increased risk of cancer; a second mechanism, on the

other hand, involves the intestinal microbiota activated by metabolism, capable of producing toxins with a direct pro-carcinogenic effect or enzymes capable of activating carcinogens ingested with the diet (18,19). The microbiota performs important immune functions and metabolic functions by also regulating some inflammatory cytokines involved in the transcription pathways. Indeed, when the host's microbial communities are in perfect balance with each other, the production of anti-inflammatory and pro-inflammatory cytokines is equally balanced, while variations in the number, diversity and stability of commensal bacteria can shift this balance towards a pro-inflammatory phenotype.

The increased production of cytokines, including TNF, IL-1 and IL-17, together with the activation of TLRs by some pathogens, causes the activation of the NF-kB signal, a transcription factor of various anti-apoptotics, which induce cell proliferation and an increase in angiogenesis processes and therefore it is able to support oncogenesis (18,19).

In accordance with this, several studies have investigated the effects of dysbiosis and dysmetabolism on tumourogenesis:

• Fusobacterium nucleotum promotes infections and inflammation of the colorectal mucosa by inhibiting the host's NK cells (20, 21, 22, and 23)

• Escherichia Coli during inflammatory states in the presence of IL-10 is protected by the same oxidative through over-expression of the heat shock proteins Ibp A and B, releasing the toxins colibactin and CDT (24, 25)

• Helicobacter Pylori and Bacteroides Fragilis cause damage to the Ros-DNA induced through the activation of the host's stermin-oxidase (23, 26 and 27)

• Enterococcus Faecalis causes DNA mutations through the production of extracellular superaxis and oxygen-derived species

• Shigella Flexneri by inducing the degradation of p53 in host cells interferes with the DNA damage repair pathway (28,29)

• Clostridium Leptore and Coccoides through the production of the enzyme β-glucuronidase promotes the activation of estrogen receptors and cell proliferation in estrogen-sensitive tissues (30,31 and 32)

1.2 Probiotic-cancer interactions

The complicated interactions between the intestinal microbiota and the host are expressed in a bidirectional relationship in which some components of the microbiota are closely connected to the phenomena underlying the development of diseases and carcinogenesis; other components may have this role if certain environmental conditions exist, sometimes favoured if not determined by their actions as well as other components capable of determining the well-being and protection of the organism (33). This class includes probiotics, that is, live non-pathogenic microbes, which play a central role in the health of the host by strengthening the intestinal ecosystem when administered in sufficient quantities.

Commensal microorganisms have certain characteristics to be called probiotics; that is, they must be (13):

• Safe for human administration; the European Food Safety Authority (EFSA) defines as safe those bacterial species that are not carriers of acquired and / or transmissible antibiotic resistance.

• Active and vital in the gastrointestinal tract, in quantities that justify any benefit

• Able to persist and multiply in the human intestine

• Able to confer physiological benefits observable through studies

• Capable of adhesion to the cells of the mucosa or epithelium

• With antimicrobial resistance and resistance to hydrolase of bile salts (34)

• Endowed with antagonistic activity against pathogens,

• Capable of mediating immunostimulation and modulation, antimutagenic and anticarcinogenic activities (35)

On the basis of the foregoing, for example Lactobacilli, Lactococci, Bifidobacteria, Enterococci, including their bio-products, have such properties that they can be defined as probiotics (36).

Table 1 shows several in vitro studies on the anticancer effects of live and dead probiotics and their active metabolites.

Table 1. Preclinical studies of the efficacy of probiotics on the modulation of the intestinal microbiota in oncology

Author	*Probiotic*	*Target*
M.Thirabunyanon et al. (2009)	E.Faecium RM11 e L. Fermentum RM 28	CRC
Y. Rahbar et al. (2020); L. Wang et al. (2019)	Mix di Bifidobacterium e Lactobacillus	Pelvic neoplasms
M. Mego et al. (2015)	Mix 10 probiotics	Metastatic CRC
G.E.Theodoropoulos et al (2016); M.L. Consoli et al. (2016); A.A. Hibberd (2017); A.T Flescch et al. (2017)	Mixture	CRC
H.A. Lee et al. (2015)	L. Plantarum	CRC
F. Maghsood et al (2020)	Polysaccharide, protein secretory, nucleic acid macromolecules of L. Reuteri	CRC
T.L. Bedadaa et al.(2020)	LPS, EPS	Metastatic CRC
T. Cd et al. (2007)	SCFAs	CRC
N.M. El-Debb et al. (2018)	EPS 20079 of L. Acidophilus	CRC
Y. Rahbar et al. (2020)	EPS, MCF 7	CRC, head and neck squamous carcinoma, pancreatic tumour
L.D.Lagodicgossmann et al. (2007)	SCFAs of Propionibacteria	CRC
I. Kahouli et al. (2016)	L. Reuteri	CRC
J. Escamilla et al. (2012)	Cell-free supernatan of L. Casei and L.R GG	Metastatic CRC
M. Pancione et al. (2019)	MPL A of Salmonella	CRC
L. Giannotti et al. (2010)	L. Jonhsoni	CRC

Lactobacilli are part of the lactic acid bacteria family and derive almost all of their energy from the fermentation of glucose and lactose into lactic acid, generating ATP through the non-oxidative phosphorylation of the substrate. Lactobacilli and their pro-bioactive cellular materials (LPS, MPL A) are known to produce several beneficial effects in the gastrointestinal tract and release several enzymes that establish potential synergistic effects on digestion:

• Improve the state of lactose intolerance thanks to the production of the enzyme β-galactosidase, capable of breaking down lactose into glucose and galactose, which are better digestible (37)

• Effectively block antibiotic-associated diarrhoea, a pathological condition caused by alterations in carbohydrate metabolism, with reduced absorption of short-chain fatty acids and consequent osmotic diarrhoea (38, 39 and 40)

• Valid adjuvant therapy in many gastrointestinal diseases such as irritable bowel syndrome, lymphomas and obesity, caused by the alteration of the microbiota (41 and 42)

• modulation of immune responses mediated activation of the reticule-endothelial system, increase of cytokine pathways and regulation of interleukins and tumour necrosis factors and activation on the cell surface of the host of TLR-4 correlated to the activity of the response mediated by T cells (43 and 44)

• An antimutagenic effect, presumably due to the ability to bind heterocyclic amines, which, after cooking the meat, are carcinogenic products (45)

