UNIVERSITÀ DEGLI STUDI DI CATANIA

DOTTORATO DI RICERCA IN BASIC AND APPLIED BIOMEDICAL SCIENCES

XXXIII CICLO

Dipartimento di Scienze Biomediche e Biotecnologiche (BIOMETEC)

Dott.ssa Rossella Salemi

Anti-proliferative Effects of Cell-Free Supernatants from Lactobacillus Rhamnosus GG in Cancer Cells

TESI DI DOTTORATO DI RICERCA

Coordinatore:

Ch.ma Prof. Stefania Stefani

Relatore:

Ch.mo Prof. Massimo Libra

ANNO ACCADEMICO 2019/2020

INDEX

ABSTRACT

The gut microbiome is not a silent and isolated ecosystem but exerts several physiological and immunological functions into a complex 'super-organism' with which has co-evolved.

The lactobacilli, protagonists of this ecosystem, have been used as an effective therapy for treatment of several pathological conditions displaying an overall positive safety profile. The lactobacilli administration has shown to induce qualitative and quantitative modifications in the human gastrointestinal microbial ecosystem with encouraging perspectives in counteracting pathologys.

Despite great attention, the understanding of the biological processes involved in the beneficial effects of lactobacilli strains is still limited. To this end, the present study investigated the growth-inhibitory effects of Lactobacillus rhamnosus GG against tumour cell lines. Administration of cell-free supernatant from Lactobacillus rhamnosus GG on human (HT29, HCT-116, Caco-2) colon carcinoma cell lines and human (A375) melanoma cells raised a significant concentration-dependent anti-proliferative effect, determined by cell viability assays. Specifically, cell-free supernatant from Lactobacillus rhamnosus GG did not induce apoptotic cell death in tumour cell lines as revealed by annexin V and propidium iodide staining but rather induced G2/M cell cycle arrest. In addition, CFS-LGG administration combined with chemotherapy drugs has potentiated their action. Taken together, these findings provide evidence for beneficial anti-proliferative and tumour inhibitory effects derivated by Lactobacillus rhamnosus GG.

1. INTRODUCTION

Millions of years of evolution have seen the microbiota and host co-evolve into a complex ‘super-organism’. The commensalism, mutualism and parasitism are some of the most important relationships established within this pacific ecosystem.

Following the benefits to many key aspects of host life, microbial communities, which do not harm but either benefit the host, have an evolutionary advantage at reside a permanent niche establishing a state of immune tolerance with their host. While, microbial entities, that enter the ecosystem activating innate and adaptive immune responses, may disturb this symbiotic relationship and promote disease. The delicate balance that allows the symbiotic coexistence between these two worlds is maintained thanks to the anatomical separation of the microbial communities from the host compartment by well-conceived, multi-level barriers.

In the complex 'super-organism', the microbial communities are ghettoised in the gut and on the skin, in which barriers, relying on an intact epithelial lining, a mucous layer, the stratum corneum (in the skin case) and a low pH, as well as a sensing systems that detect invading bacteria, guarantee the host tolerance and a mutual benefit.

However, perturbation of these barriers, through environmental changes including infection, diet or lifestyle, may modify the microbiome, disturb this symbiotic relationship and promote inflammation and diseases, including cancer (Feng, Q. et al., 2018).

Consistent with symbiotic evolution host–microbiota interactions, the human being must be considered as a complex unity-totality with the microbiota which can intervene to safeguard the ‘super-organism’.

Already since the late nineteenth century it is known that the commensal bacteria and other microorganisms that colonize the epithelial surfaces of our body have been prove to activate the innate immunity triggering the antitumor immune responses.

To date, more studies have follow one another to identify correlative relationships between microbial species and cancer phenotypes and these have

encouraged to understand the profound influence of the microbiota on the efficacy of cancer treatments. Now, the current challenge in microbiome research is to identify individual microbial species that causally affect cancer phenotypes and unravel the underlying mechanisms to develop, in the near future, microbiota-based therapeutics (Eran Elinav, et al., 2019).

Many eminent working scientists on the cancer microbiome think that unravelling the complex interaction between gut microbiota and cancer, an integrated approaches combining microbiota, tumour and immune system are necessary.

A suitable approach may be modelled combining microbiome and host multi-omic characterization coupled with the experimentation in preclinical models.

Following methods to target the microbiome to improve therapy effectiveness, the main objectives are to identify the microbiota composition that favours therapy response. In cancer therapy studies, robust approaches are those using colonization of mice with human commensal bacteria, as well as clinical trials using faecal microbiota transplant aiming to improve the success of immunotherapy have been initiated.

The microbiome represents extraordinary opportunity as a prognostic biomarker to choose appropriate preventive and therapeutic strategies.

It should not in fact be underestimated that the human microbiome also includes proteins and metabolites produced by individual members of the microbial community and cometabolites produced by networks within humans in concert with the microbiota.

Rather than faecal transplant that may also transfer pathogens, an important goal remains to identify the mechanisms shared by different bacteria that could enhance therapy response in any clinical setting.

In this regard, trials could be started using an oral treatment with a single bacterial strain and assess the possible favourable response to the drug in patients (as was done with Bifidobacterium spp. and anti-PD1).

It could also be important to characterize the effect of the diet in improving the efficacy of therapy by modifying the composition of the microbiota.

Overall, emerging evidence of the microbiota contribution to cancer development, progression and response to treatment, make you imagine the future of cancer care as involving a treatment approach personalized to patient genetic and microbiome characteristics.

Looking to the not very far future, to date are in use new interventions altering microbiota composition and function, including prebiotic, probiotics, postbiotic interventions or personalized nutritional approaches may, altering the microbiome configuration towards one that favours cancer treatment responsiveness.

2. THE MICROBIOME AND CANCER

The mutual benefit between host and microbiota establish that they have been co-evolved in close relationship with each other (Kau A. L. et al., 2011; Consortium H. M. P., 2012).

Among the human symbiotic microbial populations, 99% is within the gastrointestinal tract, and it deeply influences host's homeostasis exerting both local and long-distance effects. It's not coincidence that the gastrointestinal microbiome is the best-investigated microbiome and serves as a model for understanding host−microbiota interactions and disease (Feng Q. et al., 2018).

The gut microbiota, indeed, performs a number of beneficial host functions, including nutrition and metabolization of dietary compounds, production of vitamins, protection against the expansion and systemic infiltration of gut pathogens (Vaishnava S. et al., 2008; Belkaid, Y. et al., 2013; Magnúsdóttir S. et al., 2015). However, this close relationship also carries risks for disease development, particularly when this delicate equilibrium is perturbed leading to an impaired microbiota (Carding S. et al., 2015).

Additionally, microbial communities have been described in other organs including the skin and vagina (Consortium H. M. P., 2012; Grice E. A. & Segre J. A., 2011).

Interestingly, microbial communities and abundance are different in each organ. These differences might be an explanation for the occurrence of inflammation, diseases, including carcinogenesis, likely organ specific

(Consortium H. M. P., 2012). Thus, it is not surprising that bacteria have been detected within lung, breast, colon, gastric, pancreatic, cholangiocarcinoma, ovarian, and prostate cancers (Urbaniak, C. et al., 2016; Ferreira, R. M. et al., 2018; Pushalkar, S. et al., 2018; Banerjee, S., et al. 2017;
Sfanos, K. S. et al., 2008; Aviles-Jimenez, F. et al., 2016; Mao, Q. et al., 2018).
In addiction, while tumours of the enteric tract, respiratory system, or reproductive tract are routinely exposed to microorganisms, many organs, for example, the liver, not containing a known microbiome, they may be exposed to bacterial metabolites through anatomical links with the gut (Zmora, N. et al., 2019; Shin, W. & Kim, H. J., 2018; Rubinstein, M. R. et al., 2013; Tahara, T. et al., 2014).
Although there is less direct evidence that intratumoral bacteria can affect patient outcomes compared with the gut microbiota, being metabolically actives, can alter the chemical structure of common chemotherapeutic agents, changing their activity and thus their effective local concentration (Panebianco, C. et al., 2018; Lehouritis, P. et al, 2015).
This omnipresence framework of bacterial communities within the host can help to understand the great impact of microbiota architecture on human health.
Consistent with the descriptive microbiome composition analyses and functional studies, among all the pathologies linked with the gut microbiome, tumourigenesis is one of the mostly studied (Goodman, B. et al., 2018).
In particular, the current interest is aimed to understanding the complexity and bidirectionality of the connection existing between microbiome and cancer.
In fact, establishing when cancer development can alter the microbiome and, in turn, changes in the microbiome can affect cancer progression, it is particularly complex (Zitvogel L. et al., 2017).
Therefore, it is absolutelly not superfluous discussing about the tight link between intestinal microbiota and tumorogenesis, and the importance of probiotics supplementation with anti-cancer therapy.

2.1 Contribution of microbiota in human tumorogenesis

The intricate relationship between the microbiota and the host in respect to our lifestyles tumour-promoting and tumour-suppressive, it is increasingly appreciated.

As common knowledge, consumption of alcohol, smoking, obesity as well as more generally unhealthy diets, can contribute to carcinogenesis and modulate microbiomes.

How much the microbial metabolism contributes to the carcinogenesis also promoted by these unhealthy lifestyles remains to be clarified (Robert F. Schwabe and Christian Jobin. 2013).

However, it is now established that human metabolism represents a combination of microbial and human enzyme activities (Belkaid Y. et al., 2013).

The bacterial metagenome is all known to be functionally far more diverse than that of humans, and is enriched for genes that are relevant for nutrient, bile acid and xenobiotic metabolism, as well as for the biosynthesis of vitamins (Gill S. R. et al., 2006; Philipp B., 2011; Vaishnava, S. et al., 2008; Magnúsdóttir, S. et al., 2015).

In fact, it has been widely studied that the gut microbiota may produce or transform molecules affecting several aspects of human health, including: gut barrier integrity maintenance and protection against gastro-intestinal pathogens, host's immune system modulation, drug metabolism, besides host's metabolism modulation (Gensollen, T. et al., 2016; Schmidt, T.S.B. et al., 2018; Bultman, S.J., 2014; Cani, P.D., 2018).

Importantly, the gastro-intestinal entero-endocrine cells secrete several peptide hormones, involved for example in gastro-intestinal motility, digestive functions and neuromodulation, and at the same time capable to adjust the gut microbiota composition, as during stress response (Ceranowicz P. et a., 2015; Sandrini S. et al., 2015).

In the same way, the gut microbial population secrete active molecules which can be sensed by the gut cells and whose effects are transduced to the nervous system through the gut-brain-axis (Sandrini S. et al., 2015).

In summary, the metabolic activities, generated by intestinal microbiota, may affect metabolic activation or inactivation of dietary phytochemicals, metabolism of hormones and the generation of tumour-promoting secondary bile acids, as well as diet-induced inflammation and regulating tumorogenesis through metabolic activation and inactivation of carcinogens (Belkaid Y. et al., 2013).

Besides the well-established cancer-promoting role of specific pathogens in certain cancers, a considerable variation of the normal microbiota composition may occur through changes in diet, innate immune responses and inflammation, or infections (Arthur J. C. et al., 2012; Holmes E. et al., 2012; Ley R. E. et al., 2006).

Given these evidence, the gut bacteria could favour the tumours onset in two different ways: a first mechanism involves activation of TLR signalling pathways, leading to gastric mucosa chronic inflammation, in turn, linked to the increased risk of cancer; a second mechanism instead involves the metabolic-activated intestinal microbiota, capable of producing toxins with a direct pro-carcinogenic effect or enzymes capable of activating the carcinogens ingested with the diet (Barbacid M., 1987; Fearon E. R., 2011).

A wealth of studies in patients and mice has ascribed the microbiota for colorectal cancer-promoting (Grivennikov, S. I., 2012). Such effects would seem to be caused, in fact, by altered host–microbiota interactions and by dysbiosis (Robert F. Schwabe and Christian Jobin. 2013).

In addiction, both local and distant tumours have been linked with gastro-intestinal dysbiosis rather than by infections with specific pathogens (Lecuit, M. et al. 2004).

A prime example of cancer that is promoted by dysbiotic microbiota through long-distance mechanisms is provided by liver that does not contain a known microbiome.

A substantial increase of bacterial metabolites, cancer-promoting, in liver disease has been observed and has been linked to intestinal dysbiosis (Wiest, R. & Garcia-Tsao, G., 2005; Seki, E. et al., 2007; Dapito, D. H. et al., 2012; Yoshimoto, S. et al., 2013). Thus, bacterial metabolites can reach the liver via the portal vein promoting liver cancer (Dapito, D. H. et al., 2012; Yoshimoto, S. et al., 2013; Yu, L. X. et al., 2010).

Following the well-established cancer-promoting role of specific pathogens in certain cancers, within a dysbiotic gut, it is likely that certain bacterial pathogens can negatively affect either the host's metabolism or the host's gut and immune system functionalities, thereby triggering tumour growth (Rea, D. et al., 2018; Bhatt, A.P. et al., 2017).

As previously mentioned, the microbiota carries out important immune and metabolic functions by also regulating some inflammatory cytokines transcription pathways. In fact, when 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 aggressive phenotype.

The increased production of cytokines, including TNF, IL-1 and IL-17, together with the activation of TLRs by some pathogens, causes the NF-kB signalling activation, a transcription factor of various anti- apoptotic, which induces the cell proliferation and the increase of angiogenesis processes and therefore it is able to support oncogenesis (Barbacid M., 1987; Fearon E. R., 2011).

In line with that, several preclinical studies performing direct manipulation of the microbial community using germ-free mice models suggested that the gut microbiome is able to deeply affect cancer genesis and progression and than dysbiosis is sufficient to promote cancer (Dapito, D. H. et al., 2012; Yoshimoto, S. et al.,2013; Yu, L. X. et al., 2010; Grivennikov, S. I. et al., 2012; Couturier-Maillard, A. et al., 2013; Hu, B. et al., 2013; Garrett, W. S. et al., 2009; Zhang, H. L. et al., 2012).

Notably, obesity, a well-established risk factor for cancer development, is one of the conditions that leads to dysbiosis (Calle, E. E. & Kaaks, R., 2004).

Besides a 'dysmetabolism' linked to decrease in microbial richness in humans, an increased population of Firmicutes and decreased populations of Bacteroidetes have been observed in the gut of both humans and mice obese (Ley, R. E. et al., 2006; Ley, R. E. et al., 2005; Cotillard, A. et al.,2013; Le Chatelier, E. et al., 2013).

Also in liver cancer, obesity cancer-promoting dysbiosis-associated, leads to an increased prevalence of Clostridia (Yoshimoto, S. et al., 2013).

Moreover, microbial dysbiosis in the luminal or mucosal compartment of CRC patients has been reported from correlative studies (Chen, W. et al., 2012; Sanapareddy, N. et al., 2012; Sobhani, I. et al., 2011; Wang, T. et al., 2011), in which Fusobacterium nucleatum was highlighted as a potential candidate for CRC susceptibility (Kostic, A. D. et al., 2012; Kostic, A. D. et al., 2013; McCoy, A. N. et al., 2013; Rubinstein, M. R. et al., 2013).

Interestingly, Fusobacterium nucleatum can stimulate cancer formation by blocking immune-effectors that normally inhibit tumorigenesis. This bacterium, indeed, inhibits for its own advantage host's Natural Killer (NK) cells, in order to recruit at the site of the infection myeloid suppressor cells, therefore promoting inflammation and indirectly helping cancer genesis (Gur C. et al., 2015).

Although the mechanisms that contribute to dysbiosis are not yet understood, host-derived inflammatory responses, when altered, unequivocally may contribute to dysbiosis.

Inflammation it is known to contribute creating to a milieu that favours the outgrowth of specific bacteria, also through specific metabolites (Halazonetis, T.D., 2004; Yao, Y. et al., 2014; Frisan, T., 2016).

For example, inflammation alters the production of nitrate that is derived from the activity of inducible nitric oxide synthase providing a unique source of energy for facultative anaerobic bacteria, as Enterobacteriaceae, at the expense of obligate anaerobic bacteria (Lupp, C. et al., 2007).

Additionally, inflammation induces expression of stress-response genes in bacteria; for example, Escherichia coli from Il10−/− mice with intestinal inflammation show an increased expression of small heat shock proteins IbpA and IbpB, which protects this bacterium from oxidative stress (Patwa, L. G. et al., 2011).

Accordingly, enhancement of the inflammation or the inhibition of the host's immune response, helping the tumour immune-escape. For example, Helicobacter pylori or Bacteroides fragilis are both able to cause active oxygen species (ROS)-induced accumulation of DNA damage, following the activation of the host's spermine oxidase (Murata-Kamiya, N. et al., 2007; Rubinstein, M.R. et al., 2013; Wu, S. et al., 2007). While, Enterococcus faecalis produces extracellular superoxide and derivative oxygen species is capable to diffuse into host's cells, increasing the possibility of host's cellular DNA mutations.

Moreover, the well-established cancer-promoting role of specific pathogens in certain cancers must be careful.

In fact, during pathogenic infections, bacterial pathogens can expand and release a large amount of toxins which, in turn, induce host's DNA breaks, thus contributing to genomic instability, tumour initiation and progression.

Although only Helicobacter pylori is included among class I carcinogens by the World Health Organization (WHO) (Moss, S.F., 2017), additional bacterial populations may affect host's DNA replication and integrity. This is the case of Escherichia coli, which produces two toxins, colibactin and cytolethal distending toxin (CDT), both capable to generate DNA double-strand breaks within the host's epithelial cells, thus promoting a transient cell cycle arrest, allowing for genomic mutations to arise (Lara-Tejero, M. et al., 2000).

Moreover, gut pathogenic bacteria, as Shigella flexneri, can also interfere with DNA damage response and repair pathways, therefore inducing host's cells p53 degradation and increasing the probability of introducing mutations during the DNA damage response in infected cells (Bergounioux J. Et al., 2012). In the same way, Helicobacter pylori, induces the proteasome-mediated degradation of p53 in gastric epithelial cells, by interfering with the host's AKT pathway, thus promoting the rise of gastric cancer (Buti L. et al, 2011).

Furthermore, Fusobacterium nucleatum effector adhesin A (FadA) and Bacteroides fragilis metalloproteinase toxin (MP toxin) are all capable to interact (directly or indirectly) with the host's epithelial E-cadherin, activating β-catenin signalling, that, in turn, triggers several host's cellular proliferative and pro-survival pathways and therefor the potential cancerogenic transformation of those affected cells (Rubinstein M. R. et al., 2013).

Consistent with the tremendous bacterial microbiota ability, certain bacteria species may interfere with host's hormones metabolism.

Clostridium leptum e Clostridium coccoides, are an example of bacteria that promote estrogenic receptors activation and cell proliferation in tissues responding to oestrogens, as breast and endometrium, through the secretion of β-glucuronidase (Plottel C.S. et al., 2011). This enzyme, in fact, de-conjugates liver-catabolized and plant-derived oestrogens, enabling them to bind and activate the estrogenic receptors expressed by target cells (Doisneau-Sixou S.F. et al., 2003; Fernández M.F. et al., 2018).

Accordingly, the evidences that the gut microbiota composition of women with breast cancer differs from that from healthy controls, link, this augmented intake of oestrogen hormones, with an increased risk of developing breast cancer.

Although there are notable examples of pathogenic microbiota capable of promoting oncogenesis through the modulation oncogenic host's cell pathways or by interfering either with the host's hormonal or the host's immune system, it is difficult to clearly determine whether microbiota changes may affect cancer genesis or the contrary (Kilkkinen A. et al., 2008; Conlon M.A. et al., 2014).

It is now established that, besides changes in the host's lifestyle, diet and immune system, the anti-cancer treatment might shape the patient's microbiome and, at the same time, host's specific microbiome can deeply affect patient's response to therapy (Gopalakrishnan V. et al., 2018).

2.2 Microbiota impact in tumour suppression and cancer therapy

Studying the microbiota effects on cancer involves, considering "two sides of the same coin". Given the intricate relationship between the microbiota and the host, it is not surprising that the gut microbial population may affect pathological processes, such as cancer genesis and development, either in a positive or in a negative way.

The dual role of the intestinal microbiota in host's health protecting arise through the production of various metabolites and bio-products (C. Manichanh et al., 2012).

First among all, the bacterial lipopolysaccharide (LPS), major component of the outer membrane in gram-negative bacteria, is one widely studied of the probiotics' derived bio-products. The researchers have indeed highlighted that LPS is able to modulate host's immune system, thereby triggering an indirect immune-mediated response against tumour development. In fact, it has been shown that is able to active the host's cell surface receptor toll-like receptor 4 (TLR4), belonging to the family of pattern recognition receptors (PRRs), activating immune T cell-mediated response against cancer cells (Paulos, C.M. et al., 2007).

In the same way, the monophosphoryl lipid A (MPL) from Salmonella enterica and bacterial derived pyridoxine (a group B vitamin) have been respectively used as adjuvant in the vaccine formulation used against anti-cervical carcinoma and as host's antitumor immune-surveillance stimulant (Paavonen, J. Et al., 2009; Aranda, F. et al., 2015).

Regularly, the gut microbial population affect host biological processes but it has been also demonstrated that many microorganisms derived molecules have implicated in anti-cancer properties (G.B. Gorbach et al., 1987; J. Fu, 2015).

Such mechanism is observable in microbial-derived SCFAs behaviour, hormone-like metabolites, resulting of dietary fibers fermentation in the large intestine (B.D. Muegge, 2011).

Besides for use as a major source of energy by the liver, microbial-derived SCFAs, including butyrate and propionate, are able to inhibit host's tumour

cells histone deacetylases with a general anti-cancer effect. Such mechanism has been observed in preclinical and clinical studies that have achieved antitumor effect of butyrate in both colorectal cancer (CRC) and lymphoma (Jan, G. et al., 2002; Wei, W. et al., 2016).

