Susceptibility of lactic acid bacteria, bifidobacteria and bacteria of intestinal origin to antitumor compounds used in breast and lung cancer

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Abstract

Chemotherapy is a cornerstone in the treatment of cancer, even though it often causes harmful side effects on the mucosa characterized by inflammation and ulceration of the epithelial gastrointestinal tract (known as mucositis). In an attempt to identify microorganisms that could prevent or treat mucositis symptoms, this work reports on the susceptibility-resistance profiles of a set of 23 lactic acid bacteria (LAB) and bifidobacteria strains to the commonest chemotherapeutic antitumorals used to treat lung and breast cancer. The minimum inhibitory concentration (MIC) of each antitumoral to these strains was compared to that obtained for eleven strains of representative species from the human gastrointestinal tract. All strains proved to be resistant up to the highest concentration assayed (MIC >128 µg/ml) to apecitabine, cyclosphosphamide, docetaxel, erlotinib, gefitinib, irinotecan and placitaxel. Variability in MICs among species and strains was recorded for afatinib, doxorubicin, 5-fluorouracil, gemcitabine and pemetrexed. The highest inter-species variability of MICs was observed for pemetrexed and afatinib. Doxorubicin was the compound showing the lowest MICs for LAB and bifidobacteria species, as only two strains showed a MIC >16µg/ml. Bifidobacteria strains were also very susceptible to pemetrexed (MIC ≤0.5µg/ml), except for strains of *Bifidobacterium adolescentis* and *Bifidobacterium longum* subsp. *longum*. In order to assess the intra-species and inter-strain variability, MICs of pemetrexed and afatinib to 32 strains belonging to four *Bifidobacterium* species were analysed. The distribution of MICs to these two compounds showed a bimodal curve for pemetrexed (<2-8 µg/ml; 256 µg/ml) and unimodal for afatinib (128 µg/ml). Among the more resistant strains to afatinib, *B. longum* L43 was selected and the protective effect of UV-killed bacteria to maintain the Cell-Index (CI) of a human cell line (HT29) monolayers during growth in the presence of this antitumoral was

analysed by using a Real-Time Cell Analyzed (RTCA). A significant maintenance of the CI in the cell cultures was recorded, which suggest a protective effect of L43 against the citotoxicity exerted by afatinib. Altogether, the results argue for a harmful impact of some chemotherapeutical compounds on LAB and bifidobacteria species from the gastrointestinal tract. Further, they suggest that selected strains resistant to high antitumoral concentrations could be used to counteract antitumoral-induced damage, which might include the relief of mucositis symptoms.

1. Introduction

Cancer remains a major cause of death worldwide, being lung, breast, prostate and colorectal cancer among the commonest diagnosed (Ferlay et al., 2010; Ferlay et al., 2013). Chemotherapy is the first line of defence in cancer therapy. This includes treatment with natural compounds, DNA-alkylating agents, antimetabolites, etc., attacking the rapidly cancer dividing cells. Although effective, all these compounds show an insufficient selectivity failing to distinguish between normal and neoplastic cells. Consequently, development of a variety of side effects induced by chemotherapy is rather frequent (Sonis et al., 2004). Pathophysiological symptoms, such as nausea, bloating, vomiting, abdominal pain and severe diarrhoea are commonly diagnosed in patients undergoing chemotherapy (Sonis et al., 2004). Currently, no effective treatments for amelioration of chemotherapy side effects exist.

One of the most debilitating effects of antitumor compounds is the damage they cause to the mucosa cells lining the gastrointestinal tract. Antitumorals targeting proliferating cells cause a loss of the gastric basal epithelium, hampering its renewal and contributing to early cell death, atrophy and ulceration of the mucosa (Sonis et al.,

2004). These harmful side effects are known as mucositis. Throughout chemotherapy, the small intestine, oesophagus, stomach, and the large intestine are mucositis most affected areas. Depending on the dose and type of antitumor agent, large percentage of patients, between 40 % (receiving a standard dose) and 100% (receiving a high dose), develop gastrointestinal mucositis (Keefe et al., 2000; Stringer et al., 2009). Bacteraemia, malnutrition, and other clinical symptoms are usually associated with chemotherapy-induced mucositis, which significantly impairs quality of life of patients (Sonis et al., 2004). Pathophysiological and clinical symptoms frequently lead to reducing dosage of antitumorals or to postpone chemotherapy treatments, which entails serious implications for the progression of cancer (Sonis et al., 2004; Elting et al., 2003). Therefore, the development of new therapies protecting or reducing the severity of mucositis would enable to improve the quality of life for patients undergoing chemotherapy and would surely increase tolerance to higher chemotherapeutics doses, contributing to raise rates of cancer survival.

Anticancer treatments have also a damaging (antimicrobial) effect on components of the intestinal microbiota (Stringer, 2013), which plays an homeostatic regulatory role in mucosal tissue by several mechanisms, including control of inflammatory processes, reduction of intestinal permeability, maintenance of the integrity of the mucus layer (which enhance the resistance towards harmful compounds and improve epithelial mechanisms of repair), and activation of the release of immune effector molecules (for a review see van Vliet et al., 2010). The combined use of antitumorals and antibiotics to combat chemotherapy-induced bacterial infection during cancer treatment, are associated with an overall reduction of the microbial diversity in the gut (Zwielehner et al., 2011; Perez-Cobas et al., 2013).