• Antitumour activity through: binding, degradation and inhibition of the mutagen; pro-carcinogenic prevention and conversion of harmful, toxic and highly reactive carcinogens; lowering of intestinal pH by short-chain fatty acids (SCFA) formed during the breakdown of non-digestible carbohydrates; host-modulation and enhancement of innate immunity through the secretion of anti-inflammatory molecules (46)

For what has been said, it is not surprising that the scientific path pursued is aimed at finding potential probiotic strain-doses of effective administration and molecular mechanisms of cancer prevention and treatment. Radiotherapy, chemotherapy and immunotherapy treatments can all modify the microbiome of patients but, at the same time, the composition of the microbiome has the great potential to profoundly influence patients' response to such therapies (47). In fact, it is known that interventions on the microbiome could be fundamental for improving the toxicity related to anticancer therapy, as well as improving the efficacy of the therapy itself (48 and 49). In particular, the regulation of the therapeutic outcome is strictly connected with the ability of the intestinal microbiota to metabolise the antitumour compounds to modulate the host's immune response and inflammatory pathways (50). In fact, the ability of probiotics in modulating the composition of the gut microbiota has shown that they are useful for the safety of traditional anticancer therapies such as chemotherapy and radiotherapy (51). Although chemotherapy, immunotherapy and radiotherapy are the mainstays of currently available anticancer treatments, these same treatments can cause adverse side effects in patients (52, 53, 54, 55,

56, 57 and 58). The purpose of administering probiotics to cancer patients, mainly lactobacilli, is to:

• Repopulate the intestinal microbiota of compromised patients, thus restoring the levels and functionality of commensal bacteria

• Decreasing the risk and severity of such anticancer treatments, diarrhoea and mucositis (51, 59, 60 and 61).

Conversely, possible side effects of administering probiotics in immunocompromised patients are reduced to (62):

• Risk of opportunistic infections

• Transfer of antibiotic resistance

1.3 Lactobacillus Rhamnosus GG in anticancer therapy

Lactobacillus Rhamnosus GG (LGG) occupies a prominent place in the wide range of probiotics thanks to its anti-inflammatory properties within the intestinal microenvironment and its elective activities on the host's immune system; therefore it is one of the most studied and well characterized both, in in vitro and in vivo studies, specifically in oncology (4, 63, 64 and 65). Gram positive bacterium of the lactic acid bacteria family was isolated in 1985 by Gorbach and Goldin who patented it in 1989 (65 and 66).

LGG is endowed with all the peculiar characteristics of a perfect probiotic (67):

• Resistance to gastric and biliary juices

• Strong adhesive properties to intestinal cells thanks to the presence of a surface molecule called SpaC that binds the kitchen creating biofilm (68)

• Production of antimicrobial substances against anaerobic bacteria

Consistent with animal model studies that have demonstrated a favourable effect of LGG in maintaining intestinal microbiota balance and intestinal epithelial barrier function when administered as an adjuvant to 5-FU chemotherapy and radiotherapy, numerous focused clinical trials are currently underway on establishing the role of LGG supplementation in preventing or limiting the toxic effects of anticancer therapies (7). Regarding LGG's ability to counteract cancer growth, it is capable of exerting antiproliferative or antimetastatic effects (69,70,71,72,73), probably through the direct modulation of different host proliferation pathways, including mTOR or WNT, as has been highlighted in several in vitro tumour models (74,75). While LGG has been shown to affect the host's immune system, killing newly developed cancer cells, in a rat colon cancer model (76). Indeed, LGG can trigger the immune system response even within the normal untransformed intestinal epithelium, thus protecting it from inflammation, which can support the formation of an environment conducive to the genesis of tumours. (77) Overall, currently, LGG it is a suitable candidate to be further characterized as a possible adjuvant in integrated anticancer therapies and above all deserving of further studies to support its candidacy as a direct cancer modulator. The table shows the preclinical studies on the use of LGG in oncology.

Effect mediated by LGG	Experimental model	Target Cell	Tumour	Year
Anti-proliferative effect on tumour cells	cell cultures	tumour cells	Colorectal	2016
Anti-inflammatory and anti-proliferative effect on tumour (DMH model)	rat	tumour cells	Colorectal	2016
Anti-metastatic and anti-proliferative effect on tumour cells	cell cultures	tumour cells	Colorectal, Cervix	2016
Modulation of mTOR and Wnt / β-catenin pathways in tumour cells	cell cultures	tumour cells	Colorectal, Cervix, Breast	2016
Prevention of polyp formation in the colon (APC / min model)	mouse	tumour cells	Colorectal	2017
Secretion of p40 (bacteriocin) which upregulates APRIL and IgA production in intestinal epithelial cells	cell cultures; mouse	tumour cells, normal cells	Colorectal	2017
Colitis-associated cancer reduction (DMH model)	mouse	tumour cells	Colorectal	2018
Reduction of tumour growth in combination with celecoxib	rat	tumour cells	Colorectal	2018
Ag-LGG nanoparticles induce apoptosis of cancer cells	cell cultures	tumour cells	Colorectal	2020
Reduction of tumour growth through stimulation of CD8 T cells	mouse	tumour cells	Colorectal	2021

Increased anti-tumour activity of anti-PD-1 (stimulation of IFN-β production by DCs)	mouse	tumour cells	Colorectal, Melanoma	2021
Alleviation of NLRP6 inflammasome in the intestine	pig	normal cells	n.a.	2017
Reduction of 5-FU-induced cytotoxicity selectively in normal cells	mouse	tumour cells, normal cells	Colorectal	2018
Gene expression change in intestinal cells (anti-inflammatory profile)	cell cultures	normal cells	n.a.	2018
Selectively anti-inflammatory effects on normal (non-cancerous) cells	cell cultures; mouse	normal cells	n.a.	2018
Protection of the intestinal barrier and intestinal eubiosis	pig	normal cells	n.a.	2018
Selective protection of normal cells from damage induced by radiotherapy	mouse	tumour cells, normal cells	Colorectal	2019
Secretion of p40 (bacteriocin) induced by intestinal epithelial cells	cell cultures	normal cells	n.a.	2019
Prevention of chemotherapy induced hepatotoxicity	rat	tumour cells, normal cells	Colorectal	2021

In addition, some of the most authoritative clinical studies on the use of the effects of LGG for therapeutic purposes in neoplastic pathology are reported below.