Being there a strong relationship between diet, lifestyle and gut microbiota composition it is not surprising that several commensal bacteria play a probiotic role thanks to their capability to confer health benefits, either protecting against gut dysbiosis or enhancing host's immune defence mechanisms or prevent the development of CRC through the gut microflora balance (Fulbright, L.E. et al., 2017; Zitvogel, L. et al., 2017).

A similar behaviour is observed when the administration of such probiotics is combined with the intestinal antibiotics, as for example Mutaflor (Escherichia coli Nissle 1917) with the intestinal antibiotic rifaximin; Mutaflor, in fact, enhancing the anti-inflammatory effect of rifaximin in a rat model of inflammatory bowel disease, demonstrated a clear anti-inflammatory activity (Dembi ´nski, A. et al., 2016).

Regarding probiotics antineoplastic activity, one good example of how probiotics or probiotics-derived metabolites can to inhibit tumour growth is given by ferrochrome metabolite secreted from Lactobacillus casei. This, indeed, administered to mice, is able to trigger apoptosis in tumour cells via JNK pathway direct activation.

Moreover, it has been described that Lactobacilli may stimulate host's immune cells such as NK cells or dendritic cells (DC) or TH1 response, which, in turn, leads to the elimination of cancerous or precancerous cells, although the exact bacterial bio-product mediating such stimulatory effect still needs to be identified (Lenoir M. et al., 2016; Lee J.W. et al., 2004; Baldwin C., et al., 2010; Takagi A. et al, 2008).

Given what has been said the microbiota is clear to can deeply influence both cancer pathogenesis and its therapeutic outcome.

Although the main goal of anti-cancer therapies is the eradication of the targeted malignancy, almost every available anti-cancer treatment is toxic also

towards normal cells and their use may be coupled with side effects, some of which can compromise the overallsurvival of the patients (Dy G.K. et al., 2013).

Moreover, radiotherapy, chemotherapy and immunotherapy treatments can all modify patients' microbiome but,

at the same time, microbiome composition has the great potential of deeply affect patients' response to such therapies (Roy S. et al., 2017).

In fact, interventions on microbiome it is known may to be pivotal to ameliorate anti-cancer therapy-related toxicity, as well as to improve anti-cancer therapy efficacy (Nayak R.R. et al., 2016; Fessler J.L. et al., 2017).

In particular, the regulation of therapeutic outcome is tightly connected with the ability of the gut microbiota to metabolize anti-tumour compounds, as well as to modulate host's immune response and inflammation pathways (Schwabe R.F. et al., 2013).

These two effects combined together may explain the strong involvement of the patient's microbiome composition in affecting the efficacy of both chemotherapy and immunotherapy (Gopalakrishnan V. et al., 2018).

It is therefore fundamental to identify which are the factors able to influence the gut microbiome and, in turn, to find novel strategies to manipulate the gut microbiome, with the main goal of improving patients' therapeutic outcome.

The first attempts to use bacteria in cancer therapy date back to 1890 when two heat-inactivated microorganisms were injected intratumorally in humans (McCarthy E.F., 2006; Nauts H.C. et al., 1946). Several decades later, injecting Mycobacterium bovis into the bladder of patients, it was possible to observe, following the resection of bladder tumour, that the bacteria, by inducing a local immune response, had helped to reduce the relapse of the tumour (Zbar B. et al., 1970). Additionally, a study conducted around the 1970s highlighted that the administration of Lactobacillus casei decreased superficial bladder cancer recurrence (Aso Y. et al., 1992). Several years later it emerged that the mechanism underlying these anti-cancer effects involves the direct bacterial stimulation of host's NK cells and macrophages, in turn responsible of a strong antitumoral immune response (Hoesl C.E. et al., 2005).

These and other observations have paved the way for many clinical studies based on the use of attenuated strains of gut bacteria in anti-cancer therapy, in an attempt to shed light on the key role of some bacteria on triggering antitumor immune response (Felgner S. et al., 2016).

To date, several clinical studies have shown the intradermal injection of Mycobacterium obuense in melanoma and in pancreatic ductal carcinoma activates antitumor immune response, acting on host's antigen presenting cells (APCs) and cytotoxic T cells (Stebbing J., 2012; Dalgleish A.G.; Stebbing J. Et al., 2016).

Additionally, attenuated bacteria injected directly into the tumour mass are able to both stimulate antitumor immune response and also have a direct cytotoxic effect on the tumour cells, thanks to their capability of colonizing tumours, as observed in several different refractory solid tumour studies (Toso J.F. et al., 2002; Nemunaitis J. Et al., 2003; Kramer M.G. et al., 2018).

Regarding the efficacy of chemotherapy, studies on tumour-bearing mice, free of germs or with a depleted intestinal microbiota after antibiotic therapy, have shown that the lack of response oxaliplatin drug treatment, could be due to decreased microbiota-dependent ROS production.

A possible explanation to the less effective chemotherapy response is that commensal microbiome members within the gut of the mice are able to produce TLR agonists, thus promoting the increase of an oxidative stress environment and the death of cancer cells (Iida N. et al., 2013).

Consistently, mice bearing lung tumours treated with cisplatin coupled with antibiotics, survive less and develop bigger tumours. Importantly, combined administration of cisplatin and probiotics, such as *Lactobacilli*, ensures a better response to therapy in these mice. The mechanism on which this effect is based involves the induction of pro-apoptotic genes within the tumour mass and the enhancement of the host's immune response (Gui Q.F. et al., 2015).

Similarly, combined administration of cyclophosphamide, a widely used anti-cancer molecule, and *Lactobacillus johonsoni* or *Enterococcus hirae*, improve drug efficacy in tumour-bearing mice, with a mechanism involving the

conversion of T cells from naïve to pro-inflammatory T helper 17 (TH17) (Viaud S. et al., 2013; Daillère R. et al., 2016).

Also with regard to immunotherapy, the probiotic bacteria administration has given excellent results. Given the effects that gut microbiome may play on the host's immune system, the probiotic bacteria would seem capable of inducing TNF production from tumour infiltrating myeloid cells which, in turn, reduce the growth several types of tumours in mice (Iida N. et al., 2013). It has in fact been shown that the administration of a specific bacteria, *Alistipes shahii*, to antibiotic-treated tumour bearing mice, as well as the intra-tumour injection of CpG oligodeoxynucleotides (synthetic molecules mimicking bacterial DNA) administered together with an anti-interleukin-10 receptor (IL-10R) antibody, restores TNF production with a notable improvement in the therapeutic outcome (Iida N. et al., 2013; Jahrsdörfer B.et al., 2008).

Following on from the microbiome effects on the host's immune system, it is conceivable that patients' microbiome composition strongly linked with the intrinsic efficacy of immune checkpoint inhibitors-based immunotherapy

Considering that the immune checkpoint inhibition consists in the administration of monoclonal antibodies targeting CTLA4 or PD-1, these therapeutic agents are able to block the immune-inhibitory pathway, respectively regulating T-cells proliferation early in the immune response within the lymph nodes and suppressing T-cell activation later, within the body periphery (Chen Q. et al., 2018; Buchbinder E.I. et al., 2016).

It is not surprising, in fact, that the potential involvement of the gut microbiome in modulating the efficacy of such anti CTLA4 and anti-PD1 based therapies has been brought to light, inspiring the succession of fairly recent studies that support the pivotal role of the gut microbiome in modulating the response to immune checkpoint blockade.

In fact, several studies have shown that the efficacy of anti-CTLA4 antibodies and / or anti-PD-L1 antibody in reducing mice's tumour growth, significantly increase when the gut microbiome is enriched in *Bacteroides fragilis* and *Burkholderia*, in *Bifidobacterium* species or with cocktail of Bifidobacterium species (Vétizou M. et al., 2015; Sivan A. et al., 2015).

In addiction, multiple translational studies, availing itself metagenomics analysis of patients' faecal gut microbiome, strongly support the pivotal role of the gut microbiome in modulating the response to immune checkpoint blockade (Routy B. et al, 2018; Gopalakrishnan V. et al., 2018; Matson V. et al., 2018). The metagenomics analysis of patients' fecal gut microbiome showed a difference in the composition of their gut microbiome, corresponding to a different response to therapy.

The authors observed in patient's gut microbiome an increase in the abundance of Clostridiales, Ruminococcaceae and Faecalibacteriae, which used together with anti-PD1 therapy, in functional studies performed with FMT in germ free mice, have increased anti-cancer effects and have reduced tumour growth (Gopalakrishnan V. et al., 2018).

Similarly, other researchers found that melanoma patients treated with antibiotics along with the anti-PD1/anti-PD-L1 immunotherapy had a lower survival rate. Following metagenomics analysis of patients' faecal gut microbiome showed a difference in the composition of their gut microbiome, anti-PD1 responders were enriched in Akkermansia and Alistipes species. Interestingly, even in this case, performing FMT from patients to germ free mice, the authors found that *Akkermansia muciniphila* was able to increase intra-tumour cytotoxic T cell infiltrates, thus increasing the PD-1 blockade response in mice (Rout B. et al, 2018).

Consistent with the aforementioned scientific evidence, Matson et al. have identified and functionally proven in vivo the importance of *Bifidobacterium longum*, *Enterococcus faecium* and *Collinsella aerofaciens* in ameliorating anti-PD-L1 efficacy (Matson V. et al., 2018).

Unfortunately, the significant number of patients that can use such therapy only for a limited amount of time, given the occurrence of strong toxic side effects, including gut inflammation, nullifies the great immune checkpoint inhibitors success in treating malignancies (Larkin J. Et al., 2015).

Fortunately, however, various experimental evidences provide a strong evidence of the role of gut microbiota composition in modulating the effect of

both immunotherapy response and toxicity ((Chaput N. et al., 2017; Frankel A.E. et al., 2017).

Although the gut microbiota provides a number of solutions useful in cancer treatment, it could represent a double-edged sword for tumour progression and therapy, depending on its composition and prevalent species.

Thus, the translation of the in vitro and in vivo results to the clinic needs to be carefully evaluated, so not to risk that any one antibacterial therapy, altering the intestinal equilibrium, facilitate the development of potential pathogens.

In this regard, a personalized approach, based on the specific patient's microbiome composition may be the best way forward in anti-cancer therapy using for example the probiotics, given the enormous beneficial effects that they even demonstrate to carry out in anti-cancer therapy.

3. PROBIOTICS

Probiotics are live non-pathogenic microbes, that play a central role in the host`s health benefits by strengthening the intestinal ecosystem when administered in sufficient quantities.

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

- safe for in 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 observable benefits.
- able to persist and multiply in the human intestine.
- able to confer physiological benefits observable through studies carried out following the FAO / OMS guidelines.

In addiction, other characteristics recognized as typical of probiotics are mucosal or epithelial cells adhesion, antimicrobial resistance, bile salt hydrolase potential (World Health Organization-Food and Agricultural

Organization, 2002), immunostimulation, antagonistic activity against pathogens, antimutagenic and anticarcinogenic activities (R. Crittenden et al., 2002).

Based on these characteristics, microorganisms categorized into bacterial or lactic acid and non-lactic acid bacteria strains and yeasts, including Lactobacillus, Lactococcus, Bifidobacterium and Enterococcus are considered probiotic microorganisms (K. Georgiev et al., 2015).

Biological effects produced by probiotics is important to underline that are strain-specific, so much so that the use of a new bacterial strain, even if belonging to a species already used, requires a new evaluation of safety and efficacy.

Taken as a whole, probiotics performs many and important functions in maintaining the good state of host health (Macpherson A. J. et al., 2001).

Probiotics and their pro-bioactive cellular materials is known that produce several beneficial effects in the gastrointestinal tract, and release different enzymes establishing potential synergistic effects on digestion.

Lactobacilli, for example, are able to convert lactose into lactic acid; their intake can help lactose intolerant individuals digest it more than they be able to otherwise in their absence. This beneficial effect carries through with Lactobacilli thanks to the production of the enzyme β-galactosidase, capable to break down lactose into, better digestible, glucose and galactose (Sanders ME. Et al., 2000).

Probiotics effectively block antibiotic-associated diarrhoea, a pathological condition that, by causing an alteration of the intestinal microbiota, produces changes in the carbohydrates metabolism, with reduced absorption of short-chain fatty acids and consequent osmotic diarrhoea (Mcfarland LV. Et al., 2006; Szajewska H. et al., 2006; Sazawal S. et al.,2006).

Furthermore, probiotics administrated with food are a valid adjuvant therapy in many gastrointestinal diseases such as irritable bowel syndrome, lymphomas and obesity, caused by the alteration of the microbiota (P. Marteau, 2010; R. Rastmanesh, 2011).

Overall, specific cellular constituents in probiotic lactic acid bacteria induce potential adjuvant effects such as cell mediated immune responses modulation, reticulo-endothelial system activation, cytokine pathways augmentation, and interleukins and tumour necrosis factors regulation (M. Kumar et al., 2010).
It has also been shown that some Lactobacilli have an anti-mutagenic effect, presumably due to the ability to bind heterocyclic amines, which, following cooking, are produced by meat-contained carcinogenic substances (Wollowski I. et al., 2001).
Conclusively, probiotics action mechanisms recognized in anti-tumour and anti-mutagenic activity are: binding, degradation and inhibition of mutagen by probiotics; pro-carcinogen prevention and conversion of harmful, toxic and highly reactive carcinogens; gut pH lowering by short chain fatty acids (SCFAs) formed during degradation of non-digestible carbohydrate; host's innate immunity modulation and enhancement through secretion of anti-inflammatory molecules (M. Raman et al., 2013).
For what has been said, it is not surprising that the scientific path pursued is aimed to finding potential strains of probiotics, effective administration dose and prevention and cancer treatment molecular mechanisms.

3.1 Probiotics for the prevention and treatments of cancer

Probiotics, being mostly considered as safe, affordable than standard drugs and having a long history of usage, have been widely used in vitro study, in vivo animals and clinical trials in humans, all of which have demonstrated the importance of probiotics in protecting host's health (S.S. Choi et al., 2006; J.E. Kim et al., 2007; M. Eslami et al., 2019).
Actually, probiotics ability in the modulating of gut microbiota composition has shown that they are useful for the safety of traditional anticancer therapies such as chemotherapy and radiotherapy (S. Shamekhi et al., 2020).
Although chemotherapy, targeted therapy, immunotherapy and radiotherapy represent the pillars of the currently available anti-cancer treatments, such treatments may cause diverse and even drastic side effects in patients (Bajic

J.E. et al., 2018; Scaife J.E. et al., 2015; Griggs J.J., 1998; Samaan M.A. et al., 2018; Dong J. Et al., 2018; Shahid F. et al., 2018; Lawrie T.A. et al., 2018).

The aim of administering probiotics to cancer patients, principally Lactobacilli, is to re-populate the compromised patients' gut microbiota, thus re-establishing the levels and functionality of the commensal bacteria, decreasing the risk and the severity of such anti-cancer treatments, diarrhoea and microsites related (Alexander, J.L. et al., 2017; Sokol, H. et al., 2018; Zitvogel, L. et al., 2018).

Given that consuming traditional fermented foods with sufficient dose of probiotics can reduce the risk of tumour development, supporting the fundamental role of microbiome in cancer (S. Shamekhi et al., 2020), many clinical studies are currently ongoing with the common aim of highlighting therapeutic of manipulating gut microbiota prospective in cancer patients.

In vitro experiment have shown that live whole probiotic strains of Enterococcus faecium (E. faecium) RM11 and Lactobacillus fermentum (L. fermentum) RM28 from fermented milks can be used for CRC treatment or prevention or in functional food. Both probiotics triggered colon cancer cells anti-proliferation and also showed anti antimicrobial activities against the growth of pathogens (M. Thirabunyanon et al., 2009).

Recently, some clinical trials evaluated the administration effects of a mixture of two probiotic species Bifidobacterium and Lactobacillus in cancer patients, demonstrating for the first time, the interaction between administration of probiotics, variation of the gut microbiota composition and regulation of intestinal immune functions in cancer patients as well as significant reducing moderate and severe treatment-induced diarrhoea during pelvic radiation (Y. Rahbar et al., 2020; L. Wang et al., 2019).

Importantly, a clinical trial evaluated the probiotic mixture of 10 different probiotic strains in the prevention of diarrhoea in patients with metastatic CRC, treated with irinotecan-based chemotherapy, suggesting that the administration of such probiotics is safe and leads to a reduction in the incidence and severity of diarrhoea and chemotherapy induced gastrointestinal toxicity (Mego, M. et al., 2015).

Contextually, many clinical studies, manipulating gut microbiota in cancer patients through administration of the probiotics, have shown that probiotics are able to alleviate irritable bowel syndrome, following the operation; down-regulate pro-inflammatory cytokines in treated patients; change the epigenetic patterns of tumour tissue from its baseline, with potential therapeutic benefits in CRC; reduce postoperative infection rates in patients with CRC (Theodoropoulos G.E. et al., 2016; Consoli M.L. et al., 2016; Hibberd A.A. et al., 2017; Flesch A.T. et al., 2017).

Noteworthy, although in contrary to the general definition of probiotics (J.M. Wells, 2011), is the evidence that dead probiotics or its cell components can combat cancer effectively.

In a study done by Lee et al., dead L. plantarum demonstrated superior suppressive potentials to those of pure live probiotic, and especially, dead probiotic administration at high dose reduced a considerable number of tumours compared with pure live probiotic (H.A. Lee et al., 2015).

Moreover, combined administration of pure live and dead probiotics significantly reduced pro-inflammatory cytokines and inflammatory genes overexpression, and suppressive potentials than separate administration of either groups.

In fact, L. plantarum administration suppressed the development of neoplasy significantly, in all the experimental control animals group, possessing colon tumours.

Apparently, dead probiotic sustains the status of mucosal immune system by increasing levels of secretory IgA, thus indicating the antitumor property of dead probiotic is related with the easier uptake of dead probiotic by M cells than pure live probiotic and than the stronger secretory immune responses (H.A. Lee et al., 2015).

Besides dead probiotic bacterium, secretory molecules of cell free supernatant from probiotics play an important role by exerted anti-metastatic and anti-proliferative effects.

Besides dead probiotic bacterium, secretory molecules of cell free supernatant from probiotics play an important role by exerted anti-metastatic and anti-proliferative effects.

Polysaccharide, protein secretory or nucleic acid macromolecules, in fact, was demonstrated that, exerting inhibitory properties, involved in vitro cell growth inhibition and cells apoptosis increment, through regulating gene profile expression related to the progression of cell cycle and growth of tumour (F. Maghsood et al., 2020).

3.2 Probiotics metabolites for the prevention and treatments of cancer

Since probiotics are biologically active, their evolution, metabolism, physiology and probiotic properties will be appreciated (K. Papadimitriou et al., 2011), in long term effect studies, in the direction of future methodologies standardization.

To date, probiotics strains are known to be responsible for some important effects, including gene transfer, metabolic activities and immune stimulation in the host.

As well as it has now been established that molecules and metabolites derived from probiotics including, Lipopolysaccharide, exopolysaccharide (EPS) and polysaccharides, SCFAs and lipid, protein and other metabolites like nucleic acid, can prevent tumour development through modulation of immune systems of the host, also inducing apoptosis in cancer cells, or preventing metastasis of colon cancer cells (Tesfaye Legesse Bedadaa et al., 2020).

For instance, bacterial lipopolysaccharide (LPS), a key component of gram-negative bacteria outer membrane, activates toll like receptor 4, consequently activating immune T cell mediated response against tumour cells (T. Cd et al., 2007).

While EPS extracted from probiotics plays a fundamental role in prevention and treatment of cancer.

Several scientific data indicate that lactic acid bacteria found in the gut have a role in regression of cancer through their effect on immunomodulation,

activating phagocytes to remove early stage tumour cells (A. Górska et al., 2019; A. Borowicki et al., 2012), or have a direct cytotoxic effect on the tumour cells by mechanisms of apoptosis, immune response stimulation and NF-κB inflammatory pathway inactivation, as demonstrated for L. acidophilus 20,079 EPS (N.M. El-Deeb et al., 2018). In fact, cell wall components of lactic acid bacteria, including L. acidophilus and L. casei, L. plantarum, L. rhamnosus E9, L. brevis LB63, act respectively, as anticancer substances, prevents the proliferation of hepatocellular carcinoma cell line and has an anticancer effect on colon cancer cells (HT-29) (N.M. El-Deeb et al., 2018; I.G. Bogdanov et al., 1975; C. Wang et al., 2015).

As pointed out by several authors EPS constitutes rhamnose, galactose, glucose, arabinose, and mannose, showing anticancer property against head and neck squamous cell carcinoma cell line, inhibit various colon cancer cell lines (Y. Rahbar et al., 2020), and breast cancer cell line, MCF7, prevents the proliferation of human pancreatic tumour cell line (Y. Rahbar et al., 2020; G. Zhang eta al., 2014).

Other Lactobacilli extracts, as SCFAs, conjugated linoleic acid, induce apoptosis in cancer cells. Several probiotic strain, in fact, are potential candidates for the treatment of CRC thanks to their ability to produce SCFAs which prevent the proliferative activity of in vitro tumor cells.

Probiotics L. fermentum NCIMB 5221, 2797 and 8829, Pediococcus pentosaceus FP3, Lactobacillus salivarius FP35, Lactobacillus salivarius FP25, and E. faecium FP5, has been reported that are able to produce this effect (I. Kahouli et al., 2015; M. Thirabunyanon et al., 2013). While, SCFAs metabolites produced by probiotic Propionibacterium freuden reichii damage colorectal adenocarcinoma cells by producing apoptosis in vitro (L.D. Lagadic-gossmann et al., 2007).

Similarly, conjugated linoleic acids produced by probiotic bacteria have the ability to form anticarcinogenic effects in vitro and in vivo in mice (J.B. Ewaschuk et al., 2006), as demonstrated by the Probiotic L. reuteri capable to prevents CRC causing lesions by producing propionate (I. Kahouli et al., 2016).

Notably, compounds produced by probiotics, having beneficial effects against tumour cells, might be a protein, polysaccharide or a nucleic acid (M. Kleerebezem et al., 2010).