Lactic acid bacteria (LAB) and bifidobacteria species are common inhabitants of the human gastrointestinal tract, where they contribute to the microbial intestinal balance for a healthy state (Ohashi and Ushida, 2009). In fact, these bacteria have a generally regarded as safe (GRAS) status based on a long history of safe use without reported adverse effects, and hence species and strains of LAB and bifidobacteria are frequently used as probiotics (Saxelin, 2008). Numerous scientific and clinic reports have evidenced beneficial effects exerted by certain probiotic strains to reduce the risk or symptoms of diseases such as severe diarrhoea, lactose intolerance, allergies or inflammatory diseases (for a review see Mayo et al., 2008). Furthermore, a role of probiotics on the modulation of diseases such as diabetes, obesity and autism has also been suggested in recent studies (Isolauri et al., 2015; Adams et al., 2011). However, the prevention of chemotherapy-induced gastrointestinal disorders through probiotic intervention has scarcely been investigated. To our knowledge, just a couple of reports have been published analyzing the protection exerted by *Streptococcus thermophilus* TH4 (Wang et al., 2013) and the commercial probiotic mixture VSL#3 (Bowen et al., 2007) against methotrexate and irinotecan induced mucositis, respectively.

In this work we addressed for the first time the resistance-susceptibility levels of a collection of LAB, bifidobacteria and bacteria of intestinal origin to twelve common antitumor compounds currently in use to combat lung and breast cancer. In addition, the susceptibility to afatinib and pemetrexed of 32 *Bifidobacterium* spp. strains, isolated from the human gut was also evaluated. Finally, the effectiveness of *Bifidobacterium longum* L43 for maintaining the Cell-Index of a human-derived cell line during culture to counteract the decrease caused by addition of the antitumoral afatinib was assessed *in vitro* by the use of a Real Time Cell Analyzer (RTCA) system.

2. Material and methods

2.1. Bacterial strains, growth media and culture conditions

The Minimum Inhibitory Concentration (MIC) of twelve antitumor compounds to a collection of 34 bacterial strains belonging to several species (Tables 1 and 2) was analysed. The collection included 23 type strains of LAB and bifidobacteria species from the BCCM/LMG Bacterial Collection (Ghent University, Ghent, Belgium), seven intestinal species obtained from the DSMZ (Leibniz institute, Germany), and four Gram-negative strains from our laboratory collection. The MIC of afatinib and pemetrexed was also assayed on a laboratory collection of 32 bifidobacteria strains isolated from the human gut (Delgado et al., 2008).

Lactococci were grown in M17 agar (Oxoid) supplemented with 1% glucose (VWR International) at 32ºC for 48 h in aerobic conditions. *Streptococcus thermophilus* was cultured in M17 agar (Oxoid) supplemented with 1% lactose (VWR International) at 37ºC for 48 h in anaerobic conditions. Heterofermentative lactobacilli were recovered on de Man, Rogosa and Sharpe (MRS) agar plates (VWR International) and incubated for 48 h at 32ºC or 37ºC in aerobic or anaerobic conditions depending on the species. Homofermentative lactobacilli and bifidobacteria were recovered in MRS agar supplemented with 0.25% L-cysteine (MRSc) and incubated at 37ºC for 48 h in anaerobiosis. Intestinal anaerobic strains were streaked in the following solid media: *Bacteroides* spp. in Gifu Anaerobic Medium (GAM) agar (Nissui), *Faecalibacterium prausnitzii* in Reinforced Clostridial Medium (RCM) agar (VWR International), *Ruminococcus obeum* and *Blautia coccoides* in 50% of RCM and Brain Heart Infusion (BHI) (VWR International) plates, and *Slackia* spp. was grown in GAM agar supplemented with 0.5% arginine. Strains of these species were incubated at 37ºC for