Study Code	Tumour	Microbiome modulation	Treatment	Outcome	Year
NCT01410955	Colorectal	Probiotics (B short, B bifidum, B longum, B infantis, L acidophilus, L brevis, L casei, L rhamnosus, L plantarum, S thermophilus)	Irinotecano	Microbiota modulation; Gastro-intestinal toxicity; Adverse events	2011
NCT03742596	Colorectal	Probiotics (L rhamnosus, L acidophilus, L reuteri, L paracasei, L casei, L gasseri, L plantarum, B lactis, B breve, B bifidum, B longum, B infantis)	Radiotherapy	Microbiota modulation; inflammatory markers; Gastro-intestinal toxicity; Adverse events	2018
NCT03705442	Colorectal	Probiotics (L acidophilus, L Rhamnosus, L salivarius, L plantarum, L paracasei, E faecium, B bifidum, B lactis, B longum)	Chemotherapy (FOLFIRI)	Microbiota modulation; inflammatory markers; Gastro-intestinal toxicity; Adverse events	2018

NCT03140878	Healthy subjects at risk of colorectal cancer	Probiotic (L Rhamnosus GG)	n.a.	Microbiota modulation	2018
NCT04874883	Colorectal; Head-neck	Synbiotics (L casei, L Rhamnosus, L Acidophilus, B bifidum; fructooligosaccharide)	Surgical resection	Microbiota modulation; Gastro-intestinal toxicity; Adverse events	2019
NCT01790035	Colorectal; Stomach	Probiotic (L Rhamnosus GG)	Chemotherapy; Radiotherapy	LGG safety and tolerability; Gastro-intestinal toxicity	2020
NCT02819960	Colorectal	Probiotics (L Rhamnosus GG; B short)	Irinotecano	LGG safety and tolerability; Gastro-intestinal toxicity	2021

2. AIM OF THE STUDY

Currently the clinical uses and indications for administration of LGG are (78, 79, 80 and 81):

• Treatment of infectious diarrhoea associated with antibiotics, traveller's diarrhoea, diarrhoea and C. difficile colitis

• Adjuvant of immunodeficiencies and allergies

• Obesity

• Respiratory infections

• Irritable bowel syndrome

On the basis of this and encouraged by the numerous studies in favour of the effects of prevention and reduction of gastrointestinal toxicity deriving from radio-chemo and immunotherapy treatments, as well as by the possible antiproliferative and antimetastatic effects, the purpose of this study is to evaluate the effectiveness of daily oral administration of Lactobacillus Rhamnosus GG in the maintenance of normal gastrointestinal function in patients undergoing abdominal-pelvic radiotherapy and cytotoxic chemotherapy. Therefore:

• Primary end-point: assessment of grade 3-4 diarrhoea event rate

• Secondary endpoints: diarrhoea event rate of all grades

Hospitalization rate due to gastrointestinal toxicity

General toxicity rate

Compliance with oncological treatment

Plasma modification in mRNA profiling

Modification of the intestinal microbiome

The expected clinical impact involves the determination of the epigenetic changes induced by treatment with LGG in the modulation of the intestinal microbiome and in the modulation of the expression levels of specific miRNAs associated with inflammatory processes and the patient's response to the treatments administered to them

3. METHODS

3.1 Study design

Task 1: effect of LGG on in vitro models of colorectal carcinoma (CRC)

In the first phase, the effects of LGG on cell viability, apoptosis and cell proliferation were analyzed in in vitro models of CRC (CaCO-2, HT-29, HCT-116) by carrying out cell-bacterium co-culture or treatment with mediums conditioned by LGG. Furthermore, the expression levels of proinflammatory cytokines involved in the activation processes of the immune system (IL-6, IL-10, IL12 p40, IL17A, IL-22, IL-23 p19, TNFα) were evaluated. The cell viability assay by MTT assay and the use of cell counting chambers with vital dyes (Trypan blue) were carried out from the different cultures, respectively; the study of changes in apostolic processes through the use of staining with Propidium Iodide and flow cytometric analysis using Amnis Flow Sight Technology and Western Blot. Finally, the cell proliferation assay was carried out by means of a bromodeoxyuridine (BrdU) assay.

Task 2: validation of in vitro results in mouse models of CRC

With the authorization of the Catania Ethics Committee 1, this phase was not carried out as it was a parapharmaceutical already on the market (Dicloflor Plus, UNIFARM).

Task 3: Enrolment of cancer patients for the analysis of primary and secondary endpoints after daily oral administration of lactobacillus Rhamnosus GG. This phase is the subject of this paper.

3.2 Analytical workflow

As part of the collaboration established between the BIOMETEC Department of the University of Catania and Dicofarm SpA, two clinical studies were proposed and launched, respectively called LRadio / 10/2017 and LRchemio / 10/2017, with the aim of determining the effect of administering the probiotic Lactobacillus Rhamnosus GG (LGG) in reducing the gastrointestinal adverse effects induced by chemotherapy and radiotherapy anti-tumour treatments, respectively. Objective pursued by hitting the primary and secondary endpoints set out above.

The dietary supplementation, in the form of buccal sachets 10x109 u.f.c., contains the probiotic Lactobacillus Rhamnosus GG (ATCC53103); other components present in the formulation are: erythriol, xylitol, natural flavours, citric acid, magnesium salts of fatty acids, silicon dioxide, steviol glycosides.

The study consists of a multi-centre, randomized, placebo-controlled, double-blind trial of an initial duration of 18 months in patients undergoing chemotherapy and / or radiotherapy for primary abdominal-pelvic neoplasia. Due to the global emergency Covid-19 and the consequent slowdown, up to real periods of blocking of the study, the initial duration was extended up to 30 months with the end of the study for the radiotherapy protocol in February 2021. Currently it is not to be excluded a possible reopening of the enrolment phase, according to the will of UNIFARM Spa

The study centres involved in patient enrolment are:

- The Radiotherapy Operational Unit of the Cannizzaro Emergency Hospital

- The Radiotherapy Section of UOC Radiology 1 of the AOU Policlinico G.Rodolico-San Marco of Catania

- The Medical Oncology Division of the Cannizzaro Emergency Hospital,

- The Complex Operating Unit of Medical Oncology of the San Vincenzo Hospital in Taormina

- The Medical Oncology Unit of the AOU Policlinico G. Rodolico-San Marco of Catania.