Altogether, the cell-free supernatants are fighting tumour cells effectively when using colon cancer cell line, as SW742 or HCT-116 (G. Mcintosh, 1996; J. Escamilla et al., 2012).

Interestingly, cell free supernatants containing secreted bioactive macromolecule from L. casei and L. rhamnosus LGG probiotics prevented metastasis of in vitro human colon carcinoma cell line (HCT-116) (J. Escamilla et al., 2012).

This surprising effect has been observed to reduce the activity of matrix metalloproteinase-9 (MMP-9), and enhanced zona occludens-1 (ZO-1) levels. Considering that MMP-9 is a proteolytic enzyme used to digest colon extracellular matrix, a reduction in matrix MMP-9 levels indicating a decrease in MMP-9 potential due to reduced synthesis of protein, supporting the cell invasion reduced process during metastasis (O.R.F. Mook et al., 2004).

Similarly, the enhance of ZO-1 protein that is down-regulated (S. Ohtani et al., 2009) in metastatic tumours, supports the function of healthy colonic epithelial barrier (K.A. Donato et al., 2010).

To date, research revealed varied probiotic derivatives with anti-cancer therapeutic effects as p8 protein which arresting the induction of cell cycle in in vivo experiments, monophosphoryl lipid A derived from Salmonella enterica using as adjuvant in anti-cervical carcinoma vaccine formulation (M. Pancione et al., 2012) as well as a group B vitamin pyridoxine from bacteria stimulates anti-tumour host immune-surveillance
(Q. Wu et al., 2012).

3.3 Lactobacillus rhamnosus GG: a star of the landscape probiotic against cancer

Lactobacilli are part of the lactic acid bacteria family and derive almost all their energy from the fermentation of glucose and lactose to lactic acid. Their

metabolism generates ATP through non-oxidative phosphorylation of the substrate. Some bacteria belonging to the Lactobacillus genus are able to produce, through their metabolism, small quantities of H2O2 as well as other bioactive molecules.

The probiotics, including Lactobacilli, are studied as supportive treatment for chemotherapy-associated gastrointestinal toxicity, thanks to their ability to restore gut microbial balance (H.A. Lee et al., 2015).

Lactobacillus rhamnosus GG (LGG) occupies a prominent place in the panorama of bacteria. In fact, due to its anti-inflammatory properties, the probiotic LGG is one of the most studied and well characterized among probiotics.

LGG is a gut resident bacterium known to have several anti inflammatory effects within the intestinal microenvironment (Khailova, L. et al., 2017; Wang, Y. et al., 2017; Fong, F.L. et al., 2016), and among probiotic species, it is one of the first studied specifically in oncology (Goldin, B.R. et al., 1996).

Consistently with in animal models studies that demonstrated a beneficial effect of LGG in the preservation of the gut microbiota balance and the intestinal epithelial barrier functionality when administrated as adjuvant in 5-FU and radiation treatment, a number of ongoing clinical trials are currently focused on establishing the role of LGG administration in preventing or ameliorating the toxic effects of anti-cancer therapies (S. Vivarelli, et al., 2019).

Interestingly, two ongoing clinical trials are evaluating the efficacy of LGG daily oral administration in the maintenance of normal gastro-intestinal functions in cancer patients treated either with chemotherapy and/or targeted therapy or abdominal/pelvic radiotherapy.

The big goal, which many groups, are trying to achieve is that to demonstrate the LGG potential role in the direct modulation of cancer development.

To date, LGG has been observed to exert its effect either directly on cancer cells or indirectly through the modulation of the immune system, both in vitro and in vivo.

Regarding to LGG capacity of counteracting cancer growth, this is able to exert either anti-proliferative effects or anti-metastatic effects (Orlando, A. et al., 2016; Nouri, Z. et al., 2016; Zhao, B.B. et al., 2017; Behzadi, E. et al., 2017; Cheng, Z. et al., 2017), probably through the direct modulation of several host's proliferation pathways, including mTOR or WNT, as it has been highlight within several in vitro tumour models (Ni, Y. et al., 2017; Mendes, M.C.S. et al., 2018).

While, LGG has been demonstrated that can influence host's immune system, eliminating newly developing cancer cells, in a rat colon cancer model (Gamallat, Y. et al., 2016); but not only. In fact, LGG can triggers the immune response also within the normal not transformed gut epithelium, thus protecting towards inflammation, which can support the formation of a cancer-favourable milieu (Suzuki, C. et al., 2017).

Overall, the currently ongoing clinical studies became on item with the in vitro and in vivo studies, make LGG a suitable candidate to be further characterized as possible adjuvant in integrated anti-cancer therapies and above all make it worthy of further study supporting its candidature as direct cancer modulator.

4. AIM OF THE STUDY

Considering the complex relationship between gut resident microbiota and their host, now, there is a growing attention towards the characterization of the gastro-intestinal microbiota composition and functionalities.

Even if some bacterial subpopulations are able to trigger the formation of an inflammatory and pro-cancerogenic environment, on the other side, many gut derived probiotics are able to protect the host, re-establishing the conditions of a healthy intestinal microbiota, including in cancer patients.

Moreover, the commensal bacteria and other microorganisms that colonize the epithelial surfaces of our body have been shown to produce small molecules and metabolites that have both local and systemic effects on cancer onset, progression and therapy response.

Related to the profound influence of the microbiota on the efficacy of cancer treatments, LGG is a very good example of a probiotic well studied in cancer, often administered as complementary therapeutic to cure dysbiosis but that has also proved anti-inflammatory functions and anti-cancer in both cellular and animal models.

In line with that observations the purpose of this work is identify correlative relationship between Lactobacillul rhamnosus GG microbial specie and some cancer phenotypes.

Specifically, in this work the use of the Cell-Free Supernatant from Lactobacillus Rhamnosus GG is proposed to understand how and if the metabolites and bioactive molecules produced from LGG have anti-tumour effects and its possible candidature as an adjuvant in integrated anticancer therapies.

5. MATERIALS AND METHODS

5.1 Bacterial Species and experimental design

In this study, the probiotic strain Lactobacillus rhamnosus GG (ATCC 53103) was used, kindly granted by Dicofarm Spa, as part of the collaboration between Department of Biomedical and Biotechnological Sciences (BIOMETEC) of the University of Catania and Dicofarm Company.

L. rhamnosus GG was chosen as a probiotic Lactobacillus because of broad presence in the human intestinal microbiota, easy cultivation and growth, previously established anti-inflammatory effects and preventing human diseases such as diarrhoea.

The study focusing on the effect of LGG cell-free supernatants on in vitro tumour cells.

Considering these points, in this study, we evaluated levels of apoptosis mediators and cell cycle following treatment with L. rhamnosus GG cell-free supernatant on HT-29, Caco-2, HCT-116 and A375 cell lines.

5.2 L. rhamnosus GG Culture Supernatants Preparation

L. rhamnosus GG, 200 µl of "starter " (1x 10^8 CFU/ml) was cultured in in 50 ml of Man Rogosa Sharpe (MRS) broth media (pH=6.5) at 37 °C without agitation. Following incubation for 20 h, bacterial cultures reached late-log/early stationary phase of growth with an optical density (OD) 2.43 nm, which complies with bacterial numbers of approximately 2.9 $\times 10^9$ cfu/ml. The CFS pH decreased from 6.5 to 4. Bacterial cultures were centrifuged at 7000 RPM for 30' min at 4 °C, washed twice with sterile phosphate-buffered saline (PBS) and cultured in in 50 ml of RPMI-1640 medium (pH=7.5) at 37 °C without supplemented with L-glutamine, penicillin, and fetal bovine serum (FBS). Following incubation for 5 or 20 h, bacterial cultures reached phase of growth, respectively with an optical density (OD) 1.88 (which complies with bacterial numbers of approximately 2.4 $\times 10^{10}$ cfu/ml) and 1.83 nm (which complies with bacterial numbers of approximately 2.1 $\times 10^{11}$ cfu/ml), with for

both the CFS pH decreased from 7.5 to 6.5 (Table 1) . Following centrifugation CFS was filtered by a 0.22 μm membrane filter to exclude the bacteria cells and the debris. The CFS, thus obtained, was supplemented with 2 mmol / L L-glutamine and 100 IU penicillin and 10% FBS, used on subsequent cancer cells tests.

Table 1. Cell-Free Supernatants from Lactobacillus Rhamnosus GG

Medium	D.O. a 600nm after 5 h of growth	D.O. a 600nm after 20 h of growth	Bacterial counts on plate	pH
MRS-LGG (20hrs)		1,91	$2,9x10^{9}$ CFU/ml	4
RPMI-LGG (5hrs)	1,88		$2,4x10^{10}$ CFU/ml	6,5
RPMI-LGG (20hrs)		1,83	$2,1x10^{11}$ CFU/ml	6,5

The optical density D.O. (measure of bacterial growth) in the different media and after the different incubation times with LGG is comparable. LGG, as demonstrated by the bacterial counts on the plate, is vital after incubation in both MRS and RPMI (both after 5 hours and after 20 hours)

5.3 Cell Culture

Human colon cancer HT-29, HCT-116, CAco-2 and melanoma A375 cells were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, United States). The cell lines were cultured in RPMI-1640 medium containing 2 mmol/L L-glutamine, 100 IU penicillin, 100 mg/ml streptomycin and 10% FBS and incubated at 37 °C in a humidified incubator with 5% CO2. Mediums and all the supplements were provided by Lonza (Walkersville, USA).

Cells were seeded in 25cm2 flasks with vent cap and they were passaged by 1:10 ratios using trypsin–EDTA 0.05% every 72 h. For all the experiments, the cells were later cultivated in LGG-CFS (as reported above); cells density and plates or dishes used, will be indicated in the following sections.

5.4 MTT Assay

Cytotoxicity was measured using the MTT assay. 3 ×103 cells, for each cell line, were cultivated into 96-well culture plates in complete RPMI-1640 media (with L-glutammine, penicillin and FBS) and incubated for 24 h.
Afterward, cells were stimulated for 72 h with increasing doses of LGG-CFS (10-20-30-40-50-60-70-80-90% v/v), and incubated in 5% CO2 at 37 °C in a humidified incubator. The media was removed, and 20 μL of MTT working solution [5 mg/ml in PBS] was supplied to each well for 4 h at 37 °C in a humidified incubator. After aspirating the medium, 100μL of the solution of 2-propanol and hydrochloric acid (50 mL + 167 μL) was added to each well to solubilize formazan blue crystals. The absorbance was measured at 590 nm with the ELISA Tecan Sunrise Reader according to the manufacturers' instructions. Cell viability was calculated as follows:

Cell viability percentage = [(sample OD − blank OD⁄control OD − blank OD)] × 100

5.5 Proliferation Assay

Proliferation measured using the BrdU assay. All cell lines were seeded in 96-well plates (3.000 cells/well; except Caco-2, 6000 cells/well), each treated for 48h with different percentage of LGG-CFS (0%, 20%, 50% e 90% vol.). The cells were plated in 20 μL of FBS (100% FBS) in order to obtain a final concentration of 10% of FBS in 200 μL, the same for all treatment conditions.
BrdU Cell Proliferation Assay involves incorporation of BrdU Reagent into cells cultured. During the final 2 to 24 hours of culture 1X BrdU Reagent is added to wells of the plate. BrdU Reagent will be incorporated into the DNA of dividing cells. To enable antibody binding to the incorporated BrdU, cells must be fixed, permeabilized and the DNA denatured. Prediluted Anti-BrdU Detector Antibody is pipetted into the wells and allowed to incubate for one hour, during which time it binds to any incorporated BrdU Reagent. Unbound

Prediluted Anti-BrdU Detector Antibody is washed away and 1x Peroxidase Goat anti-Mouse IgG is added, which binds to the Prediluted Anti-BrdU Detector Antibody. The 1x Peroxidase Goat anti-Mouse IgG catalyzes the conversion of the TMB Substrate (chromogenic substrate tetra-methylbenzidine) from a yellow solution to a blue solution, the intensity of which is proportional to the amount of incorporated BrdU Reagent in the cells. The colored reaction product is quantified using a spectrophotometer. All reagents were used according to the manufacturer's instructions (BrdU Cell Proliferation Assay - Millipore). The optical density (OD) of each sample was measured with a microplate reader, at 450/550 nm.

5.6 Clonogenic assays

Approximately 72 hours post treatment with LGG-CFS 90% v/v, the melanoma cells (used as model cells) were seeded at 200 cells per well onto 24-well culture plates and allowed to grow for a week in complete RPMI-1640 media. Cells were then washed twice with ice cold PBS and fixed with ice-cold methanol for 1 minute.

After aspirating methanol from plates, 0.5% crystal violet solution was added and the plate was incubated at room temperature for 5 minutes. Distilled water was used to rinse the plate. Colony-forming assays were performed at least twice in quadrupled.

5.7 Apoptosis Assay

All cells were cultivated at a density of 1×10^6 cells/well in dish (10 cm^2) and maintained for 24 h at 37 °C in a 5% CO2 incubator. The cells were seeded for 48 h with 90% (v/v) of LGG-CFS, witheout LGG-CFS and Vincristine 0,250 mM (positive control for apoptosis). Subsequently, the cells were trypsinized by Trypsin EDTA 0.05% and centrifuged.

Then, cell pellets were suspended in 100 µl Annexin V binding buffer in double-labelled with low concentration propidium iodide (1-5 µg / mL) (Alexa

Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI, Thermo Fisher Scientific) in accordance with the manufacturing protocol. The necrosis, early apoptosis and late apoptosis percentage was quantified in the cells population using the Amnis FlowSight flow cytometer (Luminex, USA). IDEAS 6.1 software was used to analyse the FACS data. All data from the LGG-CFS group were compared with untreated cells (negative control group), and Vincristine group (positive control group).
A part of the pellet was stored at -80 ° for subsequent western blotting analyses and further applications.

5.8 Western Blot

Proteins were extracted from cellular pellets lysed in cell lysis buffer (NP40) (150 mM NaCl, 1.0% NP40, pH 8.0 50mM Tris), supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). After the lysis, the supernatant enriched of interest proteins was separated through centrifugation. The Quick Start™ Bradford 1X Dye Reagent (Biorad Laboratories, Inc., Hercules, CA, USA) assay was performed on properly diluted proteins. This colorimetric assay exploits a dye, able to bind to alkaline residues of the proteins leading to a bathochromic shift, from 465 nm to 595 nm. A standard curve was produced using progressively higher known concentration of bovine serum albumin (BSA) di (2.5, 10, 50, 100, 250, 500, 750, 1,000, 1,500, 1,750, and 2,000 ng/µl) and a blank with NP40 diluted 1:10 with water. Sample's proteins concentration was determined by placing in separate wells different dilution of the protein extract. Each well contained 5µl of diluted protein + 250 µl of dye reagent. After 5 minutes of incubation at room temperature, the absorbance was detected with the Tecan Sunrise ELISA reader at the wavelength of 595nm and the concentration calculated.
For each sample, 30 µg of proteins were separated through vertical electrophoresis using 4–15% Mini Protean TGX Precast Gels (cat. n. 4561083 - Bio-Rad Laboratories, Inc., Hercules, CA, United States). Bio-Rad Trans-

Blot Turbo was used to transfer the gel proteins into a nitrocellulose membrane (Bio-Rad Laboratories, Inc.).

The transfer of protein was assessed with the Red Ponceau dye and after being rinsed with TBS-T (0.1% Tween 20, 20 mM Tris–HCl pH 7.6, 137 mM NaCl), the membrane was left in 5% semi-skimmed milk for one hour.

Primary antibodies were incubated overnight. The anti- Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (Cell Signaling, #9664) and Anti-PARP (46D11) Rabbit mAb #9532 (Cell Signaling) were used to detect two apoptosis markers, cleaved Caspase-3 and total / cleaved PARP proteins. Anti-Actin antibody [ACTN05 (C4)] housekeeping protein was detected by using Anti-Actin antibody [ACTN05 (C4)] antibody mause Ab (Abcam, ab3280).

Afterward, the membrane was rinsed three times with TBS-T solution for ten minutes. Subsequently, the membrane was incubated for one hour at room temperature with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) (diluted 1:10000). Again, the membrane was rinsed three times with TBS-T.

Chemiluminescent detection was performed using Clarity Western ECL Substrate (cat. n. 1705060 - Bio-Rad Laboratories, Inc., Hercules, CA, United States). Western blot images were collected by Bio-Rad ChemiDoc Touch Imaging System and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, United States).

All Western Blot experiments were performed in triplicate.

5.9 Cell Cycle analysis

The tumor cells and healthy cells under study were seeded respectively in 3 dishes (10 cm2): HT-29 and HCT-116 at the density of 650,000 cells / dish (dish volume: 10 mL); Caco-2 and Fribroblasts at the density of 600,000 cells / dish (volume dish: 10 mL); A375 at the density of 500,000 cells / dish (dish volume: 10 mL).

The treatment was carried out by culturing the cells without LGG conditioned medium (control) and with LGG conditioned medium 90% vol. and with Vincristine (0.250 mM) for 48hrs.

At the end of 48hrs the cells were collected and cell count was performed in Trypan blue using a Burker chamber. After counting the cells were fixed in 70% ethanol by gentle swirling. The cell pellets thus prepared can be stored at +4 ° C for a few days. The cell pellets in ethanol were then centrifuged at 1200 rpm for 5 min at room temperature, resuspended in PBS twice. Therefore, the cell pellets were prepared for cell cycle analysis using propidium iodide (PI) 1mg / mL, RNase 100 mg / mL, TRITON 10% and PBS 1X. After incubation for 10 min at 37°C in the dark, the cell samples were ready to be analyzed using the Amnis FlowSight flow cytometer (Luminex, USA). IDEAS 6.1 software was used to analyze the FACS data. All data from the LGG-CFS group were compared with untreated cells (negative control group), and Vincristine group (positive control group).

5.10 RNA Extraction, cDNA Synthesis and Real-Time Quantitative RT-PCR

All cell lines were processed as previously described in section 5.5.

Total RNA extraction was carried out by Invitrogen™ PureLink™ RNA Mini Kit from treated cells according to the manufacturers' instructions. The ratio of absorbance at 260 nm and 280 nm was studied using a Nanodrop 1000 spectrophotometer (Thermo

Fisher Scientifc, Canada) to assess the RNA concentration and purity spectrophotometrically in molecular-grade water. Then, for each sample, 2 µg of total RNA were treated with DNase I, RNase-free (Cat. N. EN0525 - Thermo Fisher Scientific™) to remove possible DNA contamination. The cDNA was converted from the isolated total RNA by SuperScript™ IV Reverse Transcriptase kit (Cat. N. 18090050 - Thermo Fisher Scientific™). In brief, 1 µg RNA from each sample was added to 50 µM Random hexamers, 2.5 mM dNTPs and the reaction volume become 13 µL with RNase free water and mixed gently. Next, the mixtures were incubated at 65 °C for 5 min. After, the

mixtures were supplemented with 4 µL 5x Super Script Bufer, 100 mM DTT, 1 µL Super Script IV Enzyme and RNase free water to obtain 20 µL reaction volume. The whole were incubated at 23 °C for 10 min, 55 °C for 10 min in order to start the reverse transcriptase enzyme and incubated in 85 °C for 5 s to block the reaction. After reverse transcription reactions, cDNA was applied for realtime quantitative RT-PCR on 7300 Real-Time PCR System (Applied Biosystems). The RT-PCR was carried out in a fnal volume of 20 µL containing 10 µL SYBR green master mix, 50 ng cDNA, 0.18 µL forward primer (10 µM), 0.18 µL reverse primer (10 µM), and nucleasefree water to bring at volume (Luminaris Color HiGreen qPCR Master Mix, high ROX, Cat. N. K0362 - Thermo Fisher Scientific™). Sequences of primers are provided in Table 1. The thermal cycling program for all target and reference genes was as follows: pre-denaturation (2 min. at 50 °C), denaturation (10 min. at 95 °C), annealing, and extension (15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) for 50 cycles. The melting curve analysis condition was as follows: 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. Duplicate experiments were carried out for each data set. GAPDH mRNA was amplifed as a reference gene, and fold changes in each target mRNA expression were calculated relative to GAPDH mRNA expression via 2−ΔΔCT method.

Table 1 Sequence of the primers applied for qRT-PCR

Primer	Forward	Reverse
Cyclin-D	*5′-CCGAGAAGCTGTGCATCTAC-3′*	*5′-GGCGGTAGTAGGACAGGAAG-3′*
Cyclin-B	*5′-AAGAGCTTTAAACTTTGGTCTGGG-3′*	*5′-CTTTGTAAGTCCTTGATTTACCATG-3′*
Cyclin-A	*5′-CCAGTCCACGAGGATAGCTC-3′*	*5′-GCCTGCGTTCACCATTCATG-3′*
GAPDH	*5′-AGAAGGCTGGGGCTCATTTG-3′*	*5′-AGGGGCCATCCACAGTCTTC-3′*

5.11 Pharmacologic treatment

The pharmacologic treatments with Irinotecan and 5-Fluorouracil (5-FU) were performed to evaluate the possible candidature of LGG-CFS as an adjuvant in integrated anticancer therapies. All cell lines were seeded in 96-well plates (3.000 cells/well; except Caco-2, 6000 cells/well), each treated for 96h with different concentration of single therapeutic agents. The cells were plated in 20 µL of FBS (100% FBS) in order to obtain a final concentration of 10% of FBS in 200 µL, the same for all treatment conditions.
The drug treatments were carried out following two different approaches:

- by culturing all cells in fixed percentage of LGG-CFS: each cell line was maintained in culture in RPMI LGG-conditioned medium (50%), treating with decreasing doses of drug (serial dilutions 1: 4 in DMSO)
  - *5-FU*: 500 µM; 125 µM; 31.25 µM; 7.8125 µM; 1.953125 µM; 0.48828125 µM; 0.1220703125 µM; 0.030517578 µM; 0.00762939453125 µM; DMSO 0.25% for all points of the treatment.
  - *IRINOTECANO*: 200 µM; 50 µM; 12.5 µM; 3,125 µM; 0.78125 µM; 0.1953125 µM; 0.048828125 µM; 0.01220703125 µM; 0.0030517578125 µM; DMSO 1% for all points of the treatment.