147 48 h under anaerobic conditions. Finally, strains of all other species were grown in BHI
148 agar at 37ºC for 24 h in aerobiosis.
149
150 2.2. Determination of MICs
151 MICs of 12 different antitumor compounds to bacterial species and strains were
152 determined by a broth microdilution test. The antitumor compounds evaluated were
153 afatinib, docetaxel, erlotinib, gefitinib, gemcitabine, irinotecan, pemetrexed (used to
154 treat lung cancer) and capecitabine, cyclophosphamide, doxorubicin, 5-fluorouracil, and
155 paclitaxel (used to treat breast cancer).
156 Individual colonies from the recovering plates (as listed above) were suspended
157 in 5 ml of a sterile saline solution (0.9% NaCl; VWR International) to a turbidity of 1 in
158 the McFarland's scale or its spectrophotometric equivalent (approx. 3×10^8 cfu/ml). The
159 inoculated saline solution was then diluted 1:1000 in the test media (see below) to
160 obtain an approximate final concentration of 3×10^5 cfu/ml. Iso-sensitest (IST) broth
161 (Oxoid) was used for lactococci, *Escherichia coli, Klebsiella pneumoniae,*
162 *Pseudomonas aureginosa* and *Serratia marcescens*, IST supplemented with 1% lactose
163 was used for *Streptococcus thermophilus*, LSM broth (IST:MRS, 9:1) was used for
164 heterofermentative lactobacilli and LSM broth supplemented with 0.03% L-cysteine
165 was used for homofermentative lactobacilli and bifidobacteria, while LSG broth
166 (IST:GAM, 9:1) supplemented with 0.25% L-cysteine was used for anaerobic species.
167 Aliquots of 100 µl of the diluted cell suspensions were added to microplate wells with
168 two-fold increasing antitumor concentrations (from 0.0625 to 128 µg/ml). MICs were
169 established as the lowest antitumoral concentration at which no growth was observed by
170 visual inspection.
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2.3. Growth conditions of HT29 cells

The HT29 cell line (ECACC 91072201) used in the protection assays was purchased from the European Collection of Authenticated Cell Cultures (ECACC). HT29 cells were cultured in McCoy's Medium (MM) (Sigma) supplemented with 10% heat-inactivated foetal bovine serum (Sigma), 3 mM L-glutamine (Sigma) and a mixture of antibiotics (50 µg/ml streptomycin-penicillin, 50 µg /ml gentamicin and 1.25 µg /ml amphotericin B; Sigma). Incubations took place at 37ºC, 5% CO_2 in a SL Waterjacked CO_2 Incubator (Sheldon Manufacturing). Culture medium was changed every two days and the cell line was trypsinized weekly using a 0.25% trypsin-EDTA solution (Sigma) following standard procedures. For the cell line experiments, $2x10^5$ HT29 cells/ml were seeded in 16-well E-plates which were connected to a real time cell analyser (RTCA) (XCELLigence equipment; ACEA Bioscience). Before testing cells were incubated for approximately 18 h until they reach a confluent and differentiated state.

2.4. *In vitro* interaction between cell-bacteria-afatinib

Bifidobacterium longm L43 strain was cultured overnight in MRSc, harvested by centrifugation, and washed twice with PBS buffer (VWR international). Aliquots of this culture were treated three times for 30 min with UV light (254 nm). Dead bacteria were frozen in liquid-nitrogen and preserved at -80ºC until use. Afterwards, 200 µl of an L43 cell suspension in McCoy's medium without antibiotics, containing 10^8 or 10^9cfu/ml (as determined before the UV light treatment by plate counting), and different afatinib concentrations (from 16 to 128 µg/ml) were added to wells that contained confluent and differentiated HT29 cells. The E-plates were then incubated for 24 h at 37ºC, 5% CO_2 in a Heracell 240 incubator (Thermo). The Cell-Index given by the RTCA apparatus was measured during a 24 h incubation period every 15 min. Duplicated wells from three

independent assays were measured for each afatinib concentration. As controls, HT29 cells in McCoy's medium without afatinib (reference control) and without afatinib but with bacteria (experimental control) were used.

3. Results

3.1. Bacterial susceptibility to antitumor compounds

A total of 34 strains were tested for their susceptibility to twelve antitumor compounds used in clinic for treating lung and breast cancer. The MIC values obtained after 48 h of incubation are summarized in Table 1. All strains grew at the highest concentration of seven of the antitumorals (capecitabine, cyclosphosphamide, docetaxel, erlotinib, gefitinib, irinotecan and placitaxel) (MIC >128µg/ml), whereas, though small in some cases, differences in MICs between species were scored for all other compounds (afatinib, doxorubicin, 5-fluorouracil, gemcitabine and pemetrexed). Differences in MICs were observed between and within the different bacterial groups.

Most LAB and bifidobacteria species were susceptible to low levels (MICs <16 µg/ml) of doxorubicin, an anthracycline antitumor-antibiotic, with the exception of *Lactococcus lactis* subsp. *cremoris* LMG 6987^T and *Lactobacillus sakei* subsp. *sakei* LMG 9468^T (MICs of 32 and 64 µg/ml, respectively). The MICs observed of afatinib, a compound that inhibits autophosphorylation of tyrosine kinases, to LAB species ranged from 32 to 128 µg/ml, except for *Lactobacillus rhamnosus* LMG 6400^T, *Lactobacillus gasseri* LMG 9203^T, and *Lactobacillus johnsonii* LMG 9436^T, which grew in the presence of this antitumoral at the highest concentration assayed (MICs >128 µg/ml). A class of chemotherapeutical drugs called antimetabolites (5-fluorouracil, gemcitabine and pemetrexed) showed the greatest degree of variability in their effect against LAB