As regards the number of subjects, considering that in previous studies the rate of grade 3-4 diarrhoea event was respectively 0.37 in patients treated with placebo and 0.22 in patients treated with probiotic among those undergoing chemotherapy; and 0.54 in patients treated with placebo and 0.37 in patients treated with probiotic among subjects undergoing radiotherapy with abdominal-pelvic irradiation; furthermore, considering a drop-out of 10%, the minimum number of subjects to be enrolled, for each study, is 128, randomized with a 1: 1 ratio through an appropriate randomization list.

Patients were selected according to compliance with the inclusion and exclusion criteria:

1. LRadio / 10/2017 inclusion criteria:

• Male and female patients over 18 years of age

• Patients receiving abdominal or pelvic RT with genitourinary and gastrointestinal tract cancers

• Characteristics of the radiant treatment:

- Type of radiation: photons X 6-18 MV

- Target volume: abdomen, pelvis

- Radiation fields: multiple, coplanar, static and dynamic

- Techniques: 3D-cRT, IMRT, VMAT

- Organs at risk: small intestine V45 <195cc, rectum V50 <50%, V65 <25% according to QUANTAC

• Performance status 0-2

• Patients capable of understanding the full nature and purpose of the study, including possible risks and side effects

• Subjects available for the entire study period by providing informed consent

2. Inclusion criteria LRchemio / 10/2017:

• Male and female patients over 18 years of age

• Cancer patients to undergo cytotoxic chemotherapy and / or target therapy at high risk of diarrhoea (fluoropyrimidine chemotherapy with / without monoclonal antibodies, taxanes, irinotecans, tyrosin kinase inhibitors)

• Performance status 0-2

• Patients capable of understanding the full nature and purpose of the study, including possible risks and side effects

• Subjects available for the entire study period by providing informed consent

3. Exclusion criteria LRadio / 10/2017:

• Known or potential hypersensitivity to one of the components of the food supplement and / or history of allergic reaction in general

• Patients receiving RT with electrons

• Small volume irradiation (5-10cc)

• Unconventional radiotherapy fractions

• Stereotaxic radio-surgery

• Lack of QUANTEC criteria

• Any evidence of severe or uncontrolled systemic disease including uncontrolled hypertension and active haemorrhagic diathesis

• Treatment with any antibiotic therapy performed within 10 days of the first administration of LR GG

• Women with potential for pregnancy (i.e. who have not been surgically sterilized or in menopause for less than 1 year)

• History of alcohol or drug abuse

• History of IBD

• Intestinal malabsorption syndromes

• Presence of enterostomy

• Concomitant consumption of other probiotics

• Any other significant disorder which, in the judgment of the investigator, could affect study participation or alter study results

4. Exclusion criteria LRchemio / 10/2017:

• Known or potential hypersensitivity to one of the components of the food supplement and / or history of allergic reaction in general

• Any evidence of severe or uncontrolled systemic disease including uncontrolled hypertension and active haemorrhagic diathesis

• Treatment with any antibiotic therapy performed within 10 days of the first administration of LR GG

• Women with potential for pregnancy (i.e. who have not been surgically sterilized or in menopause for less than 1 year)

• History of alcohol or drug abuse

• History of IBD

• Intestinal malabsorption syndromes

• Presence of enterostomy

• Concomitant consumption of other probiotics

• Any other significant disorder which, in the judgment of the investigator, could affect study participation or alter study results

The enrolment, after obtaining appropriate written informed consent, provided for the delivery of the dietary supplement in the study and the self-administration, by the patients, of 2 daily buccal sachets, from the first day of treatment until the end of the oncological treatment. According to the randomization list, each patient was administered the product according to the BATCH number 8932412 or 8932410.

The evaluation of the study objectives was carried out by taking blood biological material (1 tube of serum of 8.5ml, 2 tubes of blood count of 4 ml) and fecal at baseline, i.e. before the start of treatment and at the end of treatment for patients undergoing radiotherapy; at baseline and three months after treatment for patients receiving chemotherapy only. All the samples collected were received in the laboratories of the Department of Biomedical and Biotechnological Sciences of the University of Catania where they were processed as follows:

- Faecal samples are aliquoted into 1.5 ml tubes, each containing approximately 250 mg of sample. The aliquots obtained are stored at -80 ° C with the exception of one which is immediately processed using the QIAamp PowerFecal Pro DNA Kit in order to extract the fecal bacterial DNA to be analyzed in NGS to determine the intestinal microbiota before and at the end of the treatment with LGG. The quantity of DNA obtained so far from the fecal samples extracted varies from 12 to 50 ng / uL, a quantity sufficient to carry out the subsequent analysis in NGS.

- The blood samples are centrifuged at 2000 x g for 10 minutes in order to separate the various blood components. 4 serum cryovials of 1 ml each are

recovered from the serum tube. From the two blood count tubes, 4 plasma cryovials of 1 ml each, 2 cryovials of buffy coat of 1 ml each containing the polymorphonuclear cells and 2 cryovials of red blood cells are obtained. All the aliquots obtained are finally stored at -80 ° C until their next use.

- as regards the analysis of miRNAs from the serum or plasma samples obtained, a protocol for the amplification of specific miRNAs in droplet digital PCR (ddPCR) was developed, which allows to determine the levels with a high degree of accuracy expression of circulating miRNAs potentially modulated by LGG treatment.

During the study period, a chronogram of weekly visits was followed from the start of the probiotic until the end of the intake itself. Finally, a follow-up visit followed approximately 1 month after the end of the treatment. The purpose of the visits was to assess the continued suitability for the study, compliance with the intake of the probiotic and the verification of any adverse events experienced by patients during the trial.

All data was collected on Castor's e-CFR platform.

4. RESULTS

At present, 82 patients have been enrolled for the LRadio / 10/2017 study and 73 for the LRchemio / 10/2017 study with a drop out of 20.7% respectively, equal to 17 patients, for the first study and 45, 2, 33 patients, for the second study. Causes of after out:

1. Protocol violation:

- Occurrence of a condition listed in the exclusion criteria which makes the subject unsuitable for the study

- Poor compliance to the experimental procedures

- Use of prohibited pharmacological treatments

- Subject erroneously enrolled, failing to meet eligibility criteria

2. Spontaneous subject's withdrawal

3. Adverse event requiring subject's withdrawal

4. Other

With reference to the achievement of the primary end-point, the grade 3-4 diarrhoea event rate was 0% in both cases and controls of both studies.