- by culturing the cells in variable percentage of LGG-CFS per cell line: each cell line was kept in culture at LGG-conditioned medium percentage corresponding to its IC50 (determining 50% reduction in cell viability). Specifically, the HT-29 cells were grown in 70% LGG-CFS; HCT-116 and A375 cells were grown in 60% LGG-CFS; le Caco-2 in 90% LGG-CFS. Each cell line was then treated with increasing doses of the drug (serial dilutions 1: 4 in DMSO):
  - 5-FU: 500 µM; 125 µM; 31.25 µM; 7.8125 µM; 1.953125 µM; 0.48828125 M; 0.1220703125 µM; 0.030517578 µM;

0.00762939453125 μM; DMSO 0.25% for all points of the treatment.

- IRINOTECANO: 200 μM; 50 μM; 12.5 μM; 3,125 μM; 0.78125 μM; 0.1953125 μM; 0.048828125 μM; 0.01220703125 μM; 0.0030517578125 μM; DMSO 1% for all points of the treatment.

5.12 Statistical Analysis of Data

GraphPad Prism 6.0 software was applied for statistical data analysis. The Mean±SEM of three independent tests are shown for each group. $P < 0.05$ was considered meaningful. One-way analysis of variance (ANOVA) and Student t test were performed for determining statistical differences among groups. Dunnetts' adjustment was used for multiple comparisons.

6. RESULTS

6.1 Assessment of Cell-Free Supernatants from Lactobacillus rhamnosus GG in cancer cells viability

The growth-inhibitory effect of Lactobacillus rhamnosus GG (LGG) was examined in vitro against human colon cancer and melanoma cells. All cell lines were treated with increasing concentrations of bacterial cell-free supernatants. MTT assay was used to evaluate cell survival after 72 h incubation.

The percentage of growth inhibition of increasing concentrations of Cell-Free Supernatants from LGG against human HCT-116, HT29, Caco-2 colon cancer cell lines and A375 melanoma cell line is shown in Fig 1.

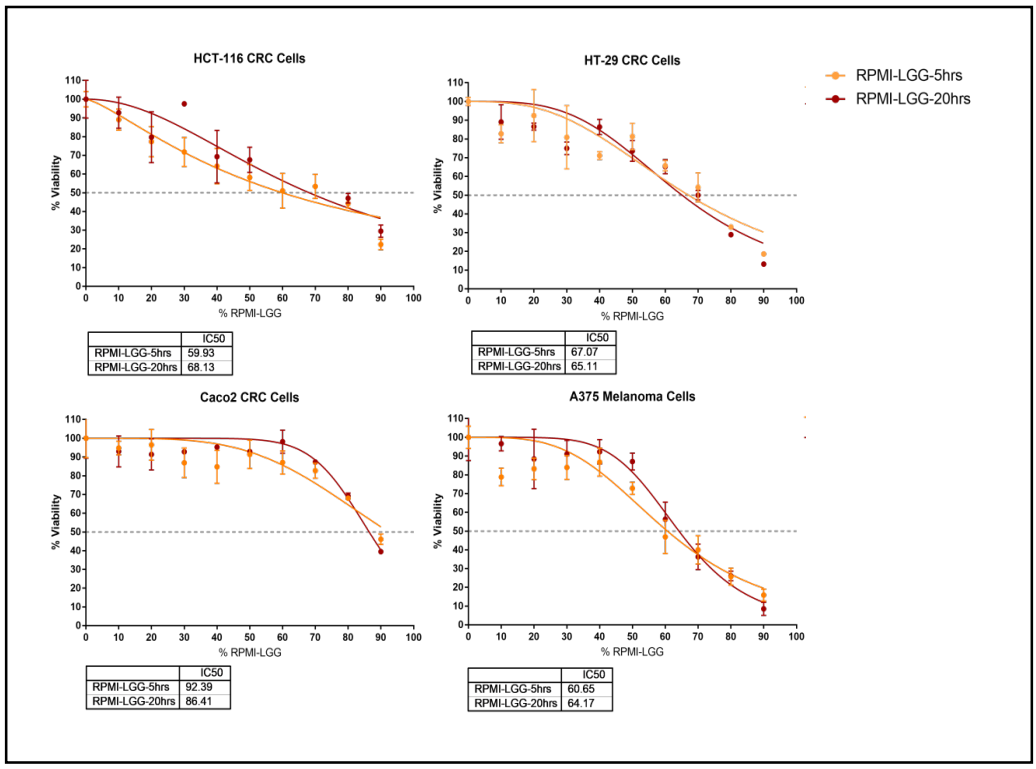
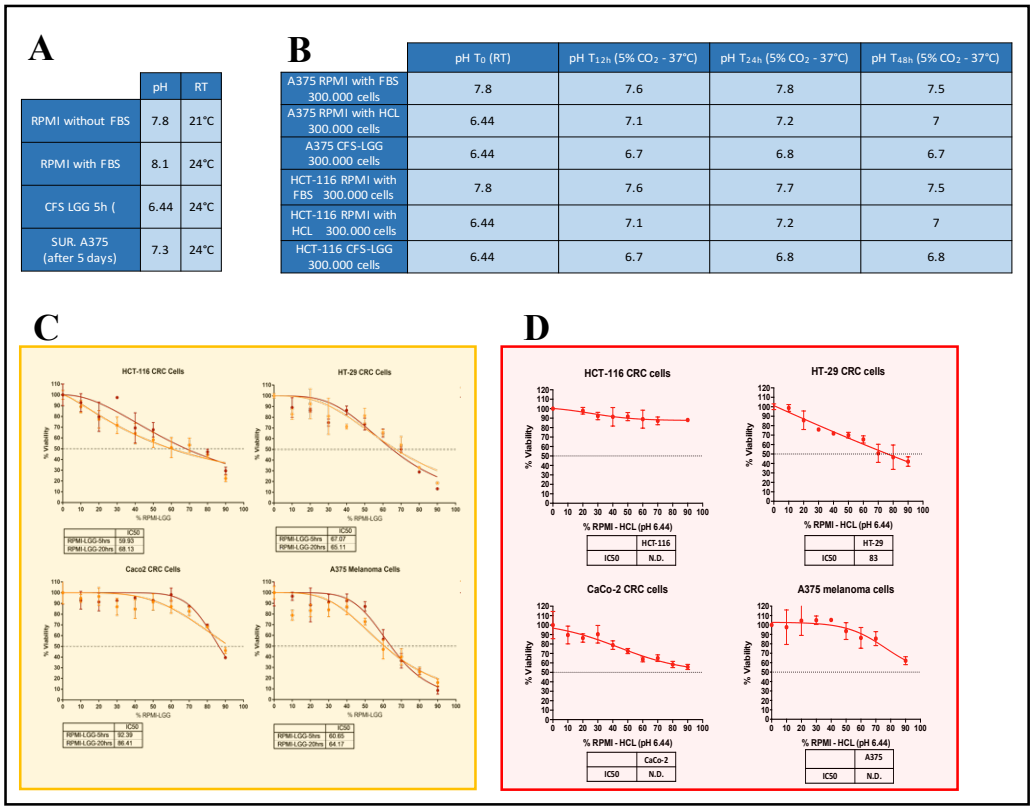


Figure 1. Inhibitory effect of Cell-Free Supernatants conditioned for 5 and 20 hrs from Lactobacillus rhamnosus GG on the growth of HCT-116, HT-29, Caco-2, A375 cell lines. Cells' viability evaluated through the MTT assay after 72 hrs of CFS-LGG at different concentration (0 - 10 - 20 - 30 - 40- 50 - 60 - 70 - 80 - 90 %/vol.). Data are expressed as mean ± SD of three separate experiments.

Both L. rhamnosus GG extracts (secreted products present in CFS), produced after 5 and 20 h, induce superimposable anti-proliferative effects in vitro and reduce the viability of human (HCT-116, HT29, Caco-2) colon cancer and human (A375) melanoma cell lines in a concentration-dependent manner.
Notably, the most pronounced anti-proliferative effect was induced by CFS-LGG on nearly all cell lines. Indeed, the concentration percentage of LGG cell free supernatants-conditioned RPMI between 65% and 70% has halved HCT-116, HT29 and A375 cell growth (IC50=59.93/68.13; IC50=67.07/65.11; IC50=60.65/64.17 respectively).
While LGG cell-free supernatant displayed no significant inhibitory effects on Caco-2 cell line. MTT analysis did not show significant differences between the different concentrations of LGG cell-free supernatant, with a slight inhibitory tendency to concentration of 90 %/vol (Fig.1).
The pH variation for each treatment point was also evaluated in order to exclude a dose response due to excessive acidification of the LGG conditioned media (pH range: 9 -› 6.5).

In order to investigate the correlation between the anti-proliferative effect of L. rhamnosus GG and pH decrease in cell culture medium due to bacterial acid production, we performed cell viability experiments culturing cells in HCL-conditioned RPMI medium to a range of predefined pH values (8–6.4), selected to correspond to pH measurements of culture medium supernatants following incubation of cells with CFS-LGG. A pH decrease below 7 did not significantly reduced the viability of cancer cells; the inhibitory effect, as compared to control cells incubated in medium with a pH of 7.8 (Fig 2D), was not proportional to the pH decrease (Fig 2C). We observed that acidic pH alone was not responsible for the anti-proliferative activity of L. rhamnosus GG against colon cancer cells and melanoma cancer cells, since, acidic pH values (HCL-conditioned RPMI medium) evoked lower growth-inhibitory effects than the incubation of cells for 72 hours with bacterial CFS (Fig 2D). Thus, for the

time period indicated, part of the growth-inhibitory effect of L. rhamnosus GG is independent of bacteria-induced pH decrease.



	pH	RT
RPMI without FBS	7.8	21°C
RPMI with FBS	8.1	24°C
CFS LGG 5h (	6.44	24°C
SUR. A375 (after 5 days)	7.3	24°C

	pH T_0 (RT)	pH T_{12h} (5% CO_2 - 37°C)	pH T_{24h} (5% CO_2 - 37°C)	pH T_{48h} (5% CO_2 - 37°C)
A375 RPMI with FBS 300.000 cells	7.8	7.6	7.8	7.5
A375 RPMI with HCL 300.000 cells	6.44	7.1	7.2	7
A375 CFS-LGG 300.000 cells	6.44	6.7	6.8	6.7
HCT-116 RPMI with FBS 300.000 cells	7.8	7.6	7.7	7.5
HCT-116 RPMI with HCL 300.000 cells	6.44	7.1	7.2	7
HCT-116 CFS-LGG 300.000 cells	6.44	6.7	6.8	6.8

Figure 2. Acidic pH in culture medium is not involved in the Lactobacillus rhamnosus GG anti-proliferative effect.
(A) pH values measured in RPMI medium with and without FBS, culture supernatants after incubation of LGG for 5 hrs and culture supernatants after incubation of A375 cell line in complete RPMI for 5 days. (B) pH values measured in RPMI conditioned medium with CFS-LGG or HCL at different time point. (C) Effect of acidic pH on the proliferation of tumour cells. The percentage growth inhibition refers to control each cell lines cultured in standard RPMI medium having a pH of 7.8. (D) Comparison of the growth-inhibitory effect of different percentage of CFS-LGG on tumour cells versus the effect of pH adjusted medium with HCL. Values of growth are not comparable to those induced by incubation with bacterial CFS concentrations at the same pH value. Results were reproduced in 3 independent experiments.

6.2 Cell-free supernatants from Lactobacillus rhamnosus GG inhibits proliferation of cancer cells

In order to be able to analyse the dose-dependent decrease of the cell population, due to an effect of the CFS of L. rhamnosus GG on the proliferative activity of tumour cells, a treatment was carried out by culturing the cells in increasing percentages of conditioned medium (RPMI) from LGG at the following concentrations: 0%, 20%, 50% and 90% vol. After 48hr the share of viable and dead cells was evaluated by cell count in Trypan blue using the Burker chamber.

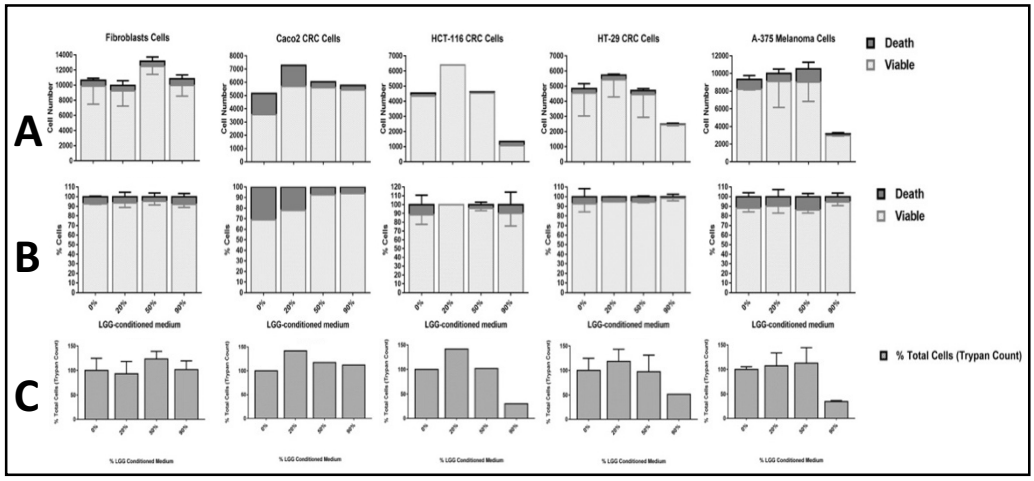


Figure 3. Trypan Count: Viable vs. Death cells (48 hrs LGG CM treatment). (row A) number of dead cells in relation to the percentage of LGG used;(row B) relative percentage of dead cells for each sample;(row C) variation in the number of total cells in relation to the percentage of LGG used in the treatment.

Overall, for each of the cell lines analysed, cell death does not increase along with the % of LGG-CM used (Fig. 3 A - B). Regarding the Total cell number, in Fibroblast (healthy cells) and Caco2 total cell number doesn't significantly change, whereas in HCT-116, HT-29 and A-375 cell number is reduced to 40-50% with 90% LGG-CM treatment (Fig 3 C).

It should be specified that trypan blue does not allow to distinguish apoptotic cells from necrotic cells, therefore this experimental approach was used in order to understand by direct observation under the microscope, how the dose-dependent effect on the viability of the treated cells could have a correlation

with cell death or with the hypothesis of an arrest of the proliferating activity of cells.

The observations reported so far helped to exclude that the dose-dependent effect on cell viability of the medium conditioned by LGG is due to cell death, in favour of the hypothesis of on proliferating activity effect of the treated cells.

About that the effects of various concentrations of bacterial cell-free supernatants on the conversion of the MTT tetrazolium salt and on DNA stain BrdU in cells after 48 hrs comparing with control groups were evaluated and presented in Table 2.

Table 2. The inhibitory effects of bacterial cell-free supernatants on the growth of five cell lines using MTT assay and proliferation assay

CFS *L. rhamnosus GG*	% Cell survival at three Concentrations (%/vol)			% Cell proliferation at three Concentrations (%/vol)			pH at 90 %/vol
	20	50	90	20	50	90	
Caco-2	88.9 ± 1.8	88.4 ± 4.2	48 ± 1.8	76.8 ± 3	101.3 ± 4	45.9 ± 10.1	6.75 ± 0.07
HT-29	99.9 ± 3.1	97.1 ± 2	24.5 ± 0.9	105.5 ± 2.1	90.4 ± 15.6	1 ± 2.8	6.75 ± 0.07
HCT-116	96.5 ± 2	79.9 ± 13.7	30.8 ± 3.3	100.6 ± 26.4	104.7 ± 0.8	1 ± 1.2	6.75 ± 0.07
A375	95.1 ± 3	98.9 ± 3.1	17.6 ± 0.4	115 ± 4.4	103.9 ± 6.8	16.7 ± 6.1	6.75 ± 0.07
Fibroblast	96.9 ± 2.8	103.3 ± 10.9	96.5 ± 4.7	71.6 ± 21	67.6 ± 26.7	94.6 ± 26.7	6.75 ± 0.07

Data are expressed as mean ± SD of three separate experiments; Control (medium without LGG-CFS) versus treatments groups.

All cell lines were treated with increasing concentrations of bacterial cell-free supernatants. MTT assay was used to evaluate cell survival and BrdU Cell Assay was used to evaluate cell proliferation after 48 h incubation.

Overall, cell proliferation has been multiplexed with viable cell percentage with comparable results (Fig. 4). For every tumour cell line (Caco2, HCT-116, HT-29, A-375) and Fibroblast, cell proliferation percentage changes proportionally to viable cell percentage.

The analysis of the data, display in Figure 4, shows a drastic dose-dependent decrease in cell viability (orange bar) and proliferation (blue bar) for HCT-116,

HT-29 and A375 tumour cell lines; the same trend cannot be described for Caco-2 cells, in which instead a halving of proliferating cells percentage is observed at the highest percentage of the medium conditioned by LGG (90%).

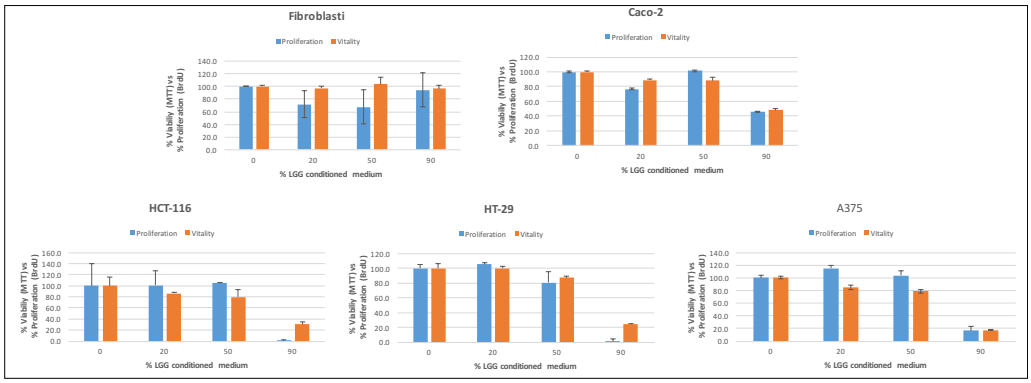


Figure 4. BrdU cell proliferation analysis compared to MTT analysis (48 hrs LGG CM treatment). Cell proliferation (BLUE bars, BrdU assay) can be multiplexed with Viable cell percentage (MTT assay, ORANGE bars) with comparable results.

Only for Fibroblast cells, proliferation % is not reduced proportionally to LGG-CM doses (compared to either Total or Viable cell %), which is very interesting.These results allow us to conclude that the LGG-conditioned medium (LGG-CM) has dose-dependent effect on the tumour cells viability under study (greater effect in cell lines HCT-116, HT-29 and A375; minor effect in the Caco-2 cell line), while it seems to have no effect in fibroblasts (healthy cells). Furthermore, the dose-dependent effect on cell viability determined by LGG-CM does not seem to be due to cell death.

Therefore, the dose-dependent decrease in LGG-CM cell viability is proportional to the decrease in proliferating activity in the HCT-116, HT-29, Caco-2 and A375 tumour cell lines, but not in the healthy cell line (Fibroblasts) in which a dose-dependent decrease in cell proliferation is not observed with respect to cell viability.

The absence reducing cell proliferation and cell viability observed for Fibroblasts after treatment with LGG-CM can be explained by considering the cell growth and duplication times of these cells and comparing them with those of tumour cells.

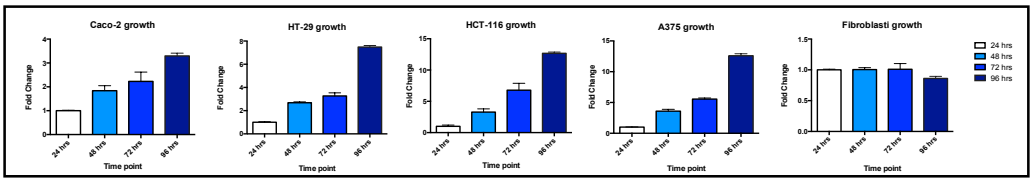
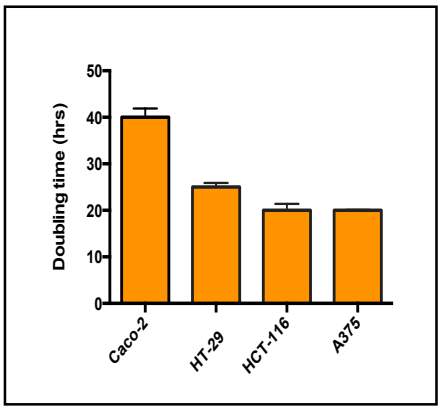


Figure 5. Cell growth of Caco-2, HT-29, HCT-116 and A375 cancer cells and Fibroblast healthy cells. Time points: 24, 48, 72, 96 hrs.

As can be seen in Figure 5, three of the tumour cell lines (HT-29, HCT-116, A375) increase their number by about 8-12 folds over the course of 96h compared to initial cells number, while the growth of Caco-2 is significantly lower with an increase of about 3 folds compared to the number of initial cells. In the healthy cell line (Fibroblasts), however, no increase in cell population is observed after 96h from seeding.

Therefore, it was possible to calculate the duplication time of the four tumour cell lines over 72h but not of the Fibroblasts for which it is greater than 96h.



The observation of cell growth and duplication time allows us to advance the hypothesis that the failure to decrease the percentage of proliferation, vitality and the total number of cells (Fig. 3-4) that is observed in Fibroblasts is not a function of a lower dose-dependent effect of LGG-CM, but rather of the longer duplication time that they need.