and bifidobacteria. Most species showed resistance up to the highest concentration of 5-fluorouracil assayed (MIC ≥128 μg/ml), but a few, such as *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T, proved to be very susceptible (MIC 0.25 μg/ml). Other strains such as *Lactobacillus pentosus* LMG 10755^T, and *Bifidobacterium adolescentis* LMG 10502^T showed intermediate resistance (MICs 8 and 32 μg/ml, respectively). Regarding gemcitabine, *S. thermophilus* and all bifidobacteria species were shown to be resistant up to the highest dose tested (MIC ≥128 μg/ml). Lactococci and lactobacilli displayed a species-specific susceptibility pattern; while some species grew at the maximum concentration (MIC of ≥128 μg/ml), MIC values of some others ranged from 0.5 to 16 μg/ml. Finally, lactococci, *S. thermophilus* and most lactobacilli were resistant to high concentration of pemetrexed (MIC of ≥128 μg/ml). However, intermediate (8-16 μg/ml) and low (0.0625-0.125 μg/ml) MIC values were observed for specific lactobacilli species. In contrast to LAB a majority of bifidobacteria species proved to be very susceptible to gemcitabine (MICs 0.0625-0.5 μg/ml), with the exception of *B. adolescentis* LMG 10502^T and *Bifidobacterium longum* subsp. *longum* LMG 13197^T (MIC ≥ 128 μg/ml).

As concerns the non-LAB bacteria assayed in this study, they all proved to be highly resistant to the twelve antitumor compounds; all strains grew well up to the maximum concentration assayed (MIC >128 μg/ml). The exception was doxorubicin, to which all strains showed MICs of 128 μg/ml of higher, except for *Faecalibacterium prausnitzii* DSM 1767 (MIC 32 μg/ml), *Ruminococcus obeum* DSM 25238^T (MIC 64 μg/ml) and *Slackia isoflavoniconvertens* DSM 22006^T (MIC 32 μg/ml).

3.2. Cut-off values for *Bifidobacterium* species to afatinib and pemetrexed

High inter-species variability of MICs for afatinib and pemetrexed in bifidobacteria species was observed (Table 1). As only the MIC of one strain per species was determined, 32 bifidobacteria strains belonging to four species (*B. animalis, B. bifidum, B. longum* and *B. pseudolongum*) were selected to assess the intra-species diversity in their susceptibility to these two compounds. The distribution of MICs for the different species and strains is summarized in Table 2. MIC values of pemetrexed spread over a broad range (from <2 to >256 μg/ml), showing a kind of bimodal distribution. A few strains proved to be highly susceptible, while most others tolerated high pemetrexed concentrations (>256 μg/ml). In contrast, MICs of afatinib ranged from 32 to 256 μg/ml showing a unimodal (128 μg/ml) normal distribution curve.

3.3. Cell-Index evaluation of HT29 cell cultures

A Real Time Cell Analyzer (RTCA) was applied for analysing *in vitro* the cytotoxicity of afatinib to an intestinal-derived human cell line (HT29). Effects were measured when cells reached confluency, trying to mimic their differentiate state in the gut epithelium. The RTCA system monitors continuously through gold-microelectrodes and in real a time manner variations in impedance [referred to as the Cell-Index (CI)]. Changes in the CI are due to attachment/detachment of cells to the microplate during growth, as well as to changes in the cell size or morphology. The CI curves along incubation of HT29 cells in the presence of various afatinib concentrations (16-128 μg/ml) are depicted in Figure 1. The CI of the cultures was normalized at a time point immediately before addition of the compound. CI values are always referred to that of the control (HT29 cells growing alone; CI=0) (Figure 1). The CI curve of 16 μg/ml afatinib was very similar to that of the control, whereas addition of concentration of 64 and 128 μg/ml caused an immediate drop of the CI that never recovers afterwards. The

effect of an afatinib concentration of 32 µg/ml caused an intermediate effect on the CI of HT29, after an initial increased, a pronounced decrease started at around 9 h of incubation getting at 24 h a negative value similar to those obtained for 64 and 128 µg/ml.

3.4. Effect of B. longum L43 on the CI of HT29 cultures

An RTCA experiment was set up in order to evaluate whether the damage(s) of afatinib to the HT29 cell line could be reduced or prevented by the presence in the system of a probiotic strain. The strain to be assayed, *B. longum* L43, was selected based on its properties of probiosis (Delgado et al., 2008) and high resistance to afatinib (MIC 128 µg/ml). Bifidobacteria grow extremely well in McCoy's medium without antibiotics producing high amounts of lactic and acetic acids, which kill HT29 cells. This causes an immediate drop in the CI similar to that of 128 µg/ml of afatinib (data not shown). For this reason, only dead bifidobacteria (UV treated) can be added in this assay. Figure 2 shows the CI values of HT29 cell cultures growing without and with three afatinib concentrations and in the absence of presence of the probiotic (at 19^8 and 10^9 cfu/ml concentrations). As can be seen in the figure, the presence of dead cells of *B. longum* L43 exerted a protective effect against the decrease of the CI. The drop of the CI was completely prevented in the presence of 10^9 cfu/ml of dead bacteria up to an afatinib concentration of 64 µg/ml.