In relation to the secondary end-points: the rate of diarrhoea event of any degree, for the study LRadio / 10/2017 was 6 patients among those who took lot 8932410 and 5 patients among those who took lot 8932412. Other adverse events observed were tenesmus and dysuria. In the LRchemio / 10/2017 study, a diarrhoea event rate of any degree was observed in 4 patients among those who

took lot 8932410 and 3 patients among those who took lot 8932412. Other adverse events observed were, neutropenia, asthenia, anaemia and constipation.

Additionally, the hospitalization rate for gastrointestinal toxicity and / or general toxicity was 0% in both studies.

As regards to compliance with cancer treatment, only 2 patients voluntarily left the study. The main causes of drop-out were cases of screening failure or taking drugs prohibited during the study and taken independently without prior communication to the Centre.

Finally, for the determination of the epigenetic modifications induced by treatment with LGG in the modulation of the intestinal microbiome and in the modulation of the expression levels of specific miRNAs associated with inflammatory processes and the patient's response to the treatments administered to them, to date, blood and faecal samples have been collected within the study LRchemio / 10/2017, for 25 of them samples are already available before the start and at the end of the treatments). As regards the LRadio / 10/2017 study, the samples obtained at the end of the radiotherapy treatment are also available for 38 of the 82 enrolled patients, and for 12 of them the samples were also collected at the follow-up, i.e. one month after the treatments end.

5. DISCUSSION

The gut microbiota is considered a truly neglected organ (82). In the last decade, new pre-clinical and clinical results, combined with the technological advancement of intestinal microbiota characterization techniques (i.e., metagenomics, metatrascriptomics, metabolomics), have made it possible to establish the key role of the intestinal microbiota in cancer. (83)

In particular, it has been observed that an eubiotic microflora is essential in order to reduce the toxic effects of anti-cancer therapies (especially at the gastrointestinal level) and moreover, especially with regard to some types of therapies (ie, immunotherapy) a healthy microbiota is associated with greater therapeutic efficacy (84).

Among the ways to effectively modulate the composition of the microbiota, there is the administration of probiotics, a controlled method that allows to enrich the microflora in specific strains of bacteria, with beneficial effects for the host (85). LGG is a model probiotic in oncology, having a double effect. On the one hand, LGG improves gastrointestinal health by promoting the secretion of mucins and positively modulating the host's immune system. On the other hand, LGG is able to selectively counteract the growth of cancer cells, although the molecular mechanism has yet to be identified (85).

These premises led to the launch of our two clinical studies (LRadio / 10/2017 and LRchemio / 10/2017) in order to study the role of LGG as a positive modulator of gastro-intestinal health in cancer patients with abdominal-pelvic involvement. . In addition to verifying the effectiveness of the treatment in terms

of reducing diarrhoea associated with the therapy and other side effects (affecting the gastro-intestinal system and general), important parameters will be evaluated, both at the blood and fecal level, still subject to processing phase.

In the blood, inflammation markers (cytokines etc.) will be evaluated, in particular their variation from the beginning of the combined treatment (baseline) to the end (endpoint). In fact, LGG is expected to positively interfere with the host's immune system, favouring the decrease of these inflammation markers.

Also at the blood level, the modulation of some miRNAs selected among those associated with carcinogenesis will be evaluated. In this context, the bioinformatics analysis allowed the identification of 4 potential miRNAs associated with the development of colorectal carcinoma and actively modulated by the intestinal microbiota and its modifications induced by the administration of probiotic or fecal transplantation, i.e. the miRNAs hsa-miR-145-5p, hsa -miR-195-5p, hsa-miR-21-5p and hsa-miR-223-3p. The analysis of the expression levels of these miRNAs in liquid biopsy samples obtained from patients with colorectal cancer and healthy controls showed that they were actually involved in neoplastic progression. Furthermore, the evaluation of miRNA expression levels hsa-miR-145-5p, hsa-miR-195-5p, hsa-miR-21-5p and hsa-miR-223-3p in a pilot series of patients with CRC and recruited within the two clinical trials LGG showed that treatment with the probiotic induces a significant reduction of up-regulated miRNAs in the tumour and a significant increase in down-regulated miRNAs in the tumour. These important preliminary results obtained represent the basis for demonstrating how enrichment of the intestinal microbiota in cancer

patients can be useful not only as a supportive therapy but also as a basic treatment for cancer.

Finally, the intestinal microbiome will be isolated from the patient's faeces (also in this case at least two samples will be taken, one at time zero or baseline and one at the end of the combined treatment or endpoint) and its composition will be characterized. This analysis will in fact allow to evaluate: (1) the possible abundance of LGG following treatment with the probiotic; (2) the qualitative and quantitative variation of the species that populate the intestine. This last point will allow us to understand the effect that the daily intake of LGG has on the modulation of the patient's microbiome, which will therefore itself be related to the therapeutic efficacy and general intestinal health of the patient during and at the end of the study.

Overall, the results of these two clinical studies will allow to confirm in an extended cohort whether LGG can be suggested as an adjuvant in cancer therapy, including colorectal cancers.

Although LGG is protective against normal intestinal epithelial cells, it is able to counteract the growth of cancer cells (85). To characterize this effect, we selected three colon cancer lines and evaluated, by viability assay, the effects of the supernatant obtained from LGG, or LGG-SN, administered alone and in combination with 5-FU or IR.

The results obtained show that LGG-SN is not only able to reduce cell viability in a concentration-dependent manner, but also to act synergistically with

chemotherapeutics, sensitizing the cells to both 5-FU and IR. Hence LGG-SN can be suggested as a therapeutic adjuvant.

These results are critical because they make it clear that LGG is capable of secreting a molecular product that interferes with cell viability. Identifying exactly this molecular mediator will be the next experimental step. It can in fact be postulated that it is, for example, a bacteriocin (such as p40 and p75) (86). Alternatively, it could be a metabolite, such as lipoteic acid (LTA) (87). Or a new molecule not yet characterized. The key experiments that will be carried out in the future are: (1) cell cycle and apoptosis measurement assays to verify whether the effect of LGG-SN is cytotoxic or cytostatic; (2) proteomic and metabolomic characterization of LGG-SN.

In conclusion, the results deriving from the two lines of research (clinical studies on the one hand and in vitro studies on the other) will make it possible to clarify whether LGG can be suggested as an adjuvant to be associated with anti-tumour treatment, especially in cases of colorectal cancer, which, given their heterogeneity, are associated with a still very low survival rate.