Figure 6. Doubling time (72 hrs)

6.3 Cell-free supernatants from Lactobacillus rhamnosus GG promotes G2 cell cycle arrest in cancer cells

In order to better assess the variation in cell proliferation, observed in the previously experimental tests mentioned, the analysis of the cell cycle was carried out using a flow cytometer.

In this regard, following to evaluate the decrease in proliferating activity in the cell lines under study, each cell lines were seeded in three dish (10 cm^2).

The treatment was carried out by culturing the cells without LGG-conditioned medium (control), with 90% vol LGG-conditioned medium and with 0.250 mM of Vincristine (positive control for apoptosis) for 48hrs.

At the end of 48hrs the cells were collected and the cells count were carried out in Trypan blue using a Burker chamber, which allowed to obtain an initial estimate of the cells state.

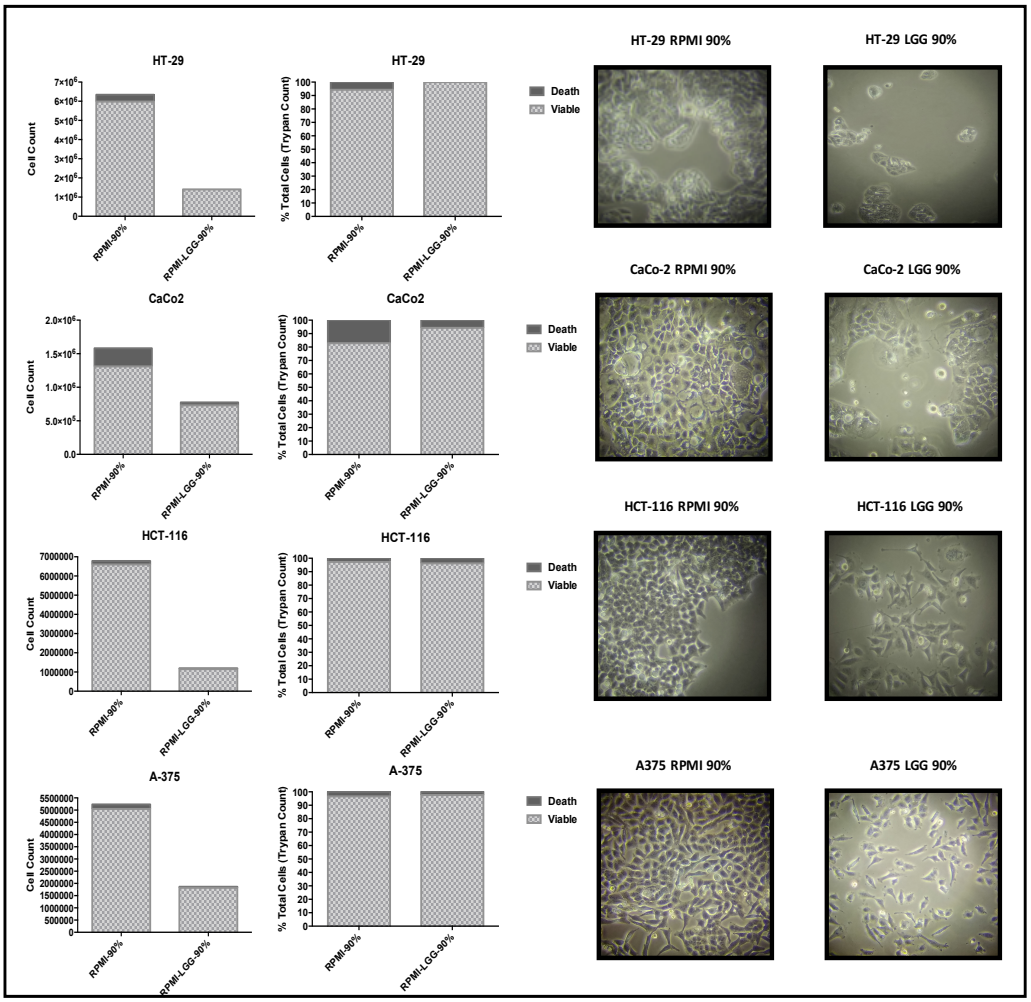


Figure 7. Trypan Count: Viable vs. Death cells (48 hrs LGG CM treatment); supported by optical microscope images.

As shown in Figure 7., the cell count in Trypan blue did not show an increase of dead number cells in the sample treated with 90% of LGG compared to the control, either as an absolute value (cell count) or as a relative percentage of live cells / dead cells (% total cells) in the four tumour cell lines. Furthermore, after treatment with LGG-CM, a reduction of the cell population of about 40-60% is observed in all four cell lines (optical microscope images).

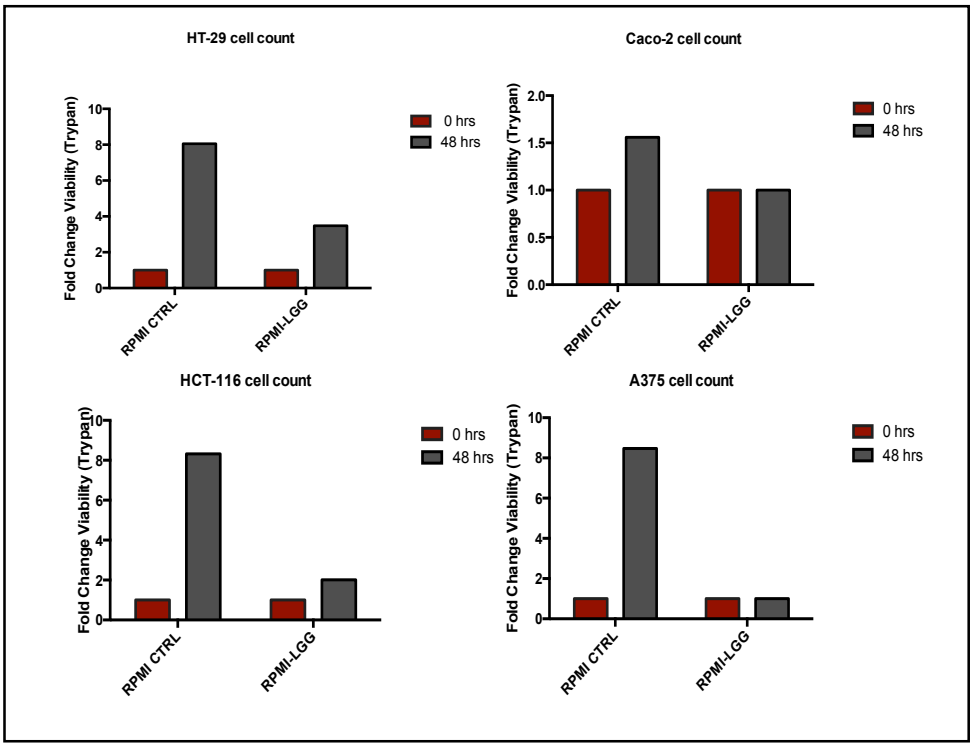


Figure 8. Trypan Count: untreated vs. treated cells (48 hrs)

This observation was confirmed by a further analysis of the trypan blue count which allowed to highlight a slight cells number increase after 48h of treatment with LGG-CM 90% compared to the initial cells number of (0hrs); on the other hand, after 48h in the control groups, an increase in the number of cells of about 8 folds for the HT-29, A375 and HCT-116 and of 1.5 folds for the Caco-2 compared to the number of initial cells was observed (Fig. 8).

Therefore, the percentages of cells in the various phases of the cell cycle (G0 / G1, S and G2 / M) were evaluate, and, in addition, the apoptotic cells percentages, positioned in the so-called hypodiploid peak, were evaluate; since the apoptotic cells, once resuspended in an appropriate buffer, lose the small fragments of DNA, undergoing a reduction of the PI fluorescence (correlated to the decrease of the DNA amount).

Overall, the analysis of the cell cycle of the tumour cells, shown in figure 9, revealed for all cell samples treated with LGG-CM 90%, a decrease in phase G0 / G1 and an increase in phase S and G2 / M compared to the untreated cells

(the percentages of cells detected by the flow cytometer in the different phases of the cell cycle are shown in the Table 3).

Only the analysis of the cell cycle of Caco-2 cells did not show a less change in the cell population in phase G0 / G1, S and G2 / M in the sample treated with LGG-CM 90%, compared to the untreated cells (the percentages of cells detected by the flow cytometer in the different phases of the cell cycle are shown in the Table 3 - Figure 9)

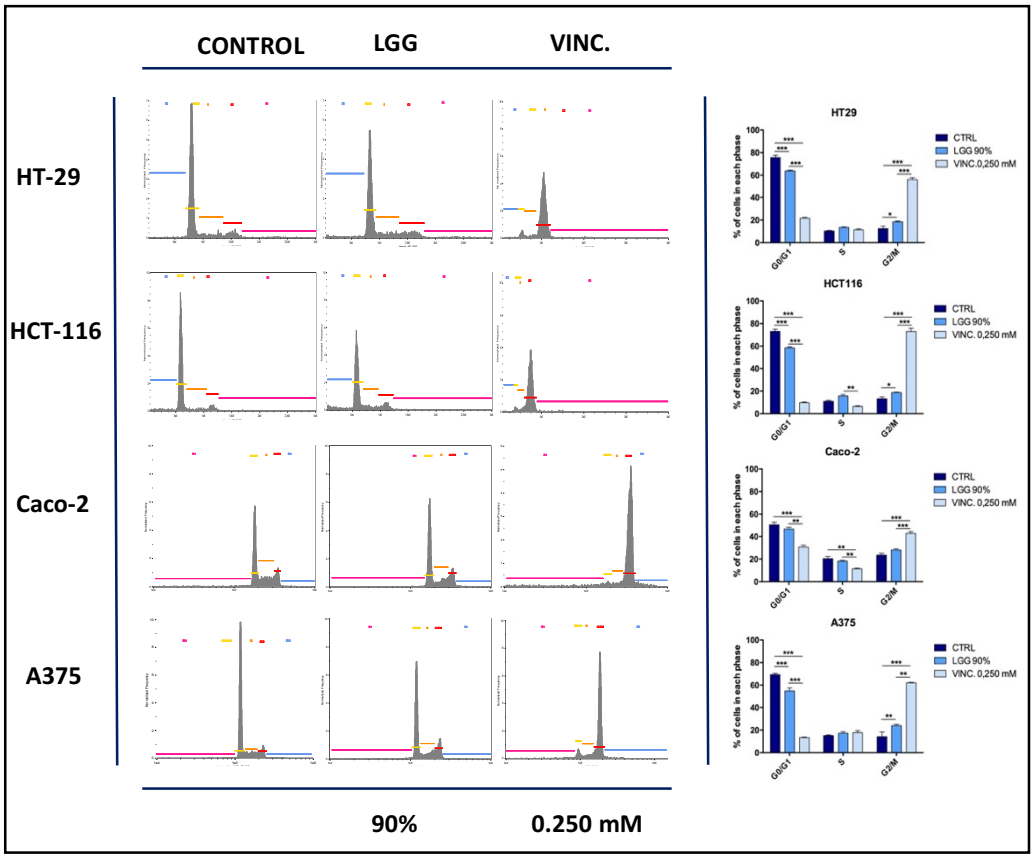


Figure 9. Cell cycle analysis: untreated vs treated (48 hrs). Cell cycle phases of untreated and treated cells was detected by staining with propidium iodide followed by flow cytometric analysis.

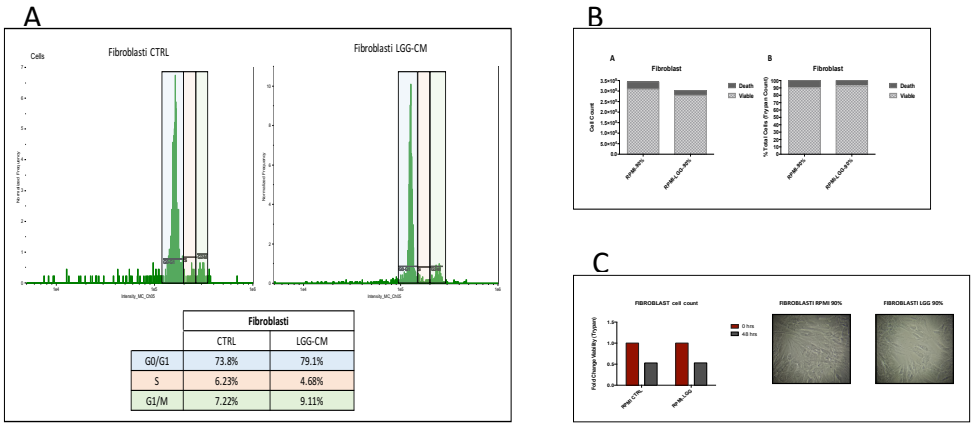
Tabella 3. Percentages of cells detected by the flow cytometer in the different phases of the cell cycle

		CONTROL MEDIUM	LGG-CONDITIONED MEDIUM	VINCRISTINE
HT-29	G0/G1	81.4%	70.1%	4.3%
	S	8.25%	11.5%	2.3%
	G2/M	8.79%	13.1%	89.3%
HCT-116	G0/G1	70.1%	64.7%	3.29%
	S	11.5%	7.96%	3.84%
	G2/M	13.1%	11%	85.6%
Caco-2	G0/G1	55.7%	60.3%	2.73%
	S	21.7%	16.9%	4.08%
	G2/M	15.3%	18.3%	77%
A375	G0/G1	72.6%	60.5%	6.74%
	S	15.5%	14.5%	7.08%
	G2/M	10.4%	20.03%	77.9%

In contrast to what was observed for tumour cells, the Fibroblasts the cell count in Trypan blue did not show an increase of dead number cells in the sample treated with 90% of LGG compared to the control, or a reduction of the cell population (optical microscope images).

Moreover, a cells number decrease after 48h of treatment with LGG-CM 90% compared to the initial cells number of (0hrs) was observed by a further analysis of the trypan blue count; on the other hand, it was comparable to a decrease of the cells number, after 48h in the control groups (Fig. 10).

Correspondingly, the analysis of the cell cycle of the healthy cells, shown in figure 10, did not reveal for the cell samples treated with LGG-CM 90%, a decrease in phase G0 / G1 and an increase in phase S and G2 / M compared to the untreated cells.



Fibroblasti		
	CTRL	LGG-CM
G0/G1	73.8%	79.1%
S	6.23%	4.68%
G1/M	7.22%	9.11%

Figure 10. (A) Cell cycle analysis: untreated vs treated (48 hrs). Cell cycle phases of untreated and treated cells was detected by staining with propidium iodide followed by flow cytometric analysis. (B-C) Fibroblast Trypan Count: Viable vs. Death cells (48 hrs LGG CM treatment); supported by optical microscope images.

The results obtained from the cell cycle analysis confirm that LGG-CFS has a dose-dependent effect on the viability of tumour cells, which is proportional to the decrease in proliferating activity in the HCT-116, HT-29, Caco-2 and A375 tumour cell lines.

In addition, the cell cycle analysis revealed that the decrease in proliferating activity would appear to be caused by a block in the G2 / M phase of the cell cycle.

However, the lower dose-dependent LGG-CM effect observed in the Caco-2 tumour cell line could be induced by the longer replication time of these cells in which consequently a lower variation of the cell cycle phases is observed compared to the untreated control.

In order to investigate the molecular events involved in cell cycle, we examined the expression of cell cycle-related genes (Cycline A, Cycline B and Cycline D). The comparative differential expression of selected genes was examined by SYBR-Green Real-time PCR in CFS-LGG-treated HT-29, HCT-116, Caco-2 and A375 cells compared to non-treated cells. Transcription levels of each gene were normalized with GAPDH Ct values.

In order to investigate the molecular events involved in cell cycle, we examined the expression of cell cycle-related genes (Cyclin A, Cyclin B and

Cyclin D). The comparative differential expression of selected genes was examined by SYBR-Green Real-time PCR in CFS-LGG-treated HT-29, HCT-116, Caco-2 and A375 cells compared to non-treated cells. Transcription levels of each gene were normalized with GAPDH Ct values.

The results reported in Figure 11 show an increase in Cyclin A and B in the four cell models after treatment with LGG compared to the untreated control.

The importance of this result lies in the regulatory nature of cyclins in the cell cycle. Their protein concentration, in fact, changes during the phases of the cell cycle as a function of the variation in gene expression and their degradation.

The observed increase in Cyclin A and Cyclin B therefore indicates that the cell populations treated with CFS-LGG were preparing for the transition from phase G0 / G1 to phase G2 / M.

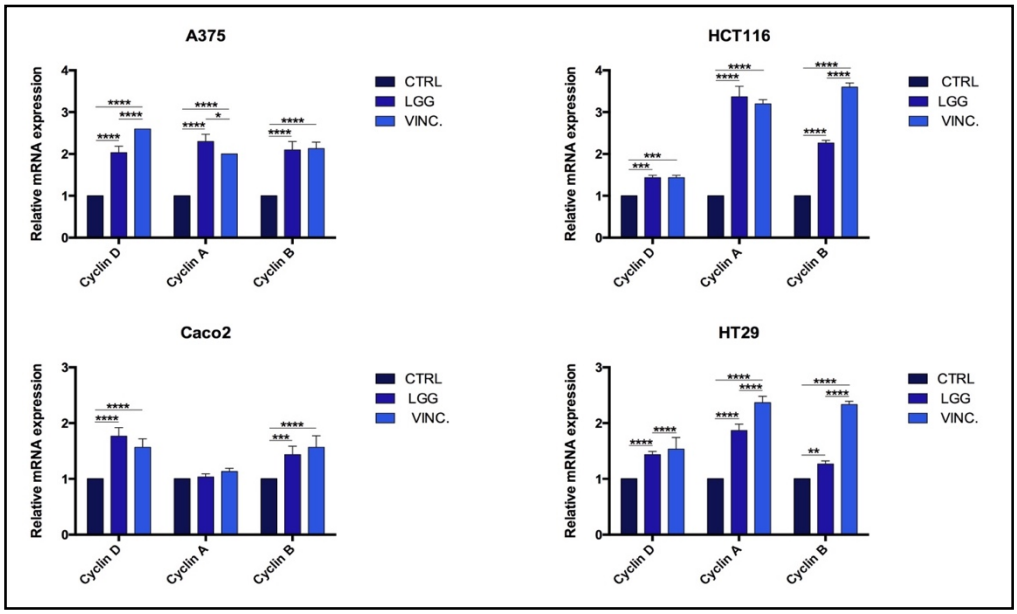


Figure 11. Cyclin A, B, D mRNA expression: untreated vs treated (48hrs). Relative gene expression (mean fold change) of Cyclin A, Cyclin B and Cyclin D1 in CFS-LGG-treated versus non-treated HT-29, HCT-116, Caco-2 and A375 cells. All cell lines were cultured with 90% CFS-LGG for 48hrs, and then RT-PCR was carried out with specific primers. For analysis, GAPDH was used as the internal reference and non-treated cells were used as the calibrator. mRNA relative expression for all genes was calculated by the comparative quantification Ct method (ΔΔCt). Results are representative of three independent experiments. Asterisks represent statistically significant differences.

Interestingly, all tumour cell lines treated with LGG-CFS 90% v / v showed a 3-90-fold increase in cell growth after discontinuation of treatment, confirming the reversible cell growth block, linked to bacterium metabolites released in the medium (Fig.12 A).

In addition, a pilot clonogenic assay on A375 melanoma cells showed that although the cells returned to proliferate after CFS-LGG treatment, colony growth was less compared to untreated cells (Fig.12 B).

Therefore, the cells free supernatant from L. rhamnosus GG, not only, is able to arrest cell cycle in treatment-dependent manner but it would seem also able to slow down, afterwards to treatment, the growth of cancer cells, which is notoriously fast.

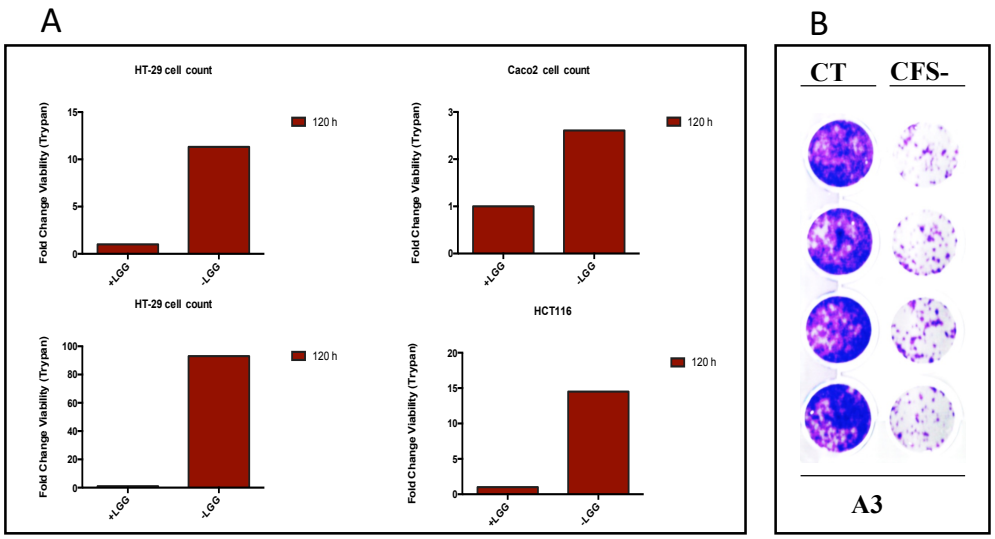


Figure 12. (A) Trypan Count after 120hrs of cells cultured with CFS-LGG and after 120hrs of cells cultured without CFS-LGG. (B) Clonogenic assay: A375 malanoma cells (used as model cells), 72 hours post treatment with LGG-CFS 90% v/v, were seeded at 200 cells per well onto 24-well culture plates and allowed to grow for a week in complete RPMI-1640 media. The cells treated with LGG-CFS 90% were compare to cells untreated.

6.4 Cell-Free Supernatants from Lactobacillus rhamnosus GG does not promote apoptotic cell death in cancer cells

As specified above, trypan blue does not allow to distinguish apoptotic cells from necrotic cells, therefore this experimental approach, through microscope direct observation requires to be confirmed with more sensitive and precise techniques.