4. Discussion

Chemotherapy has a harmful impact on the gastrointestinal mucosa, either as a direct cytotoxic effect on the cells or through changes in the microbiota lining the gut

(Yang et al., 2013; Zwielehner et al., 2011). In this work, we focused on the analysis of the resistance-susceptibility profiles of LAB and bifidobacteria species and strains to twelve chemotherapeutics commonly used to treat lung and breast cancer. The concentration levels of the different compounds assayed were higher than the physiological concentrations reached during treatment for all antitumorals (Reference Marta). The aim of this study was to assess whether selected strains resistant to the antitumorals could have presumptive protective effects against the damage caused by the chemotherapy.

Depending on the antitumoral, different resistance/susceptibility profiles were found among species and strains. Most of the species showed resistance to high levels of several antitumor agents (such as capecitabine, cyclosphosphamide, docetaxel, erlotinib, gefitinib, irinotecan and placitaxel). Nevertheless, variable susceptibility was observed to some others (such as afatinib, doxorubicin, 5-fluorouracil, gemcitabine and pemetrexed).

The taxoids docetaxel and paclitaxel share a similar mechanism of action, the promotion of microtubule assembly and inhibition of microtubule disassembly. Similarly, erlotinib and gefitinib are tyrosine kinase inhibitors of the epidermal growth factor receptor (Golan et al., 2012). The target of these four antitumor agents are eukaryotic cell structures and, therefore, high resistance of bacteria was expected. In contrast, it was surprising the high resistance displayed by bacteria to irinotecan, capecitabine and cyclophosphamide. These compounds interfere with cell division mechanisms of eukaryotes and prokaryotes, such as binding to the topoisomerase I-DNA complex preventing recognition of the DNA strand (irinotecan), inhibiting DNA synthesis (capecitabine), or creating crosslinks in the DNA by adding an alkyl group to guanine bases (cyclophosphamide) (Golan et al., 2012). Differential cell permeability of

prokaryotes as compared to eukaryotes or enhanced activity of non-specific efflux systems may account for the bacterial resistance (Blair et al., 2015).

The high resistance of lactobacilli to cyclophosphamide agrees well with reports on the literature showing an increase in *L. johnsonii* counts after cyclophosphamide treatment (Viaud et al., 2013). Our data further agree with increases for *E. coli*, *Serratia* spp. and *Bifidobacterium* spp. after irinotecan treatment, as it have been reported by Stringer et al. (2009). The effect of capecitabine on components of the microbiota has yet to be reported.

On the whole, LAB and bifidobacteria species seemed to be more susceptible than other bacterial groups to the remaining antitumor compounds tested in this study. These results agreed with articles reporting increases in counts of *Bacteroides* spp. in patients undergoing antitumoral chemotherapy (Nyhlen et al., 2002; Zwielehner et al., 2011), but desagree with others describing increases of bifidobacteria during treatment (Zwielehner et al., 2011). This variable response to anticancer agents in patients might be due, at least in part, to the highly diverse individual composition of the basal microbiota (Qin et al., 2010). Species-specific differences in the susceptibility to afatinib, doxorubicin, 5-fluorouracil, gemcitabine and pemetrexed were observed. Bacteria possess a remarkable ability to rapidly adapt and evolve in response to antibiotics and biocides (Fernández-Fuentes et al., 2012; Baquero et al., 2013). Therefore a similar plasticity response to antitumorals would be expected. As previously described for antibiotics (Blair et al., 2015), non-specific (cell-wall impermeability, activity of membrane-located efflux pumps responsible for extrusion of toxic substances) and specific (alteration of the target, enzymatic inactivation of the drug, or prevention of the drug from accessing its target) mechanisms could be responsible for the species-specific resistance patterns.

Based upon this variability, the antitumor compounds afatinib and pemetrexed were selected for a more in depth analysis of 32 strains of four *Bifidobacterium* species. This assay will allow us to estimate the inter- and intra-species variability in the susceptibility to these compounds. The distribution of MICs for afatinib follows a normal curve, which could eventually lead to the setup of microbiological breakpoints for this compound, following the procedure for determining antibiotic resistance cut-offs (EFSA, 2012). The MICs of pemetrexed were far lower in *B. animalis* and two strains of *B. longum* than in all other *Bifidobacterium* species, which could be a species-specific property. Similarly species- and strain-specific susceptibility levels of bifidobacteria to different antibiotics has been reported elsewhere (Mayrhofer et al., 2011; Flórez et al., 2008).