6. REFERENCES

1. Banna GL, Torino F, Marletta F, Santagati M, Salemi R, Cannarozzo E, Falzone L, Ferraù F, Libra M. Lactobacillus rhamnosus GG: an overview to explore the rationale of its use in cancer. Front Pharmacy. 2017 Sep 1;8:603. doi: 10.3389/
2. Miller, G. W. & Jones, D. P. The Nature of Nurture: Refining the Definition of the Exposome. *Toxicol. Sci.* **137**, 1–2 (2014)
3. Juarez, P. D. & Matthews-Juarez, P. Applying an Exposome-Wide (ExWAS) Approach to Cancer Research. *Front. Oncol.* **8**, (2018)
4. Wang, B., Yao, M., Lv, L., Ling, Z. & Li, L. The Human Microbiota in Health and Disease. *Engineering* **3**, 71–82 (2017)
5. Siroux, V., Agier, L. & Slama, R. The exposome concept: a challenge and a potential driver for environmental health research. *Eur. Respir. Rev.* **25**, 124–129 (2016)
6. Lin, L. & Zhang, J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol.* **18**, 2 (2017)
7. Vivarelli, S. *et al.* Gut Microbiota and Cancer: From Pathogenesis to Therapy. *Cancers (Basel).* **11**, 38 (2019)
8. Dieterich, W., Schink, M. & Zopf, Y. Microbiota in the Gastrointestinal Tract. *Med. Sci.* **6**, 116 (2018)
9. Visconti, A. *et al.* Interplay between the human gut microbiome and host metabolism. *Nat. Commun.* **10**, 4505 (2019)
10. Sheflin, A. M., Whitney, A. K. & Weir, T. L. Cancer-Promoting Effects of Microbial Dysbiosis. *Curr. Oncol. Rep.* **16**, 406 (2014)
11. Nichols, R. G. & Davenport, E. R. The relationship between the gut microbiome and host gene expression: a review. *Hum. Genet.* (2020). doi:10.1007/s00439-020-02237-0
12. Gomaa, E. Z. Human gut microbiota/microbiome in health and diseases: a review. *Antonie Van Leeuwenhoek* **113**, 2019–2040 (2020)
13. Almeida, A. *et al.* A unified catalog of 204,938 reference genomes from the human gut microbiome. *Nat. Biotechnol.* **39**, 105–114 (2021)

14. Vujkovic-Cvijin, I. *et al.* Host variables confound gut microbiota studies of human disease. *Nature* **587**, 448–454 (2020)
15. Zheng, D., Liwinski, T. & Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Res.* **30**, 492–506 (2020)
16. Hadrich, D. New EU projects delivering human microbiome applications. *Futur. Sci. OA* **6**, FSO474 (2020)
17. Kho, Z. Y. & Lal, S. K. The Human Gut Microbiome – A Potential Controller of Wellness and Disease. *Front. Microbiol.* **9**, (2018)
18. Barbacid M. Ras genes. Annum. Rev. Biochem. 56, 779-827 (1987)
19. Fearon E. R. Molecular Genetics of Colorectal Cancer. Annual Review of Pathology: Mechanisms of Disease 6,479-507 (2011)
20. Kostic, A. D. et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Res. 22, 292–298 (2012)
21. Kostic, A. D. et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe 14, 207–215 (2013)
22. McCoy, A. N. et al. Fusobacterium is associated with colorectal adenomas. PLoS ONE 8, e53653 (2013)
23. Rubinstein, M. R. et al. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe 14, 195–206 (2013)
24. Patwa, L. G. et al. Chronic intestinal inflammation induces stressresponse genes in commensal Escherichia coli. Gastroenterology 141, 1842–1851 (2011)
25. Lara-Tejero, M.; Galán, J.E. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science 2000, 290, 354–357
26. Murata-Kamiya, N.; Kurashima, Y.; Teishikata, Y.; Yamahashi, Y.; Saito, Y.; Higashi, H.; Aburatani, H.; Akiyama, T.; Peek, R.M.; Azuma, T.; et al. Helicobacter pylori CagA interacts with E-cadherin and deregulates the

beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene 2007, 26, 4617–4626

27. Wu, S.; Rhee, K.J.; Zhang, M.; Franco, A.; Sears, C.L. Bacteroides fragilis toxin stimulates intestinal epithelial cell shedding and gammasecretase-dependent E-cadherin cleavage. J. Cell Sci. 2007, 120, 1944–1952
28. Bergounioux, J.; Elisee, R.; Prunier, A.L.; Donnadieu, F.; Sperandio, B.; Sansonetti, P.; Arbibe, L. Calpain activation by the Shigella flexneri effector VirA regulates key steps in the formation and life of the bacterium's epithelial niche. Cell Host Microbe 2012, 11, 240–252
29. Buti, L.; Spooner, E.; Van der Veen, A.G.; Rappuoli, R.; Covacci, A.; Ploegh, H.L. Helicobacter pylori cytotoxin-associated gene A (CagA) subverts the apoptosis- stimulating protein of p53 (ASPP2) tumor suppressor pathway of the host. Proc. Natl. Acad. Sci. USA 2011, 108, 9238–9243
30. Plottel, C.S.; Blaser, M.J. Microbiome and malignancy. Cell Host Microbe 2011, 10, 324–335
31. Doisneau-Sixou, S.F.; Sergio, C.M.; Carroll, J.S.; Hui, R.; Musgrove, E.A.; Sutherland, R.L. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr. Relat. Cancer 2003,10, 179–186
32. Fernández, M.F.; Reina-Pérez, I.; Astorga, J.M.; Rodríguez-Carrillo, A.; Plaza-Díaz, J.; Fontana, L. Breast Cancer and Its Relationship with the Microbiota. Int. J. Environ. Res. Public Health 2018, 15, 1747
33. Macpherson A. J., Hunziker L., McCoy K. & Lamarre A. IgA responses in the intestinal mucosa against pathogenic and nonpathogenic microorganism. Microbes and Infection. 3, 1021-1035 (2001)
34. World Health Organization-Food and Agricultural Organization, Guidelines for the Evaluation of Probiotics in Food, FAO/WHO joint report., London, (2002)
35. R. Crittenden, G. Mogensen, Technological challenges for future probiotic foods, Int. Dairy J. 12 (2002) 173–182