Thus, to further investigate the anti-proliferative effects of L. rhamnosus GG, whether the cells free supernatant from probiotic strain can promote apoptotic cell death was examined.

Cell death was first monitored by a flow cytometry assay using Alexa Fluor® 488 annexin V / Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI and was second verify by western blotting analysis.

Annexin V belongs to the superfamily of proteins called annexins, whose main property is to bind to the cell membrane in a Ca2 + -dependent manner.

To distinguish live cells from apoptotic and necrotic cells, annexin V is used in double labeling with low concentration propidium iodide (PI) (1-5 μg / mL). The low concentration of PI stains only necrotic cells and makes it possible to distinguish necrotic cells from apoptotic ones (both positive for annexin V).

Therefore, to evaluate the variation in cell death, understood as necrosis, early apoptosis and late apoptosis after treatment with LGG-conditioned supernatant after 48h, the cells were seeded respectively in 3 dishes (10 cm2): HT-29 and HCT-116 at the density of 650,000 cells / dish (dish volume: 10 mL); Caco-2 at a density of 600,000 cells / dish (dish volume: 10 mL); A375 at a density of 500,000 cells / dish (dish volume: 10 mL).

The treatment was carried out by culturing the cells without LGG conditioned medium (control), with LGG conditioned medium 90% vol. and with 0.250 mM of Vincristine (positive control for apoptosis) for 48h.

At the end of 48h, the cells were collected, counted and a part prepared for flow cytometric analysis using a double labelling with annexin-5 and PI; the remainder was used to prepare cell pellets, duly stored at -80 ° C for subsequent western blotting analyses.

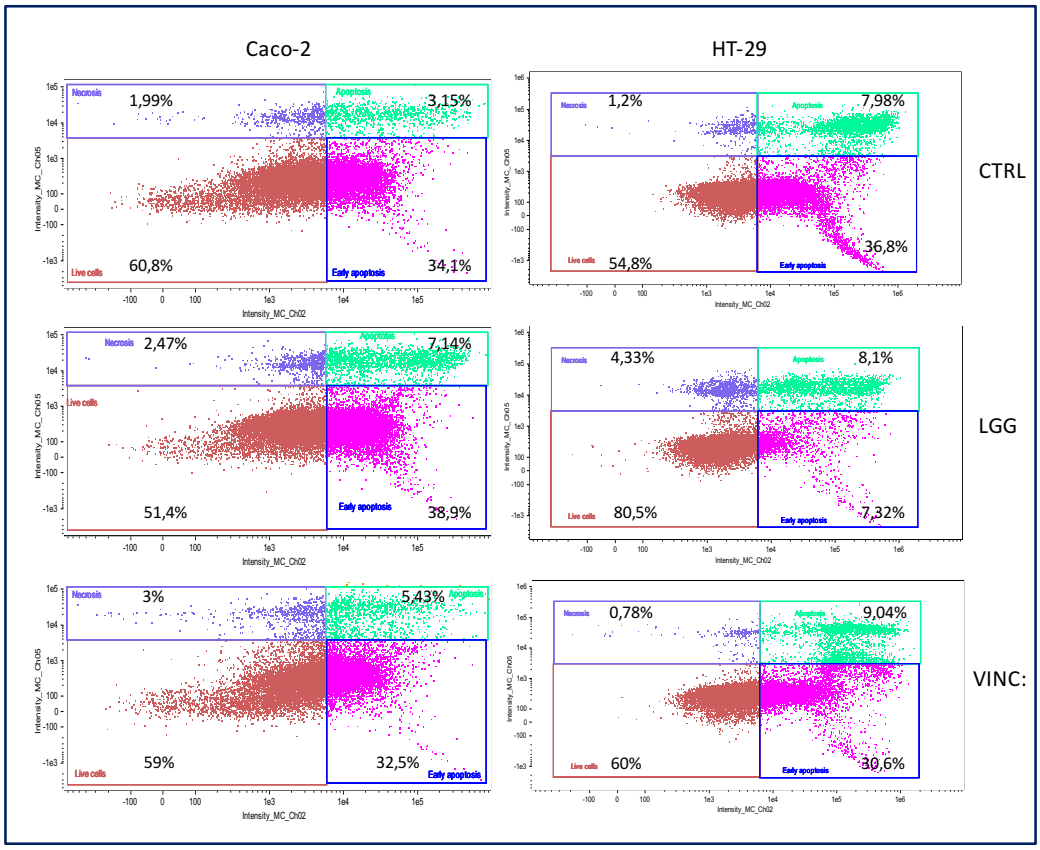


Figure 13. Caco2 and HT29 annexin V and PI for flow cytometry analysis: untreated (CTRL), LGG treated, Vincristina treated

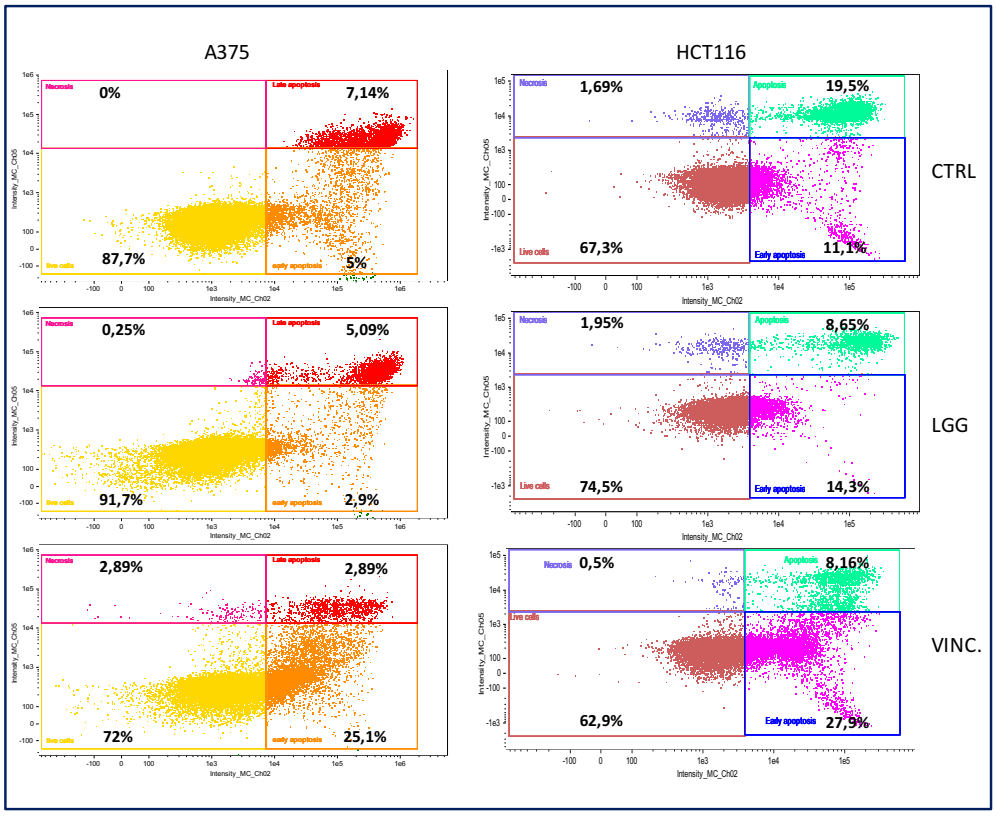


Figure 14. A375 and HCT-116 annexin V and PI for flow cytometry analysis: untreated (CTRL), LGG treated, Vincristin treated

As expected, the flow cytometry analysis with annexin -5 and propidium iodide did not show in any of the four cell lines examined, increased cell death due to necrosis, early or late apoptosis following treatment with LGG 90 conditioned supernatant. %, compared to the untreated cell sample (the percentages of live cells, early apoptosis, late apoptosis and necrosis are reported in the respective quadrants) (Fig. 13, 14).

The analysis of the protein expression of two markers of apoptosis, (cleaved Caspase-3 and total / cleaved PARP) further confirmed that the treatment with supernatant conditioned by LGG did not cause apoptosis (Fig 15).

The protein expression levels of cleaved Caspase-3 in the four tumour cell lines under study were evaluated by western blot.

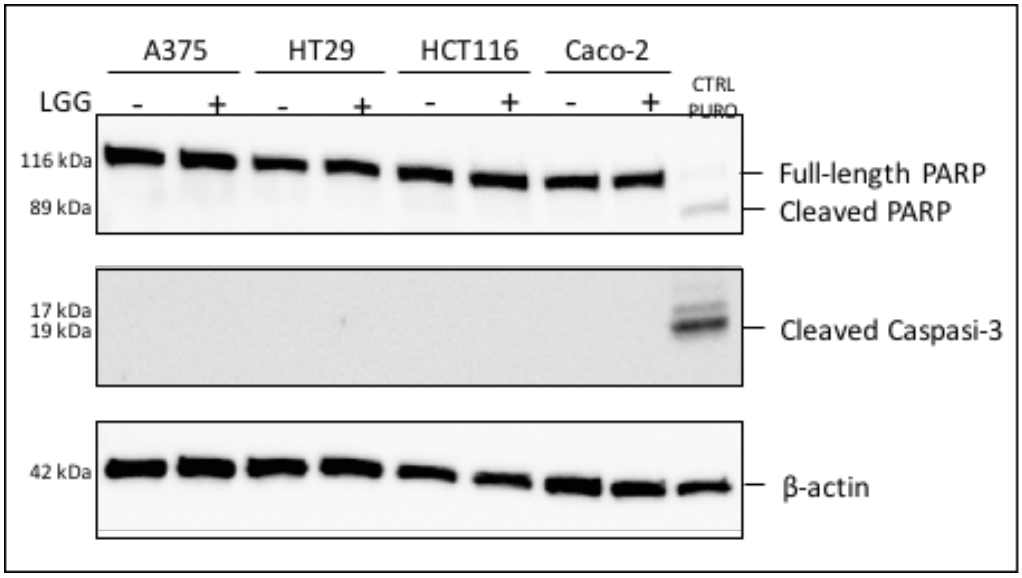


Figure 15. Effect of apoptosis in colon rectal cancer and melanoma cell lines (wester blot analysis)

A treatment with Puromycin was added in order to determine a positive control of cell death for apoptosis in order to ascertain the proper functioning of the antibody used.

6.5 Combinatorial effect of chemotherapy and cell free supernatant from L. rhamnosus GG on tumour cells viability

Consequent to observation of CFS-LGG effect on viability and proliferation tumour cells, the combinatorial effects of LGG-CM with chemotherapeutical drugs were evaluated.

For this study, two of the most common chemotherapy drugs in use in clinical practice, IRINOTECANO and 5-FLUOROURACIL, were used.

The treatment was carried out following two different approaches, culturing cells in a fixed percentage of LGG-CM and culturing cells in a variable percentage of LGG-CM (see methods).

Each figure shown below is divided into two quadrants showing the graphs (A and B) relating to the drugs dose-increasing treatment with 50% of LGG-CM and the graphs (C and D) relating to the drugs dose-increasing treatment with variable percentage of LGG-CM per cell line, as previously described.

Each quadrant contains two graphs that describe (A and C) the viability percentage of each cell line in relation to the drug dose (5-FU or Irinotecan) increase with (+ LGG) and without (-LGG) LGG conditioned medium.
In particular, histograms B and D represent the viability variation between control -LGG (cells not treated with the drug and without LGG-CM), cells not treated with the drug but with LGG-CM (+ LGG), cells treated with the drug (at the chosen dose) and without LGG-CM and cells treated with the drug (at the chosen dose) and in the presence of LGG-CM, normalized on the untreated control (-LGG).

Figure 16 (left) shows the results of HT-29 cells treatment at increase doses of 5-FU and LGG-CM 50% (A and B) or LGG-CM 70% (C and D). The treatment with low doses of 5-FU (from 0.008 μM to 2 μM) and LGG-CM 50% showed ~ 30% of cell viability reduction compared to the treatment with only 5-FU, while the effects of the two treatments were equivalent at high doses of 5-FU (from 8 μM to 500 μM) (Fig. 16 A).
Drilling down, one may note that this decrease was not due to an adjuvant effect of LGG-CM to 5-FU. In fact, considering the untreated control (light blue rectangle) versus treatment with 2 μM of 5-FU and without LGG-CM (blue rectangle), significant viability variation was not observed.
Apparently, the effect of LGG-CM 50% did not add up to the effect of the drug, increasing its action, but they seemed rather two overlapping but independent effects (Fig. 16 B).
In contrast, HT-29 cells treatment at growing doses of 5-FU and LGG-CM 70% reduced 80% of cells viability compared to the treatment with only 5-FU. Notably, uniform viability decrease at all drug doses and not dose-dependent was observed (Fig.16 C). Considering 2 M of 5-FU, LGG-CM 70% enhances the effect of the drug by reducing cell viability by 50% compared to treatment without LGG-CM (Fig. 16 C). A synergistic effect between LGG-CM 70% and 5-FU would appear to exist on the HT-29 cell lines.

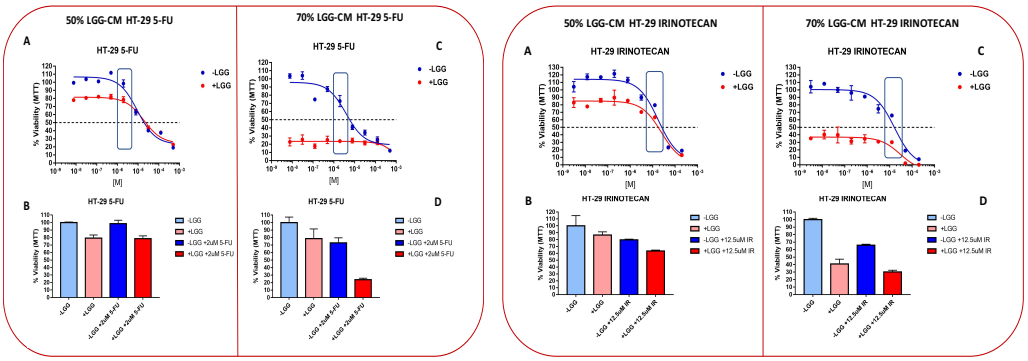


Figure 16. (To the left) HT-29 5-FU dose-response treatment with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); HT-29 5-FU dose-response treatment with 70% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D). (To the right) HT-29 IRINOTECAN dose-response treatment with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); HT-29 IRINOTECAN dose-response treatment with 70% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D).

A similar trend to that described for 5-FU in the HT-29 treatment at growing doses of Irinotecan was observed (Fig. 16 right). Specifically, Irinotecan combined with LGG-CM 50% promoted a decrease on cell viability of ~ 25% compared to the drug alone, while the combination Irinotecan and LGG-CM 70% caused a uniform viability decrease at all drug doses (~ 65%) (Fig. 16 A, C right). Interestingly, both LGG-CM 50% and LGG-CM 70% combined with Irinotecan promoted a synergic effect on viability decrease of HT-29 toumor cells, also observable at 12.5 μM of drug (Fig. 16 B, D right).

A synergic effect of combined treatment with LGG-CM and 5-FU on HCT-116 viability was also observed in witch the greatest contribution of LGG-CM 60% highlighted in order to enhance the drug effect (Fig. 17 A, B, C, D left).

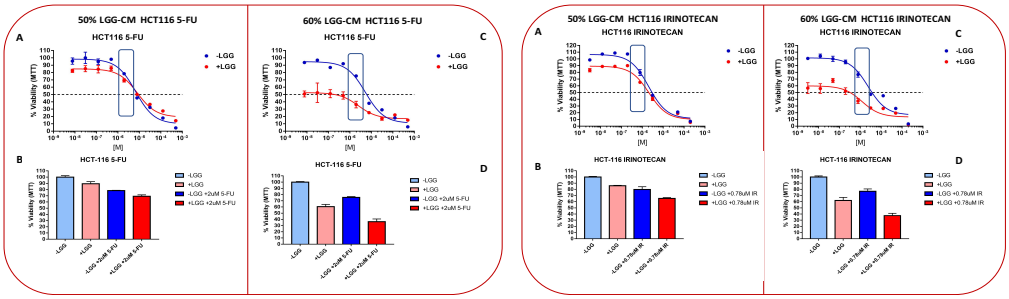


Figure 17. (To the left) HCT-116 5-FU dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); HCT-116 5-FU dose-response with 60% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D). (To the right) HCT-116 IRINOTECAN dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); HCT-116 IRINOTECAN dose-response with 60% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D).

A similar trend in the combined treatment with LGG-CM and Irinotecan was observed (Fig. 17 A, B, C, D right).

As shown so far, the greater or lesser effect of the combination LGG-CM-drugs (5-FU or Irinotecan) is not due to greater or lesser responsiveness of the tumour cell line to drug treatments but rather to the anti-proliferative effect of the cell free supernatant from L. rhamnosus GG.

The above is confirmed by analysis of the Caco-2 tumour cell line behaviour in the combinatorial treatment with drugs and LGG-CM.

In fact, the cell viability trend, shown in Figure 18 A and B (left), described a similar and superimposable effect by treating Caco-2 cells with 5-FU alone or in combination with LGG-CM 50%. However, a treatment with a higher percentage of LGG-conditioned medium (LGG-CM 90%) significantly changed the trend with a massive cell viability reduction (Fig. 18 C, D left).

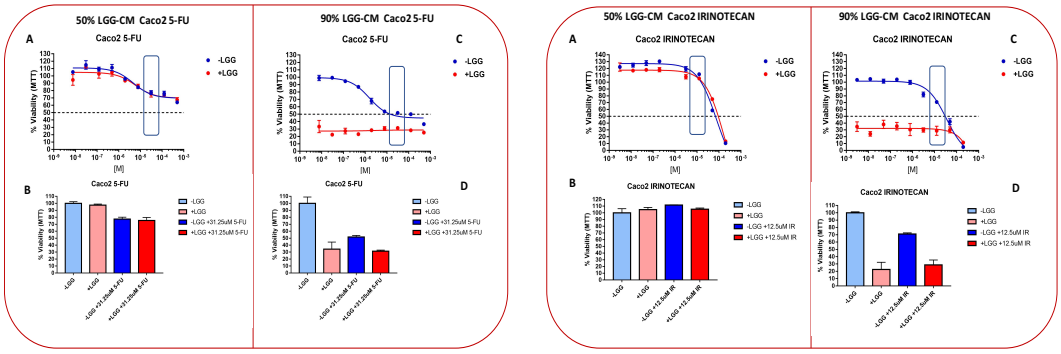


Figure 18. (To the left) Caco-2 5-FU dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); Caco-2 5-FU dose-response with 90% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D). (To the right) Caco-2 IRINOTECAN dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); Caco-2 IRINOTECAN dose-response with 90% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D).

The behaviour of the Caco-2 cells treated with Irinotecan alone and in combination with LGG-CM 50% or 90% was also perfectly corresponding to the LGG-conditioned medium trend (Fig. 18 A, B, C, D right).

Finally, also in the A375 cell line the impact of LGG-CM 50% was observed which, adding to the effect of the drugs, has enhanced its effectiveness (Fig. 19).

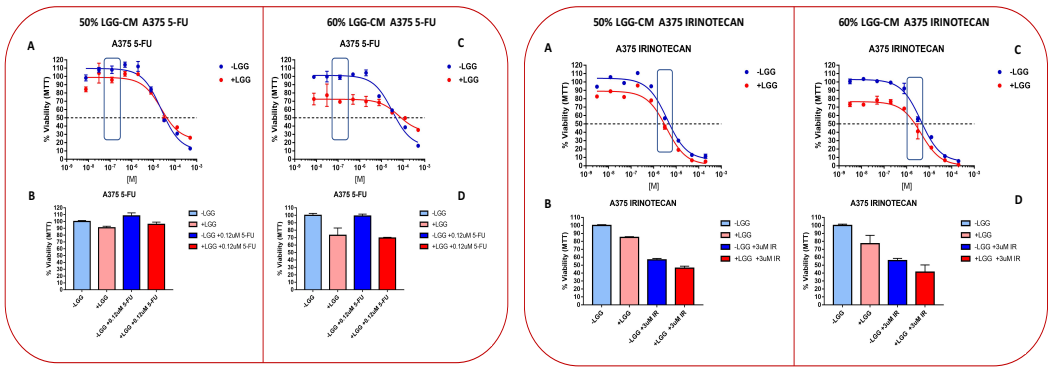


Figure 19. (To the left) A375 5-FU dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); A375 5-FU dose-response with 60% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D). (To the right) A375 IRINOTECAN dose-response with 50% LGG-CM (+LGG) and without LGG-CM (-LGG) (A-B); A375 IRINOTECAN dose-response with 60% LGG-CM (+LGG) and without LGG-CM (-LGG) (C-D).

7. DISCUSSION AND CONCLUSION

Lactobacillus rhamnosus GG is a non-pathogenic facultatively anaerobic bacterium, legally examined and approved as a human probiotic strain in many studies (Lam EK et al. 2007; Yan F. et al., 2002).

Probiotic bacteria have known to convey a wide range of beneficial effects to their hosts so that recently many studies have concentrated on the effects of probiotics in reduction of cancer cell viability and tumour size (Choi SS et al., 2006).

Generally, specific LAB strains are deemed capable to beneficially activate anticancer mechanisms, thereby regulating host's immune response (Rauch M. et a., 2010; Hirayama K. et al. 2000; Round JL et al., 2009). Probiotic soluble factors have been proposed to be important for the suppression of neoplastic cells (Oelschlaeger TA, 2010).

In a study from a few years ago, cell-free supernatants (CFS) from two LAB strains, L. casei and L. rhamnosus GG, were shown to inhibit colon cancer cell invasion by influencing matrix metalloproteinase-9 (MMP-9) activity and levels of the tight junction protein zona occludens-1 (ZO-1) in cultured metastatic human colorectal carcinoma cells (Escamilla J. Et al., 2012).