The use of animal testing in research is subjected to strict control measures and the development of alternative methods is being encouraged worldwide. In this work, an *in vitro* culture analysis using an RTCA system was employed to assess the cytotoxic effect of afatinib on an intestinal cell line and to evaluate the presumptive protection of a selected probiotic strain. The RTCA apparatus senses changes in conductivity (impedance) through the cell monolayers. Under this culture system, the addition of afatinib causes a profound immediate decrease of the so called Cell-Index (CI). Decreases in the CI are due to any effect causing cytotoxicity, structural damages, apoptosis, and/or inducing morphological modifications (in either shape or size) to the cells (Xi et al., 2008). The suitability and accuracy of measuring the CI of cell cultures by the RTCA technology for evaluating the cytotoxic degree of antitumorals has been recently reported (Salis et al., 2015; Benay et al., 2015). In this sense, the cytotoxic effect of afatinib on a human-derived cell line (HT29) through the use of an RTCA approach is being reported for the first time. Arguably, maintainance of the CI, as

compared to a control, is considered to be a positive effect that might contribute to sustain fitness/performance/viability of the cell line. The assay showed a dose-dependent decrease of the CI in afatinib-treated HT29 cultures. In the presence of a 10-fold inhibitory concentration $(IC)_{50}$ of afatinib (a dose killing 50% of cells) (Poindessous et al., 2011), *B. longum* L43 protected the CI of the HT29 culture during at least 24 h of cultivation. This suggests that, somehow, the monolayer integrity is maintained. Failure and success of the use of different probiotic strains in the recovering of chemotherapy-induced mucositis have been reported (Wang et al., 2013; Mauger et al., 2007; Justino et al., 2015). Controversial data may be due to the fact that the strains employed had been only selected on the basis of their general probiotic properties (Ciorba et al., 2015). Although further evidence would require *in vivo* experiments, our preliminary results pointed towards a promising suitability of *B. longum* L43 for ameliorating the afatinib induced mucositis in cancer patients.

5. Conclusions

Previous studies have reported negative effects of chemotherapy in the gastrointestinal tract ecosystem. Analysis of the resistance-susceptibility patterns of strains from different bacterial groups to the commonest antitumoral currently in use to treat lung and breast cancer showed variability among species and strains to some of them. This diversity may allow for the selection of strains resistant to physiological levels of specific antitumorals. In this context, a *B. longum* strain highly resistant to afatinib was selected in this study. The presence of dead cell of this strain in the cultures proved to counteract the CI decline caused by afatinib on growing HT29 cells. In conclusion, antitumoral resistance surveys may ultimately allow the identification of probiotic candidates for the relief of symptoms caused by antitumoral-induced mucositis.

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Table 1.- Minimum inhibitory concentration (MIC) values of twelve antitumor compounds to lactic acid bacteria, bifidobacteria and intestinal strains of dominant and representative bacterial groups.

Species	LMG code	MIC (µg/ml)											
		A	DC	E	GF	GM	I	PE	CA	CI	DX	F	PA
Lactococci													
Lc. lactis subsp. *cremoris*	LMG 6987^T	32	>128	>128	>128	16	>128	>128	>128	>128	32	>128	>128
Lc. lactis subsp. *lactis*	LMG 6890^T	32	>128	>128	>128	0,5	>128	>128	>128	>128	4	64	>128
Streptococci													
S. termophilus	LMG 6896^T	128	>128	>128	>128	>128	>128	>128	>128	>128	8	>128	>128
Homofermetative lactobacilli													
L. acidophilus	LMG 9433^T	64	>128	>128	>128	>128	>128	>128	>128	>128	2	>128	>128
L. delbrueckii subsp. *bulgaricus*	LMG 6901^T	128	>128	>128	>128	0.5	>128	0.125	>128	>128	1	0.25	>128
L. gasseri	LMG 9203^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	>128	>128
L. helveticus	LMG 6413^T	128	>128	>128	>128	2	>128	>128	>128	>128	2	>128	>128
L. johnsonii	LMG 9436^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	>128	>128
Heterfermentative lactobacilli													
L. brevis	LMG 6906^T	128	>128	>128	>128	>128	>128	>128	>128	>128	4	>128	>128
L. casei	LMG 6904^T	64	>128	>128	>128	16	>128	8	>128	>128	8	>128	>128
L. fermentum	LMG 6902^T	128	>128	>128	>128	16	>128	>128	>128	>128	4	>128	>128
L. paracasei subsp. *paracasei*	LMG 13087^T	64	>128	>128	>128	>128	>128	16	>128	>128	16	>128	>128
L. pentosus	LMG 10755^T	64	>128	>128	>128	0.5	>128	>128	>128	>128	4	8	>128
L. plantarum	LMG 6907^T	128	>128	>128	>128	2	>128	>128	>128	>128	16	>128	>128
L. reuteri	LMG 9213^T	64	>128	>128	>128	>128	>128	≤0.0625	>128	>128	4	128	>128
L. rhamnosus	LMG 6400^T	>128	>128	>128	>128	128	>128	>128	>128	>128	16	>128	>128
L. sakei subsp. *sakei*	LMG 9468^T	32	>128	>128	>128	1	>128	>128	>128	>128	64	128	>128
Bifidobacteria													
B. adolescentis	LMG 10502^T	128	>128	>128	>128	>128	>128	128	>128	>128	1	32	>128
B. animalis subsp. *animalis*	LMG 10508^T	64	>128	>128	>128	>128	>128	≤0.0625	>128	>128	4	>128	>128
B. longum subsp. *longum*	LMG 13197^T	128	>128	>128	>128	>128	>128	>128	>128	>128	4	128	>128
B. pseudolongum subsp. *pseudolongum*	LMG 11571^T	128	>128	>128	>128	>128	>128	≤0.0625	>128	>128	8	128	>128
B. pseudolongum subsp. *globosum*	LMG 11569^T	64	>128	>128	>128	>128	>128	≤0.0625	>128	>128	4	128	>128
B. termophilum	LMG 21813^T	64	>128	>128	>128	>128	>128	0.5	>128	>128	4	>128	>128
Other intestinal bacteria													
Bacteroides fragilis	DSM 2151^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Bacteroides thetaiotaomicron	DSM 2079^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Blautia coccoides	DSM 935^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128