36. K. Georgiev, M. Georgieva, Antiproliferative effect of bulgarian spring water probiotics (laktera nature probiotic®) against human colon carcinoma cell line, World J Pharm Pharm Sci. 4 (2015) 130–136
37. Sanders ME, Considerations for use of probiotic bacteria to modulate human health, in The Journal of Nutrition, vol. 130, 2S Suppl, pp. 384S–390S (2000)
38. Mcfarland LV., Meta-°©-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease, in Am. J. Gastroenterol., vol. 101, no 4, pp. 812–22 (2006) 121
39. Szajewska H, Ruszczyński M, Radzikowski A., Probiotics in the prevention of antibiotic- °©-associated diarrhea in children: a meta-°©-analysis of randomized controlled trials, in J. Pediatr., vol. 149, no 3, pp. 367– 372 (2006)
40. Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE.,Efficacy of probiotics in prevention of acute diarrhoea: a meta-°©-analysisof masked, randomised, placebo-°©- controlled trials, Lancet Infect. Dis.,vol. 6, no 6, pp. 374–82 (2006)
41. P. Marteau, The clinical importance of intestinal microbiota. Gastroenterol. Clin. Biol., 34 Suppl. 1, pp. S93-°©-7 (2010)
42. R. Rastmanesh, High polyphenol, low probiotic diet for weight lossbecause of intestinal microbiota interaction., Chem. Biol. Interact., vol.189, 1-°©-2, pp. 1-°©-8 (2011)
43. M. Kumar, A. Kumar, R. Nagpal, D. Mohania, P. Behare, V. Verma, P. Kumar, D. Poddar, P.K. Aggarwal, C.J.K. Henry, S. Jain, H. Yadav, Cancer-preventing attributes of probiotics: an update, Int. J. Food Sci. Nutr. 61 (2010) 473–496
44. Paulos, C.M.; Wrzesinski, C.; Kaiser, A.; Hinrichs, C.S.; Chieppa, M.; Cassard, L.; Palmer, D.C.; Boni, A.; Muranski, P.; Yu, Z.; et al. Microbial translocation augments the function of adoptively transferred self/tumor-specific CD8+ T cells via TLR4 signaling. J. Clin. Investig. 2007, 117, 2197–2204

45. Wollowski I, Rechkemmer G, Pool-°©-Zobel BL, Protective role of probiotics and prebiotics in colon cancer, The American Journal of Clinical Nutrition, vol. 73, 2 Suppl., pp. 451S–455S (2001)
46. M. Raman, P. Ambalam, K.K. Kondepudi, S. Pithva, M. Raman, P. Ambalam, K.K. Kondepudi, S. Pithva, C. Kothari, A.T. Patel, Potential of probiotics, prebiotics and synbiotics for management of colorectal cancer, Gut Microbes 4 (2013) 181–192
47. Roy, S.; Trinchieri, G. Microbiota: A key orchestrator of cancer therapy. Nat. Rev. Cancer 2017, 17, 271–285
48. Nayak, R.R.; Turnbaugh, P.J. Mirror, mirror on the wall: Which microbiomes will help heal them all? BMC Med. 2016, 14, 72
49. Fessler, J.L.; Gajewski, T.F. The Microbiota: A New Variable Impacting Cancer Treatment Outcomes. Clin. Cancer Res. 2017, 23, 3229–3231
50. Schwabe R.F. and Jobin C. The microbiome and cancer. Nat Rev Cancer. 2013 Nov;13(11):800-12. doi: 10.1038/nrc3610. Epub 2013 Oct 17
51. S. Shamekhi, H.L.J. Abdolalizadeh, E.B.N. Zarghami, An overview ofyeast probiotics as cancer biotherapeutics : possible clinical application in colorectal cancer, Clin. Transl. Oncol. (2020),https://doi.org/10.1007/s12094-019- 02270-0
52. Bajic, J.E.; Johnston, I.N.; Howarth, G.S.; Hutchinson, M.R. From the Bottom-Up: Chemotherapy and Gut-Brain Axis Dysregulation. Front. Behav. Neurosci. 2018, 12, 104
53. Scaife, J.E.; Barnett, G.C.; Noble, D.J.; Jena, R.; Thomas, S.J.; West, C.M.; Burnet, N.G. Exploiting biological and physical determinants of radiotherapy toxicity to individualize treatment. Br. J. Radiol. 2015, 88,20150172
54. Griggs, J.J. Reducing the toxicity of anticancer therapy: New strategies. Leuk. Res. 1998, 22 (Suppl. 1), S27–S33
55. Samaan, M.A.; Pavlidis, P.; Papa, S.; Powell, N.; Irving, P.M. Gastrointestinal toxicity of immune checkpoint inhibitors: From mechanisms to management. Nat. Rev. Gastroenterol. Hepatol. 2018, 15, 222–234

56. Dong, J.; Chen, H. Cardiotoxicity of Anticancer Therapeutics. Front. Cardiovasc. Med. 2018, 5, 9
57. Shahid, F.; Farooqui, Z.; Khan, F. Cisplatin-induced gastrointestinal toxicity: An update on possible mechanisms and on available gastroprotective strategies. Eur. J. Pharmacol. 2018, 827, 49–57
58. Lawrie, T.A.; Green, J.T.; Beresford, M.; Wedlake, L.; Burden, S.; Davidson, S.E.; Lal, S.; Henson, C.C.; Andreyev, H.J.N. Interventions 80 to reduce acute and late adverse gastrointestinal effects of pelvic radiotherapy for primary pelvic cancers. Cochrane Database Syst. Rev. 2018, 1, CD012529
59. Alexander, J.L.; Wilson, I.D.; Teare, J.; Marchesi, J.R.; Nicholson, J.K.; Kinross, J.M. Gut microbiota modulation of chemotherapy efficacy and toxicity. Nat. Rev. Gastroenterol. Hepatol. 2017, 14, 356–365
60. Sokol, H.; Adolph, T.E. The microbiota: An underestimated actor in radiation-induced lesions? Gut 2018, 67, 1–2
61. Zitvogel, L.; Ma, Y.; Raoult, D.; Kroemer, G.; Gajewski, T.F. The microbiome in cancer immunotherapy: Diagnostic tools and therapeutic strategies. Science 2018, 359, 1366– 1370
62. Redman, M. G., Ward, E. J. & Phillips, R. S. The efficacy and safety of probiotics in people with cancer: a systematic review. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 25, 1919–29 (2014)
63. Khailova, L.; Baird, C.H.; Rush, A.A.; Barnes, C.; Wischmeyer, P.E. Lactobacillus rhamnosus GG treatment improves intestinal permeability and modulates inflammatory response and homeostasis of spleen and colon in experimental model of Pseudomonas aeruginosa pneumonia. Clin. Nutr. 2017, 36, 1549–1557
64. Fong, F.L.; Kirjavainen, P.V.; El-Nezami, H. Immunomodulation of Lactobacillus rhamnosus GG (LGG)-derived soluble factors on antigenpresenting cells of healthy blood donors. Sci. Rep. 2016, 6, 22845
65. Goldin, B.R.; Gualtieri, L.J.; Moore, R.P. The effect of Lactobacillus GG on the initiation and promotion of DMH-induced intestinal tumors in the rat. Nutr. Cancer 1996, 25, 197– 204