Moreover, L. gasseri SF1183 has been demonstrate capable to produced molecule(s) able to interfere drastically with HCT116 cell proliferation and stimulate G1-phase arrest (Di Luccia B. et al., 2013). Notably, lactic bacteria affect intestinal microbiota by acting in the large intestine but also by mediating some mechanisms, including immunological modulation or supplying of bioactive metabolites, in other organs. Some of these interactions and beneficial effects may be mediated or influenced by the in situ production of SCFA which are been considered responsible to induce cell death from necrosis in HT-29 cells (Lan A. et al., 2007).

There are not a few studies that have highlighted the ability of L. rhamnosus GG to inhibit the viability of some cancer cell lines (Orlando A. et al., 2009).

For istance, anti-proliferative effects of L. GG on gastric and colon cancer cells have been descibed in a study in which the highest concentrations of L. GG

homogenate and cytoplasm extracts reduced the percentage of cell viability to nearly 55% and 65% in DLD-1 (colon) and HGC-27 (gastric) cancer cell lines, respectively (Choi SS et al., 2006).

Similarly, recent studies have reported that the HK cells of L. rhamnosus GG potently inhibited the viability of some cancer cell lines and than cell-free supernatant of probiotic strains were effective in the growth inhibition of cancer cells (Hojjat Sadeghi-Aliabadi et al., 2014).

In the present study, cell free supernatant from L. rhamnosus GG induced potent anti-proliferative effects to HT-29, HCT-116, Caco-2 colon cancer cells and A375 melanoma cells and reduced the cell viabilities to nearly 80%, 70%, 50% and 90% at the highest prepared concentration respectively. The results presented here are in agreement with mentioned above studies.

Moreover, the results showed that pH of cell-free supernatant of probiotic strain was suitable to tumour cell survival (Table 1); the given differences between examined pH of strain in this study and the 6.4 pH HCL-induced, has showed that growth reduction should not be attributed to the higher concentration of organic acids in the supernatant of probiotic (Fig. 2).

Thus, the inhibitory effects of supernatant can be inferred to be not resulted by the effect of organic acids, but rather antiproliferative effects of probiotic bacteria may partly be caused by the produced certain bioactive metabolites, including exopolysaccharides, such as was reported in a study by Kim et al. (Kim JU et al., 2006). In this study ha been reported that Lactobacillus rhamnosus ATCC 9595 reduced the growth of colon (HT-29) and pancreas (PANC-1) cancer cell lines. This reduction was attributed to one exopolysaccharid of bacteria, rEPS (released exopolysaccharides), which was identified to be effective in preventing cancer.

In the present study CFS of L. rhamnosus GG reduced cell viability in a concentration-dependent manner and did not induce apoptotic cell death. Although the apoptosis is a natural physiological process of programmed cell death that regulates homeostasis and represents an ideal target for anti-neoplastic strategies, some studies showed that LAB can play a role both in the regulation of apoptosis via intrinsic and extrinsic pathways, via activation of

autophagic cell death, and via gene products that regulate cell proliferation by stimulating cell cycle arrest (Zhong L. et al., 2014; Chen CC et al., 2012; Iyer C. et al., 2008; Di Luccia B. et al., 2013).

In the in vitro study proposed here, L. rhamnosus GG interfered with HCT116, HT-29, Caco-2 and A375 cell proliferation and stimulate G2/M-phase arrest.

Interestingly, the aforementioned anti-proliferative effect has been shown not to play an irreversible role in cell death but rather the effect has been shown to be reversible by removing the conditioned medium from LGG.

Notably, although the cells returned to proliferate after CFS-LGG treatment, the growth was less compared to untreated cells. Therefore, the cells free supernatant from L. rhamnosus GG is able to arrest cell cycle in treatment-dependent manner but also to slow down, afterwards to treatment, the growth of cancer cells.

Although, the overlying mechanisms deserve further analysis, the data, here reported, show clearly that cell-free supernatant from Lactobacillus rhamnosus GG exerts potent anti-proliferative and growth-inhibitory effects in vitro.

The results also became even more interesting because the combinatorial administration of CFS-LGG and chemotherapy drugs significantly expanded the drugs effect in experimental in vitro carcinoma models suggesting that use of total cell-free supernatant from this probiotic strain or its bioactive molecules in pharmacological intervention programs, may be promising, while the use of this probiotic strain in dietary intervention programs and functional foods is now established.

8. REFERENCES

- Feng, Q., Chen, W.D., Wang, Y.D. Gut Microbiota: An Integral Moderator in Health and Disease. Front. Microbiol. **2018**, 9, 151.
- Eran Elinav, Wendy S Garrett, Giorgio Trinchieri, Jennifer Wargo. The cancer microbiome. Nat Rev Cancer. 2019 Jul;19(7):371-376. doi: 10.1038/s41568-019-0155-3
- Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L. & Gordon, J. I. Human nutrition, the gut microbiome and the immune system. *Nature* **474**, 327–336 (2011).
- Consortium, H. M. P. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
- Vaishnava, S.; Behrendt, C.L.; Ismail, A.S.; Eckmann, L.; Hooper, L.V. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc. Natl. Acad. Sci. USA 2008, 105, 20858–20863.
- Belkaid, Y.; Naik, S. Compartmentalized and systemic control of tissue immunity by commensals. Nat. Immunol. 2013, 14, 646–653.
- Magnúsdóttir, S.; Ravcheev, D.; de Crécy-Lagard, V.; Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front. Genet. 2015, 6, 148.
- Carding, S.; Verbeke, K.; Vipond, D.T.; Corfe, B.M.; Owen, L.J. Dysbiosis of the gut microbiota in disease. Microb. Ecol. Health Dis. 2015, 26, 26191.
- Grice, E. A. & Segre, J. A. The skin microbiome. *Nature Rev. Microbiol.* **9**, 244–253 (2011).
- Urbaniak, C. et al. The microbiota of breast tissue and its association with breast cancer. Appl. Environ. Microbiol. 82, 5039–5048 (2016).
- Ferreira, R. M. et al. Gastric microbial community profling reveals a dysbiotic cancer-associated microbiota. Gut 67, 226–236 (2018).

- Pushalkar, S. et al. The pancreatic cancer microbiome promotes oncogenesis by induction of innate and adaptive immune suppression. Cancer Discov. 8, 403–416 (2018)
- Banerjee, S. et al. The ovarian cancer oncobiome. Oncotarget 8, 36225–36245 (2017)
- Sfanos, K. S. et al. A molecular analysis of prokaryotic and viral DNA sequences in prostate tissue from patients with prostate cancer indicates the presence of multiple and diverse microorganisms. Prostate 68, 306–320 (2008)
- Aviles-Jimenez, F. et al. Microbiota studies in the bile duct strongly suggest a role for Helicobacter pylori in extrahepatic cholangiocarcinoma. Clin. Microbiol. Infect. 22, 178 e111–178 e122 (2016).
- Mao, Q. et al. Interplay between the lung microbiome and lung cancer. Cancer Lett. 415, 40–48 (2018).
- Zmora, N., Soffer, E. & Elinav, E. Transforming medicine with the microbiome. *Sci. Transl Med.* **11**, eaaw1815 (2019).
- Shin, W. & Kim, H. J. Intestinal barrier dysfunction orchestrates the onset of inflammatory host-microbiome cross-talk in a human gut inflammation-on-a-chip. *Proc. Natl Acad. Sci. USA* **115**, E10539–E10547 (2018).
- Rubinstein, M. R. et al. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesin. *Cell Host Microbe* **14**, 195–206 (2013).
- Tahara, T. et al. Fusobacterium in colonic flora and molecular features of colorectal carcinoma. *Cancer Res.* **74**, 1311–1318 (2014).
- Panebianco, C., Andriulli, A. & Pazienza, V. Pharmacomicrobiomics: exploiting the drug-microbiota interactions in anticancer therapies. Microbiome 6, 92 (2018).
- Lehouritis, P. et al. Local bacteria afect the efcacy of chemotherapeutic drugs. Sci. Rep. 5, 14554 (2015).

- Goodman, B.; Gardner, H. The microbiome and cancer. J. Pathol. 2018, 244, 667–676.
- Zitvogel, L.; Daillère, R.; Roberti, M.P.; Routy, B.; Kroemer, G. Anticancer effects of the microbiome and its products. Nat. Rev. Microbiol. 2017, 15, 465–478.
- Robert F. Schwabe and Christian Jobin. The microbiome and cancer. Nat Rev Cancer. 2013 Nov;13(11):800-12. doi: 10.1038/nrc3610. Epub 2013 Oct 17.
- Belkaid, Y.; Naik, S. Compartmentalized and systemic control of tissue immunity by commensals. Nat. Immunol. 2013, 14, 646–653.
- Gill, S. R. et al. Metagenomic analysis of the human distal gut microbiome. *Science* **312**, 1355–1359 (2006).
- Philipp, B. Bacterial degradation of bile salts. *Appl. Microbiol. Biotechnol.* **89**, 903–915 (2011).
- Vaishnava, S.; Behrendt, C.L.; Ismail, A.S.; Eckmann, L.; Hooper, L.V. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc. Natl. Acad. Sci. USA 2008, 105, 20858–20863.
- Magnúsdóttir, S.; Ravcheev, D.; de Crécy-Lagard, V.; Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front. Genet. 2015, 6, 148.
- Gensollen, T.; Iyer, S.S.; Kasper, D.L.; Blumberg, R.S. How colonization by microbiota in early life shapes the immune system. Science 2016, 352, 539–544.
- Schmidt, T.S.B.; Raes, J.; Bork, P. The Human Gut Microbiome: From Association to Modulation. Cell 2018, 172, 1198–1215.
- Bultman, S.J. Emerging roles of the microbiome in cancer. Carcinogenesis 2014, 35, 249–255.
- Cani, P.D. Human gut microbiome: Hopes, threats and promises. Gut 2018, 67, 1716–1725.

- Ceranowicz, P.; Warzecha, Z.; Dembinski, A. Peptidyl hormones of endocrine cells origin in the gut—Their discovery and physiological relevance. J. Physiol. Pharmacol. 2015, 66, 11–27.
- Sandrini, S.; Aldriwesh, M.; Alruways, M.; Freestone, P. Microbial endocrinology: Host-bacteria communication within the gut microbiome. J. Endocrinol. 2015, 225, R21–R34.
- Arthur, J. C. et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* **338**, 120–123 (2012).
- Holmes, E., Li, J. V., Marchesi, J. R. & Nicholson, J. K. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell. Metab.* **16**, 559–564 (2012).
- Ley, R. E., Turnbaugh, P. J., Klein, S. & Gordon, J. I. Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022–1023 (2006).
- Grivennikov, S. I. Inflammation and colorectal cancer: colitis-associated neoplasia. *Semin. Immunopathol.* **35**, 229–244 (2012).
- Lecuit, M. et al. Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. *N. Engl. J. Med.* **350**, 239–248 (2004).
- Wiest, R. & Garcia-Tsao, G. Bacterial translocation (BT) in cirrhosis. *Hepatology* **41**, 422–433 (2005).
- Seki, E. et al. TLR4 enhances TGF-β signaling and hepatic fibrosis. *Nature Med.* **13**, 1324–1332 (2007).
- Dapito, D. H. et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* **21**, 504–516 (2012).
- Yoshimoto, S. et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* **499**, 97–101 (2013).
- Yu, L. X. et al. Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. *Hepatology* **52**, 1322–1333 (2010).
- Rea, D.; Coppola, G.; Palma, G.; Barbieri, A.; Luciano, A.; Del Prete, P.; Rossetti, S.; Berretta, M.; Facchini, G.; Perdonà, S.; et al.

Microbiota effects on cancer: From risks to therapies. Oncotarget 2018, 9, 17915–17927.

- Bhatt, A.P.; Redinbo, M.R.; Bultman, S.J. The role of the microbiome in cancer development and therapy. CA Cancer J. Clin. 2017, 67, 326–344.
- Grivennikov, S. I. et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491**, 254–258 (2012).
- Couturier-Maillard, A. et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J. Clin. Invest.* **123**, 700–711 (2013).
- Hu, B. et al. Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. *Proc. Natl Acad. Sci. USA* **110**, 9862–9867 (2013).
- Garrett, W. S. et al. Colitis-associated colorectal cancer driven by T-bet deficiency in dendritic cells. *Cancer Cell* **16**, 208–219 (2009).
- Zhang, H. L. et al. Profound impact of gut homeostasis on chemically-induced pro-tumorigenic inflammation and hepatocarcinogenesis in rats. *J. Hepatol* **57**, 803–812 (2012).
- Calle, E. E. & Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature Rev. Cancer* **4**, 579–591 (2004).
- Ley, R. E. et al. Obesity alters gut microbial ecology. *Proc. Natl Acad. Sci. USA* **102**, 11070–11075 (2005).
- Barbacid M. Ras genes. Annum. Rev. Biochem. 56, 779-827 (1987)
- Fearon E. R. Molecular Genetics of Colorectal Cancer. Annual Review of Pathology: Mechanisms of Disease 6,479-507 (2011).
- Cotillard, A. et al. Dietary intervention impact on gut microbial gene richness. *Nature* **500**, 585–588 (2013).
- Le Chatelier, E. et al. Richness of human gut microbiome correlates with metabolic markers. *Nature* **500**, 541–546 (2013).

- Chen, W., Liu, F., Ling, Z., Tong, X. & Xiang, C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* **7**, e39743 (2012).
- Sanapareddy, N. et al. Increased rectal microbial richness is associated with the presence of colorectal adenomas in humans. *ISME J.* **6**, 1858–1868 (2012).
- Sobhani, I. et al. Microbial dysbiosis in colorectal cancer (CRC) patients. *PLoS ONE* **6**, e16393 (2011).
- Wang, T. et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J.* **6**, 320–329 (2011).
- Kostic, A. D. et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res.* **22**, 292–298 (2012).
- Kostic, A. D. et al. *Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* **14**, 207–215 (2013).
- McCoy, A. N. et al. *Fusobacterium* is associated with colorectal adenomas. *PLoS ONE* **8**, e53653 (2013).
- 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).
- Gur, C.; Ibrahim, Y.; Isaacson, B.; Yamin, R.; Abed, J.; Gamliel, M.; Enk, J.; Bar-On, Y.; Stanietsky-Kaynan, N.; Coppenhagen-Glazer, S.; et al. Binding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity 2015, 42, 344–355.
- Halazonetis, T.D. Constitutively active DNA damage checkpoint pathways as the driving force for the high frequency of p53 mutations in human cancer. DNA Repair 2004, 3, 1057–1062.
- Yao, Y.; Dai, W. Genomic Instability and Cancer. J. Carcinog. Mutagen. 2014, 5

- Frisan, T. Bacterial genotoxins: The long journey to the nucleus of mammalian cells. Biochim. Biophys. Acta 2016, 1858, 567–575.
- Lupp, C. et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* **2**, 204 (2007).
- Patwa, L. G. et al. Chronic intestinal inflammation induces stress-response genes in commensal *Escherichia coli*. *Gastroenterology* **141**, 1842–1851 (2011).
- 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.
- Wu, S.; Rhee, K.J.; Zhang, M.; Franco, A.; Sears, C.L. Bacteroides fragilis toxin stimulates intestinal epithelial cell shedding and gamma-secretase-dependent E-cadherin cleavage. J. Cell Sci. 2007, 120, 1944–1952.
- Moss, S.F. The Clinical Evidence Linking. Cell. Mol. Gastroenterol. Hepatol. 2017, 3, 183–191.
- 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.
- 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.
- 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.

- Plottel, C.S.; Blaser, M.J. Microbiome and malignancy. Cell Host Microbe 2011, 10, 324–335
- 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.
- 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.
- Kilkkinen, A.; Rissanen, H.; Klaukka, T.; Pukkala, E.; Heliövaara, M.; Huovinen, P.; Männistö, S.; Aromaa, A.; Knekt, P. Antibiotic use predicts an increased risk of cancer. Int. J. Cancer 2008, 123, 2152–2155.
- Conlon, M.A.; Bird, A.R. The impact of diet and lifestyle on gut microbiota and human health. Nutrients 2014, 7, 17–44.
- Gopalakrishnan, V.; Helmink, B.A.; Spencer, C.N.; Reuben, A.; Wargo, J.A. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. Cancer Cell 2018, 33, 570–580.
- C. Manichanh, N. Borruel, F. Casellas, F. Guarner, The gut microbiota in IBD, Nat. Rev. Gastroenterol. Hepatol. 9 (2012) 599–608.
- 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.
- Paavonen, J.; Naud, P.; Salmerón, J.; Wheeler, C.M.; Chow, S.N.; Apter, D.; Kitchener, H.; Castellsague, X.; Teixeira, J.C.; Skinner, S.R.; et al. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): Final analysis of a double-blind, randomised study in young women. Lancet 2009, 374, 301–314.

- Aranda, F.; Bloy, N.; Pesquet, J.; Petit, B.; Chaba, K.; Sauvat, A.; Kepp, O.; Khadra, N.; Enot, D.; Pfirschke, C.; et al. Immune-dependent antineoplastic effects of cisplatin plus pyridoxine in non-small-cell lung cancer. Oncogene 2015, 34, 3053–3062.
- G.B. Gorbach, L. Sherwood, Diet and the excretion and enterhepatic cycling of estrogens, Prev. Med. 16 (1987) 525–531.
- B.D. Muegge, J. Kuczynski, D. Knights, J.C. Clemente, A. González, L. Fontana, B. Henrissat, R. Knight, J.I. Gordon, Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans, Science 332 (2011) 970–974.
- Jan, G.; Belzacq, A.S.; Haouzi, D.; Rouault, A.; Métivier, D.; Kroemer, G.; Brenner, C. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. Cell Death Differ. 2002, 9, 179–188.
- Wei, W.; Sun, W.; Yu, S.; Yang, Y.; Ai, L. Butyrate production from high-fiber diet protects against lymphoma tumor. Leuk. Lymphoma 2016, 57, 2401–2408.
- Fulbright, L.E.; Ellermann, M.; Arthur, J.C. The microbiome and the hallmarks of cancer. PLoS Pathog. 2017, 13, e1006480.
- Dembi ´nski, A.; Warzecha, Z.; Ceranowicz, P.; Dembi ´nski, M.; Cieszkowski, J.; Gosiewski, T.; Bulanda, M.; Ku´snierz-Cabala, B.; Gał ˛azka, K.; Konturek, P.C. Synergic Interaction of Rifaximin and Mutaflor (Escherichia coli Nissle 1917) in the Treatment of Acetic Acid-Induced Colitis in Rats. Gastroenterol. Res. Pract. 2016, 2016, 3126280.
- Lenoir, M.; Del Carmen, S.; Cortes-Perez, N.G.; Lozano-Ojalvo, D.; Muñoz-Provencio, D.; Chain, F.; Langella, P.; de Moreno de LeBlanc, A.; LeBlanc, J.G.; Bermúdez-Humarán, L.G. Lactobacillus casei BL23 regulates Treg and Th17 T-cell populations and reduces DMH-associated colorectal cancer. J. Gastroenterol. 2016, 51, 862–873
- Lee, J.W.; Shin, J.G.; Kim, E.H.; Kang, H.E.; Yim, I.B.; Kim, J.Y.; Joo, H.G.; Woo, H.J. Immunomodulatory and antitumor effects in vivo by

the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. J. Vet. Sci. 2004, 5, 41–48.

- Baldwin, C.; Millette, M.; Oth, D.; Ruiz, M.T.; Luquet, F.M.; Lacroix, M. Probiotic Lactobacillus acidophilus and L. casei mix sensitize colorectal tumoral cells to 5-fluorouracil-induced apoptosis. Nutr. Cancer 2010, 62, 371–378.
- Takagi, A.; Ikemura, H.; Matsuzaki, T.; Sato, M.; Nomoto, K.; Morotomi, M.; Yokokura, T. Relationship between the in vitro response of dendritic cells to Lactobacillus and prevention of tumorigenesis in the mouse. J. Gastroenterol. 2008, 43, 661–669.
- Dy, G.K.; Adjei, A.A. Understanding, recognizing, and managing toxicities of targeted anticancer therapies. CA Cancer J. Clin. 2013, 63, 249–279.
- Roy, S.; Trinchieri, G. Microbiota: A key orchestrator of cancer therapy. Nat. Rev. Cancer 2017, 17, 271–285.
- Nayak, R.R.; Turnbaugh, P.J. Mirror, mirror on the wall: Which microbiomes will help heal them all? BMC Med. 2016, 14, 72.
- Fessler, J.L.; Gajewski, T.F. The Microbiota: A New Variable Impacting Cancer Treatment Outcomes. Clin. Cancer Res. 2017, 23, 3229–3231.
- McCarthy, E.F. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. IOWA Orthop. J. 2006, 26, 154–158
- Nauts, H.C.; Swift, W.E.; Coley, B.L. The treatment of malignant tumors by bacterial toxins as developed by the late William B. Coley, M.D., reviewed in the light of modern research. Cancer Res. 1946, 6, 205–216.
- Zbar, B.; Bernstein, I.; Tanaka, T.; Rapp, H.J. Tumor immunity produced by the intradermal inoculation of living tumor cells and living Mycobacterium bovis (strain BCG). Science 1970, 170, 1217–1218.
- Aso, Y.; Akazan, H. Prophylactic effect of a Lactobacillus casei preparation on the recurrence of superficial bladder cancer. BLP Study Group. Urol. Int. 1992, 49, 125–129.