Faecalibacterium prausnitzii	DSM 17677	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	>128	>128
Ruminococcus obeum	DSM 25238^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	64	>128	>128
Slackia equolifaciens	DSM 24851^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Slackia isoflavoniconvertens	DSM 22006^T	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	>128	>128
Escherichia coli	A-15	>128	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	>128
Klebsiella pneumoniae	K-78	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Pseudomonas aeruginosa	PS-25	>128	>128	>128	>128	>128	>128	>128	>128	>128	128	>128	>128
Serratia marcescens	S-54	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128

Key of antitumorals: A, afatinib; DC, docetaxel; E, erlotinib; GF, gefitinib; GM, gemcitabine; I, irinotecan; PE, pemetrexed; CA, capecitabine; CI, cyclophosphamide; DX, doxorubicin; F, 5-fluorouracil; PA, paclitaxel.

Table 2.- Distribution of Minimum Inhibitory Concentrations (MICs) for pemetrexed and afatinib in 32 bifidobacteria strains of four species as determined by microdilution.

Species	Nº of strains	MICs (µg/ml)								
		≤2	4	8	16	32	64	128	256	>256
Number of strains with the following MICs for pemetrexed										
B. animalis	1	**1**								
B. bifidum	2					**1**	**1**			
B. longum	19		**1**	**1**		**3**	**2**	**4**	**4**	**4**
B. pseudocatenolatum	10								**4**	**6**
Number of strains with the following MICs for afatinib										
B. animalis	1							**1**		
B. bifidum	2							**1**	**1**	
B. longum	19						**3**	**14**	**2**	
B. pseudocatenolatum	10					**1**	**1**	**3**	**5**	

Figure Legends

Figure 1.- Effect of the addition of increasing afatinib concentrations on the evolution of the Cell-Index (CI) of HT29 growing cells, as recorded by a Real Time Cell Analyzer (RTCA) system. CI values were normalized against those of HT29 control cells growing in the absence of the antitumoral (CI=0).

Figure 2.- Maintenance of the Cell-Index (CI) by the addition of *Bifidobacterium longum* L43 dead cells to HT29 growing cells in the presence of various afatinib concentrations, as recorded by a Real Time Cell Analyzer (RTCA) system.

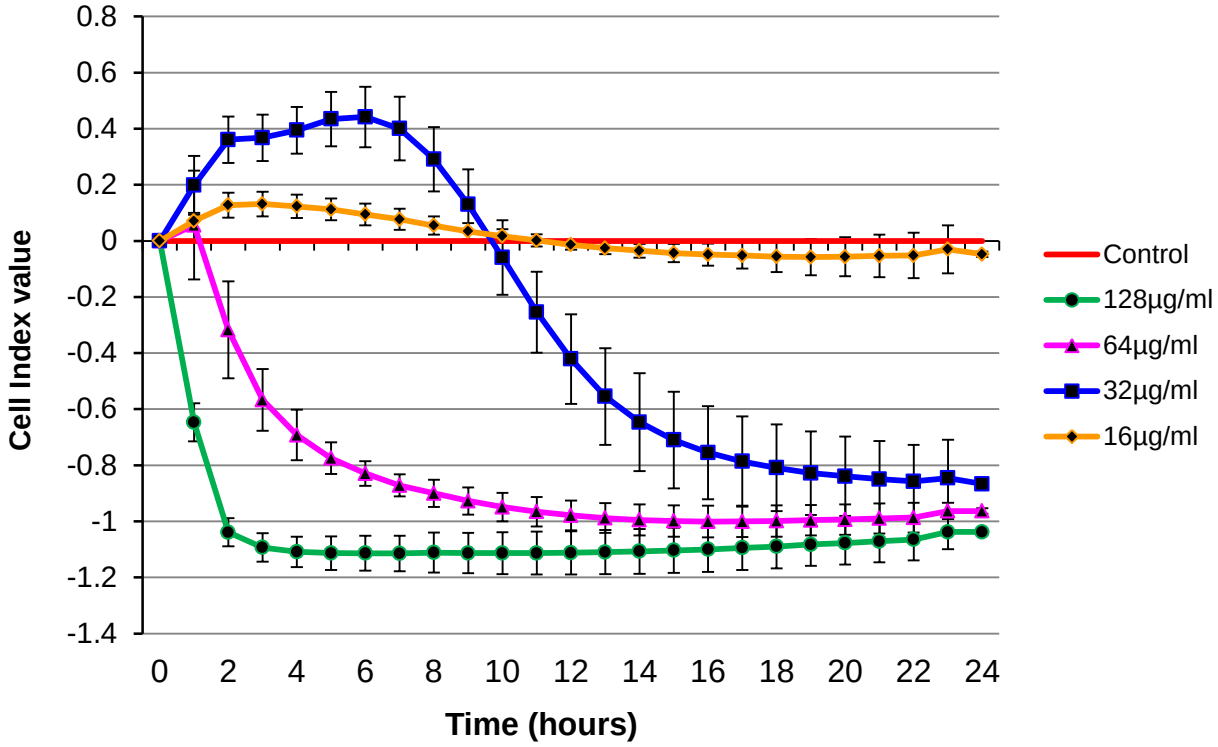
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Cell Index value
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)
Control
128μg/ml
64μg/ml
32μg/ml
16μg/ml