66. Gorbach, S., Doron, S. & Magro, F. Lactobacillus rhamnosus GG. in The Microbiota in GastrointestinalPathophysiology 79-88 (Elsevier, 2017)
67. Silva, M., Jacobus, N. V, Deneke, C. & Gorbach, S. L. Antimicrobial substance from a human Lactobacillus strain. Antimicrob. Agents Chemother. 31, 1231–1233 (1987)
68. Nishiyama, K. et al. Adhesion properties of Lactobacillus rhamnosus mucus-binding factor to mucin and extracellular matrix proteins. Biosci. Biotechnol. Biochem. 79, 271– 279 (2015)
69. Orlando, A.; Linsalata, M.; Russo, F. Antiproliferative effects on colon adenocarcinoma cells induced by co-administration of vitamin K1 and Lactobacillus rhamnosus GG. Int. J. Oncol. 2016, 48, 2629–2638
70. Nouri, Z.; Karami, F.; Neyazi, N.; Modarressi, M.H.; Karimi, R.; Khorramizadeh, M.R.; Taheri, B.; Motevaseli, E. Dual Anti-Metastatic and Anti-Proliferative Activity Assessment of Two Probiotics on HeLa and HT-29 Cell Lines. Cell J. 2016, 18, 127–134
71. Zhao, B.B.; Meng, J.; Zhang, Q.X.; Kang, T.T.; Lu, R.R. Protective effect of surface layer proteins isolated from four Lactobacillus strains on hydrogen-peroxide-induced HT-29 cells oxidative stress. Int. J. Biol. Macromol. 2017, 102, 76–83
72. Behzadi, E.; Mahmoodzadeh Hosseini, H.; Imani Fooladi, A.A. The inhibitory impacts of Lactobacillus rhamnosus GG-derived extracellular vesicles on the growth of hepatic cancer cells. Microb. Pathog. 2017, 110, 1–6
73. Cheng, Z.; Xu, H.; Wang, X.; Liu, Z. Lactobacillus raises in vitro anticancer effect of geniposide in HSC-3 human oral squamous cell carcinoma cells. Exp. Ther. Med. 2017, 14, 4586–4594
74. Ni, Y.; Wong, V.H.; Tai, W.C.; Li, J.; Wong, W.Y.; Lee, M.M.; Fong, F.L.; El-Nezami, H.; Panagiotou, G. A metagenomic study of the preventive effect of Lactobacillus rhamnosus GG on intestinal polyp formation in APC. J. Appl. Microbiol. 2017, 122, 770–784
75. Mendes, M.C.S.; Paulino, D.S.; Brambilla, S.R.; Camargo, J.A.; Persinoti, G.F.; Carvalheira, J.B.C. Microbiota modification by 86 probiotic

supplementation reduces colitis associated colon cancer in mice. World J. Gastroenterol. 2018, 24, 1995–2008

76. Gamallat, Y.; Meyiah, A.; Kuugbee, E.D.; Hago, A.M.; Chiwala, G.; Awadasseid, A.; Bamba, D.; Zhang, X.; Shang, X.; Luo, F.; et al. Lactobacillus rhamnosus induced epithelial cell apoptosis, ameliorates inflammation and prevents colon cancer development in an animal model. Biomed. Pharmacother. 2016, 83, 536–541
77. Suzuki, C.; Aoki-Yoshida, A.; Aoki, R.; Sasaki, K.; Takayama, Y.; Mizumachi, K. The distinct effects of orally administered Lactobacillus rhamnosus GG and Lactococcus lactis subsp. lactis C59 on gene expression in the murine small intestine. PLoS ONE 2017, 12, e 0188985
78. Guandalini, S. et al. Lactobacillus GG Administered in Oral Rehydration Solution to Children with Acute Diarrhea: A Multicenter European Trial. J. Pediatr. Gastroenterol. Nutr. 30, 54–60 (2000)
79. Vanderhoof, J. A. et al. Lactobacillus GG in the prevention of antibiotic-associated diarrhea in children. J. Pediatr. 135, 564–568 (1999)
80. Oksanen, P. J. et al. Prevention of Travellers Diarrhoea by Lactobacillus GG. Ann. Med. 22, 53–56 (1990)
81. Gorbach, S., Chang, T.-W. & Goldin, B. Successful treatment of relapsing Clostridium Difficile colitis with Lactobacillus GG. Lancet 330, 1519 (1987)
82. Riccio P, Rossano R. The human gut microbiota is neither an organ nor a commensal. FEBS Lett 2020;594:3262–71. https://doi.org/10.1002/1873-3468.13946.
83. Wong SH, Yu J. Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. Nat Rev Gastroenterol Hepatol 2019;16:690–704. https://doi.org/10.1038/ s41575-019-0209-8.
84. Sepich-Poore GD, Zitvogel L, Straussman R, Hasty J, Wargo JA, Knight R. The microbiome and human cancer. Science (80-) 2021;371:eabc4552. https://doi.org/ 10.1126/science.abc4552.

85. Vivarelli S, Falzone L, Basile M, Nicolosi D, Genovese C, Libra M, et al. Benefits of using probiotics as adjuvants in anticancer therapy (Review). World Acad Sci J 2019. https://doi.org/10.3892/wasj.2019.13.
86. Yan F, Polk DB. Lactobacillus rhamnosus GG: An Updated Strategy to Use Microbial Products to Promote Health. Funct Food Rev 2012;4:77–84.
87. Claes IJJ, Lebeer S, Shen C, Verhoeven TLA, Dilissen E, De Hertogh G, et al. Impact of lipoteichoic acid modification on the performance of the probiotic Lactobacillus rhamnosus GG in experimental colitis. Clin Exp Immunol 2010;162:306–14. https:// doi.org/10.1111/j.1365-2249.2010.04228.x.