- Hoesl, C.E.; Altwein, J.E. The probiotic approach: An alternative treatment option in urology. Eur. Urol. 2005, 47, 288–296.
- Felgner, S.; Kocijancic, D.; Frahm, M.; Weiss, S. Bacteria in Cancer Therapy: Renaissance of an Old Concept. Int. J. Microbiol. 2016, 2016, 8451728.
- Stebbing, J.; Dalgleish, A.; Gifford-Moore, A.; Martin, A.; Gleeson, C.; Wilson, G.; Brunet, L.R.; Grange, J.; Mudan, S. An intra-patient placebo-controlled phase I trial to evaluate the safety and tolerability of intradermal IMM-101 in melanoma. Ann. Oncol. 2012, 23, 1314–1319.
- Dalgleish, A.G.; Stebbing, J.; Adamson, D.J.; Arif, S.S.; Bidoli, P.; Chang, D.; Cheeseman, S.; Diaz-Beveridge, R.; Fernandez-Martos, C.; Glynne-Jones, R.; et al. Randomised, open-label, phase II study of gemcitabine with and without IMM-101 for advanced pancreatic cancer. Br. J. Cancer 2016, 115, 789–796.
- Toso, J.F.; Gill, V.J.; Hwu, P.; Marincola, F.M.; Restifo, N.P.; Schwartzentruber, D.J.; Sherry, R.M.; Topalian, S.L.; Yang, J.C.; Stock, F.; et al. Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma. J. Clin. Oncol. 2002, 20, 142–152.
- Nemunaitis, J.; Cunningham, C.; Senzer, N.; Kuhn, J.; Cramm, J.; Litz, C.; Cavagnolo, R.; Cahill, A.; Clairmont, C.; Sznol, M. Pilot trial of genetically modified, attenuated Salmonella expressing the E. coli cytosine deaminase gene in refractory cancer patients. Cancer Gene Ther. 2003, 10, 737–744.
- Kramer, M.G.; Masner, M.; Ferreira, F.A.; Hoffman, R.M. Bacterial Therapy of Cancer: Promises, Limitations, and Insights for Future Directions. Front. Microbiol. 2018, 9, 16.
- Iida, N.; Dzutsev, A.; Stewart, C.A.; Smith, L.; Bouladoux, N.; Weingarten, R.A.; Molina, D.A.; Salcedo, R.; Back, T.; Cramer, S.; et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science 2013, 342, 967–970.

- Gui, Q.F.; Lu, H.F.; Zhang, C.X.; Xu, Z.R.; Yang, Y.H. Well-balanced commensal microbiota contributes to anti-cancer response in a lung cancer mouse model. Genet. Mol. Res. 2015, 14, 5642–5651.
- Viaud, S.; Saccheri, F.; Mignot, G.; Yamazaki, T.; Daillère, R.; Hannani, D.; Enot, D.P.; Pfirschke, C.; Engblom, C.; Pittet, M.J.; et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science 2013, 342, 971–976.
- Daillère, R.; Vétizou, M.; Waldschmitt, N.; Yamazaki, T.; Isnard, C.; Poirier-Colame, V.; Duong, C.P.M.; Flament, C.; Lepage, P.; Roberti, M.P.; et al. Enterococcus hirae and Barnesiella intestinihominis Facilitate Cyclophosphamide-Induced Therapeutic Immunomodulatory Effects. Immunity 2016, 45, 931–943.
- Jahrsdörfer, B.; Weiner, G.J. CpG oligodeoxynucleotides as immunotherapy in cancer. Update Cancer Ther. 2008, 3, 27–32.
- Chen, Q.; Wang, C.; Chen, G.; Hu, Q.; Gu, Z. Delivery Strategies for Immune Checkpoint Blockade. Adv. Healthc. Mater. 2018, 7, e1800424.
- Buchbinder, E.I.; Desai, A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. Am. J. Clin. Oncol. 2016, 39, 98–106.
- Vétizou, M.; Pitt, J.M.; Daillère, R.; Lepage, P.; Waldschmitt, N.; Flament, C.; Rusakiewicz, S.; Routy, B.; Roberti, M.P.; Duong, C.P.; et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 2015, 350, 1079–1084.
- Sivan, A.; Corrales, L.; Hubert, N.; Williams, J.B.; Aquino-Michaels, K.; Earley, Z.M.; Benyamin, F.W.; Lei, Y.M.; Jabri, B.; Alegre, M.L.; et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science 2015, 350, 1084–1089.
- Routy, B.; Le Chatelier, E.; Derosa, L.; Duong, C.P.M.; Alou, M.T.; Daillère, R.; Fluckiger, A.; Messaoudene, M.; Rauber, C.; Roberti, M.P.; et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 2018, 359, 91–97.

- Gopalakrishnan, V.; Spencer, C.N.; Nezi, L.; Reuben, A.; Andrews, M.C.; Karpinets, T.V.; Prieto, P.A.; Vicente, D.; Hoffman, K.; Wei, S.C.; et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 2018, 359, 97–103.
- Matson, V.; Fessler, J.; Bao, R.; Chongsuwat, T.; Zha, Y.; Alegre, M.L.; Luke, J.J.; Gajewski, T.F. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science 2018, 359, 104–108
- Larkin, J.; Hodi, F.S.; Wolchok, J.D. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N. Engl. J. Med. 2015, 373, 1270–1271.
- Chaput, N.; Lepage, P.; Coutzac, C.; Soularue, E.; Le Roux, K.; Monot, C.; Boselli, L.; Routier, E.; Cassard, L.; Collins, M.; et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Ann. Oncol. 2017, 28, 1368–1379.
- Frankel, A.E.; Coughlin, L.A.; Kim, J.; Froehlich, T.W.; Xie, Y.; Frenkel, E.P.; Koh, A.Y. Metagenomic Shotgun Sequencing and Unbiased Metabolomic Profiling Identify Specific Human Gut Microbiota and Metabolites Associated with Immune Checkpoint Therapy Efficacy in Melanoma Patients. Neoplasia 2017, 19, 848–855.
- World Health Organization-Food and Agricultural Organization, Guidelines for the Evaluation of Probiotics in Food, FAO/WHO joint report., London, 2002.
- R. Crittenden, G. Mogensen, Technological challenges for future probiotic foods, Int. Dairy J. 12 (2002) 173–182.
- 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.
- Macpherson A. J., Hunziker L., McCoy K. & Lamarre A. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganism. *Microbes and Infection*. 3, 1021-1035 (2001).

- 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).
- 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.
- 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).
- Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE., Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo- controlled trials, Lancet Infect. Dis., vol. 6, nº 6, pp. 374–82 (2006).
- P. Marteau, The clinical importance of intestinal microbiota. Gastroenterol. Clin. Biol., 34 Suppl. 1, pp. S93-7 (2010).
- R. Rastmanesh, High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction., Chem. Biol. Interact., vol. 189, 1-2, pp. 1-8 (2011).
- 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.
- 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).
- 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.

- S.S. Choi, Y. Kim, K.S. Han, S. You, S. Oh, S.H. Kim, Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro, Lett. Appl. Microbiol. 42 (2006) 452–458.
- J.E. Kim, J.Y. Kim, K.W. Lee, H.J. Lee, Cancer chemopreventive effects of lactic acid bacteria, J. Microbiol. Biotechnol. 17 (2007) 1227–1235.
- M. Eslami, Importance of probiotics in the prevention and treatment of colorectal cancer, J. Cell. Physiol. 234 (2019) 17127–17143.
- S. Shamekhi, H.L.J. Abdolalizadeh, E.B.N. Zarghami, An overview of yeast probiotics as cancer biotherapeutics : possible clinical application in colorectal cancer, Clin. Transl. Oncol. (2020), https://doi.org/10.1007/s12094-019- 02270-0.
- 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.
- 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.
- Griggs, J.J. Reducing the toxicity of anticancer therapy: New strategies. Leuk. Res. 1998, 22 (Suppl. 1), S27–S33
- 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.
- Dong, J.; Chen, H. Cardiotoxicity of Anticancer Therapeutics. Front. Cardiovasc. Med. 2018, 5, 9.
- 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.
- 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

to reduce acute and late adverse gastrointestinal effects of pelvic radiotherapy for primary pelvic cancers. Cochrane Database Syst. Rev. 2018, 1, CD012529.

- 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.
- Sokol, H.; Adolph, T.E. The microbiota: An underestimated actor in radiation-induced lesions? Gut 2018, 67, 1–2.
- 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.
- M. Thirabunyanon, P. Boonprasom, P. Niamsup, Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells, Biotechnol. Lett. 31 (2009) 571–576.
- Y. Rahbar, A. Yari, A. Akbar, M. Talebi, Modulatory role of exopolysaccharides of Kluyveromyces marxianus and Pichia kudriavzevii as probiotic yeasts from dairy products in human colon cancer cells, J. Funct. Foods 64 (2020) 1–9.
- L. Wang, Y. Wang, Q. Li, K. Tian, L. Xu, G. Liu, C. Guo, Exopolysaccharide, isolated from a novel strain Bifidobacterium breve lw01 possess an anticancer effect on head and neck Cancer - genetic and biochemical evidences, Front. Microbiol. 10 (2019) 1044, https://doi.org/10.3389/fmicb.2019.01044.
- Mego, M.; Chovanec, J.; Vochyanova-Andrezalova, I.; Konkolovsky, P.; Mikulova, M.; Reckova, M.; Miskovska, V.; Bystricky, B.; Beniak, J.; Medvecova, L.; et al. Prevention of irinotecan induced diarrhea by probiotics: A randomized double blind, placebo controlled pilot study. Complement Ther. Med. 2015, 23, 356–362.
- Theodoropoulos, G.E.; Memos, N.A.; Peitsidou, K.; Karantanos, T.; Spyropoulos, B.G.; Zografos, G. Synbiotics and gastrointestinal

function-related quality of life after elective colorectal cancer resection. Ann. Gastroenterol. 2016, 29, 56–62.

- Consoli, M.L.; da Silva, R.S.; Nicoli, J.R.; Bruña-Romero, O.; da Silva, R.G.; de Vasconcelos Generoso, S.; Correia, M.I. Randomized Clinical Trial: Impact of Oral Administration of Saccharomyces boulardii on Gene Expression of Intestinal Cytokines in Patients Undergoing Colon Resection. JPEN J. Parenter. Enter. Nutr. 2016, 40, 1114–1121. [CrossRef]
- Hibberd, A.A.; Lyra, A.; Ouwehand, A.C.; Rolny, P.; Lindegren, H.; Cedgård, L.; Wettergren, Y. Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention. BMJ Open Gastroenterol. 2017, 4, e000145. [CrossRef]
- Flesch, A.T.; Tonial, S.T.; Contu, P.C.; Damin, D.C. Perioperative synbiotics administration decreases postoperative infections in patients with colorectal cancer: A randomized, double-blind clinical trial. Rev. Col. Bras. Cir. 2017, 44, 567–573.
- J.M. Wells, Immunomodulatory mechanisms of lactobacilli, Microb. Cell Fact. 10 (2011) 1–15.
- H.A. Lee, H. Kim, K. Lee, K. Park, Dead nano-sized Lactobacillus plantarum inhibits azoxymethane/dextran sulfate sodium-induced colon cancer in Balb/c Mice, J. Med. Food 18 (2015) 1400–1405.
- F. Maghsood, B. Johari, M. Rohani, H. Madanchi, Z. Saltanatpour, M. Kadivar, Anti-proliferative and anti-metastatic potential of high molecular weight secretory molecules from probiotic Lactobacillus reuteri cell-free supernatant against human colon cancer stem-like cells (HT29-ShE), Int. J. Pept. Res. Ther. (2020) 1–13, https://doi.org/10.1007/s10989-020-10049-z.
- K. Papadimitriou, J. Kok, Future challenges in lactic acid Bacteria stress physiology research, Food Microbiol. Food Saf. 21 (2011) 507–518.
- Tesfaye Legesse Bedadaa, Tatek Kasim Fetoa, Kaleab Sebsibe Awokea, Asnake Desalegn Garedewb, Fitsum Tigu Yifatb, Dagim

Jirata Birrib. Probiotics for cancer alternative prevention and treatment. Biomedicine & Pharmacotherapy 129 (2020) 110409.

- T. Cd, C.M. Paulos, C. Wrzesinski, A. Kaiser, C.S. Hinrichs, M. Chieppa, L. Cassard, D.C. Palmer, A. Boni, P. Muranski, Z. Yu, L. Gattinoni, P.A. Antony, S.A. Rosenberg, N.P. Restifo, Microbial translocation augments the function of adoptively transferred self / tumor-specific, J. Clin. Invest. 117 (2007) 2197–2204.
- A. Górska, D. Przystupski, M.J. Niemczura, J. Kulbacka, Probiotic bacteria: a promising tool in cancer prevention and therapy, Curr. Microbiol. 76 (2019) 939–949.
- A. Borowicki, A. Michelmann, K. Stein, D. Scharlau, K. Scheu, U. Obst, A. Michelmann, K. Stein, D. Scharlau, et al., Fermented wheat aleurone enriched with probiotic strains LGG and Bb12 modulates markers of tumor progression in human colon cells LGG and Bb12 modulates markers of tumor progression in human colon cells, Nutr. Cancer 63 (2012) 151–160.
- N.M. El-Deeb, A.M. Yassin, L.A. Al-Madboly, A. El-Hawiet, A novel purified Lactobacillus acidophilus 20079 exopolysaccharide, LA-EPS-20079, molecularly regulates both apoptotic and NF-KB inflammatory pathways in human colon cancer, Microb. Cell Fact. 17 (2018), https://doi.org/10.1186/s12934-018- 0877-z
- I.G. Bogdanov, P.G. Dalev, A.I. Gurevich, M.N. Kolosov, V.P. Mal'kova, L.A. Plemyannikova, I.B. Sorokina, Antitumour glycopeptides from Lactobacillus bulgaricus cell wall, FEBS Lett. 57 (1975) 259–261.
- C. Wang, T. Wu, S. Hsieh, Y. Tsai, C. Yeh, C. Huang, Antioxidant activity and growth inhibition of human colon cancer cells by crude and purified fucoidan preparations extracted from Sargassum cristaefolium, J. Food Drug Anal. 23 (2015) 766–777.
- G. Zhang, J. Zhang, X. Wang, W. Yang, Z. Sun, C.N. Kumar, H. Guan, J. Guan, Apoptosis of human tongue squamous cell carcinoma cell

(CAL-27) induced by Lactobacillus sp. A-2 metabolites, J. Appl. Oral Sci. 22 (2014) 282–286.

- I. Kahouli, M. Malhotra, C. Tomaro-Duchesneau, L.S. Rodes, M.A. AlouiJamali, Satya Prakash, Identification of Lactobacillus fermentum strains with potential against colorectal Cancer by characterizing short chain fatty acids production, anti-proliferative activity and survival in an intestinal fluid: in vitro analysis, J. Bioanal. Biomed. 7 (2015) 104–115.
- M. Thirabunyanon, P. Hongwittayakorn, Potential probiotic lactic acid Bacteria of human origin induce anti-proliferation of colon cancer cells via synergic actions in adhesion to cancer cells and short-chain fatty acid bioproduction, Appl. Biochem. Biotechnol. 169 (2013) 511–525.
- L.D. Lagadic-gossmann, C. Lemaire, C. Brenner, Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria, Apoptosis. 12 (2007) 573.
- J.B. Ewaschuk, J.W. Walker, H. Diaz, K.L. Madsen, Bioproduction of conjugated linoleic acid by probiotic bacteria occurs In Vitro and In Vivo in mice, J. Nutr. 136 (2006) 1483–1487.
- I. Kahouli, N.R. Handiri, Characterization of L. Reuteri NCIMB 701359 probiotic features for potential use as a colorectal cancer biotherapeutic by identifying fatty acid profile and anti-proliferative action against colorectal cancer cells, Drug Des. 5 (2016) 1–11.
- M. Kleerebezem, P. Hols, E. Bernard, T. Rolain, M. Zhou, R.J. Siezen, et al., The extracellular biology of the lactobacilli, FEMS Microbiol. Rev. 34 (2010) 199–230.
- G. Mcintosh, Probiotics and colon cancer prevention, Asia Pacific J Clin Nutr. 5 (1996) 48–52.
- J. Escamilla, M.A. Lane, V. Maitin, J. Escamilla, M.A. Lane, V. Maitin, Cell-Free Supernatants from probiotic Lactobacillus casei and Lactobacillus rhamnosus GG Decrease colon cancer cell invasion In Vitro, Nutr. Cancer 64 (2012) 871–878.

- O.R.F. Mook, W.M. Frederiks, C.J.F. Van Noorden, The role of gelatinases in colorectal cancer progression and metastasis, Biochim. Biophys. Acta 1705 (2004) 69–89.
- S. Ohtani, M. Terashima, J. Satoh, N. Soeta, Z. Saze, S. Kashimura, F. Ohsuka, Y. Hoshino, M. Kogure, M. Gotoh, Expression of tight-junction-associated proteins in human gastric cancer: down-regulation of claudin-4 correlates with tumor aggressiveness and survival, Gastric Cancer 12 (2009) 43–51.
- K.A. Donato, M.G. Gareau, Y.J.J. Wang, P.M. Sherman, Lactobacillus rhamnosus GG attenuates interferon-γ and tumor necrosis factor-α-induced barrier dysfunction and pro-inflammatory signalling, Microbiology 156 (2010) 3288–3297.
- M. Pancione, A. Remo, V. Colantuoni, Genetic and epigenetic events generate multiple pathways in colorectal cancer progression, Patholog. Res. Int. 2012 (2012) 1–11.
- Q. Wu, T. Ishikawa, R. Sirianni, H. Tang, J.G. McDonald, I.S. Yuhanna1, et al., 27- hydroxycholesterol promotes cell-autonomous ER-positive breast Cancer growth, Cell Rep. 5 (2012) 637–645
- 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.
- Wang, Y.; Liu, L.; Moore, D.J.; Shen, X.; Peek, R.M.; Acra, S.A.; Li, H.; Ren, X.; Polk, D.B.; Yan, F. An LGG-derived protein promotes IgA production through upregulation of APRIL expression in intestinal epithelial cells. Mucosal Immunol. 2017, 10, 373–384.
- Fong, F.L.; Kirjavainen, P.V.; El-Nezami, H. Immunomodulation of Lactobacillus rhamnosus GG (LGG)-derived soluble factors on antigen-presenting cells of healthy blood donors. Sci. Rep. 2016, 6, 22845.

- 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
- Silvia Vivarelli, Rossella Salemi, Saverio Candido, Luca Falzone, Maria Santagati, Stefania Stefani, Francesco Torino, Giuseppe Luigi Banna, Giuseppe Tonini and Massimo Libra. Gut Microbiota and Cancer: From Pathogenesis to Therapy. Cancers 2019, 11, 38; doi:10.3390/cancers11010038
- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Mendes, M.C.S.; Paulino, D.S.; Brambilla, S.R.; Camargo, J.A.; Persinoti, G.F.; Carvalheira, J.B.C. Microbiota modification by

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

- 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
- 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, e0188985.
- Lam EK, Tai EK, Koo MW, Wong HP, Wu WK, Yu L, et al. Enhancement of gastric mucosal integrity by Lactobacillus rhamnosus GG. Life Sci . 2007; 80:2128–2136.
- Yan F, Polk DB. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem . 2002; 277:50959–50965.
- Choi SS, Kim Y, Han KS, You S, Oh S, Kim SH. Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. Lett Appl Microbiol . 2006; 42:452–458.
- Rauch M, Lynch SV. Probiotic manipulation of the gastrointestinal microbiota. Gut Microbes. 2010;1(5): 335–338. pmid:21327043
- Hirayama K, Rafter J. The role of probiotic bacteria in cancer prevention. Microbes Infect. 2000;2(6): 681–686. pmid:10884619
- Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9(5): 313–324. pmid:19343057
- Oelschlaeger TA. Mechanisms of probiotic actions—A review. Int J Med Microbiol. 2010;300(1): 57–62. pmid:19783474
- Escamilla J, Lane MA, Maitin V. Cell-free supernatants from probiotic *Lactobacillus casei* and *Lactobacillus rhamnosus* GG

decrease colon cancer cell invasion *in vitro*. Nutr Cancer. 2012; 64(6): 871–878. pmid:22830611

- Di Luccia B, Manzo N, Baccigalupi L, Calabrò V, Crescenzi E, Ricca E, et al. Lactobacillus gasseri SF1183 affects intestinal epithelial cell survival and growth. PLoS One. 2013;8(7): e69102. pmid:23894414
- Lan A, Lagadic-Gossmann D, Lemaire C, Brenner C, Jan G. Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. Apoptosis. 2007;12(3): 573–591. pmid:17195096
- Orlando A, Messa C, Linsalata M, Cavallini A, Russo F. Effects of Lactobacillus rhamnosus GG on proliferation and polyamine metabolism in HGC-27 human gastric and DLD-1 colonic cancer cell lines. Immunopharmacol Immunotoxicol. 2009; 31:108–116.
- Choi SS, Kim Y, Han KS, You S, Oh S, Kim SH. Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. Lett Appl Microbiol . 2006; 42:452–458.
- Hojjat Sadeghi-Aliabadi, Fatemeh Mohammadi, [...], and Maryam Mirlohi. Effects of *Lactobacillus plantarum* A7 with probiotic potential on colon cancer and normal cells proliferation in comparison with a commercial strain. Iran J Basic Med Sci. 2014 Oct; 17(10): 815–819.
- Kim JU, Kim Y, Han KS, Oh S, Whang KY, Kim JN, et al. Function of cell-boundand released Exopoly saccharides produced by Lactobacillus rhamnosus ATCC 9595. J Microbiol Biotechnol . 2006;16:939–945.
- Zhong L, Zhang X, Covasa M. Emerging roles of lactic acid bacteria in protection against colorectal cancer. World J Gastroenterol. 2014;20(24):7878–7886. pmid:24976724.
- Chen CC, Lin WC, Kong MS, Shi HN, Walker WA, Lin CY, et al. Oral inoculation of probiotics Lactobacillus acidophilus NCFM suppresses tumour growth both in segmental orthotopic colon cancer and extra-intestinal tissue. Br J Nutr. 2012;107(11): 1623–1634. pmid:21992995.

- Iyer C, Kosters A, Sethi G, Kunnumakkara AB, Aggarwal BB, Versalovic J. Probiotic Lactobacillus reuteri promotes TNF-induced apoptosis in human myeloid leukemia derived cells by modulation of NF-kappa B and MAPK signaling. Cell Microbiol. 2008;10: 1442–1452. pmid:18331465.