Figure 1

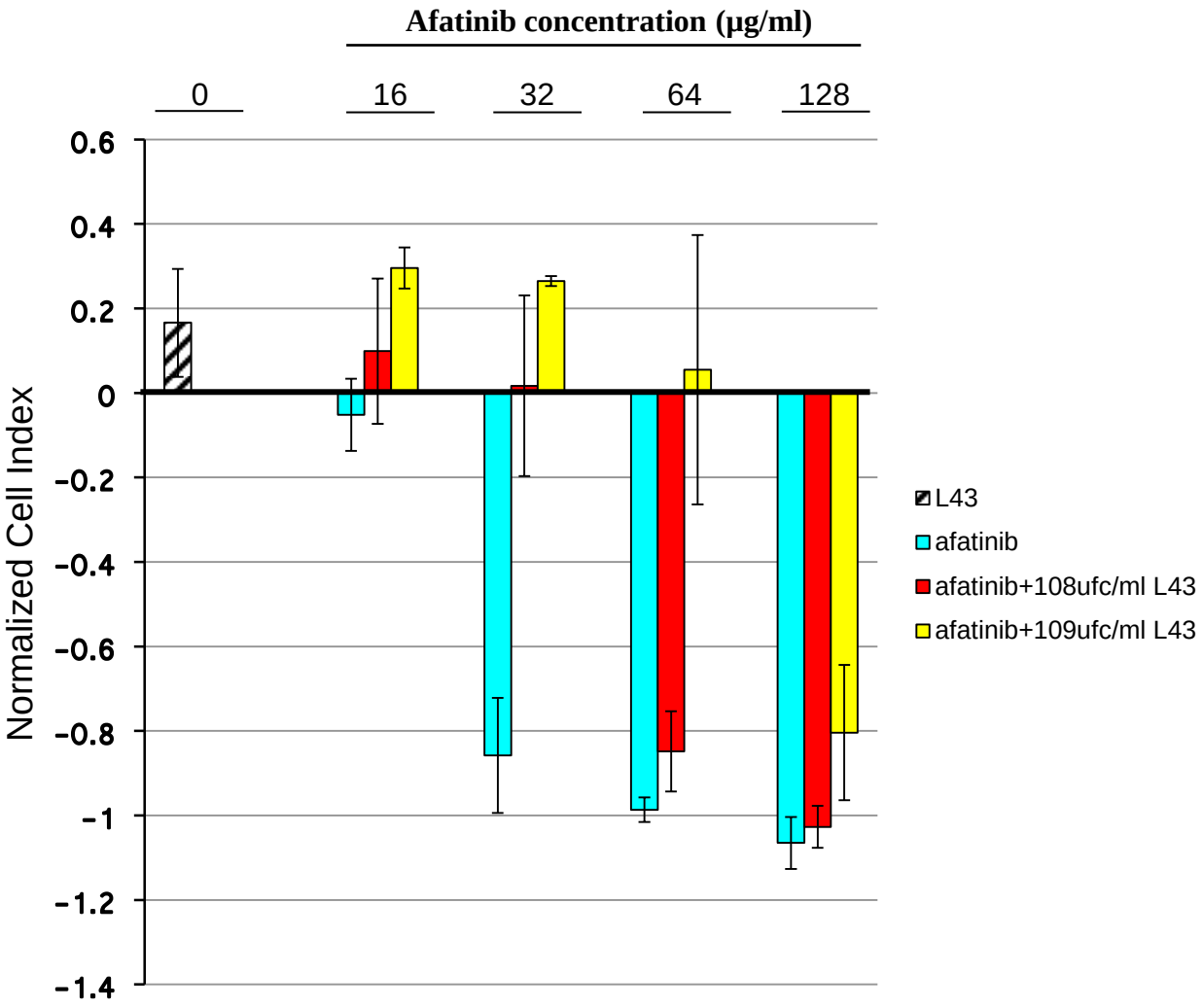
Afatinib concentration (µg/ml)
0
16
32
64
128
Normalized Cell Index
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
L43
afatinib
afatinib+108ufc/ml L43
afatinib+109ufc/ml L43

Figure 2