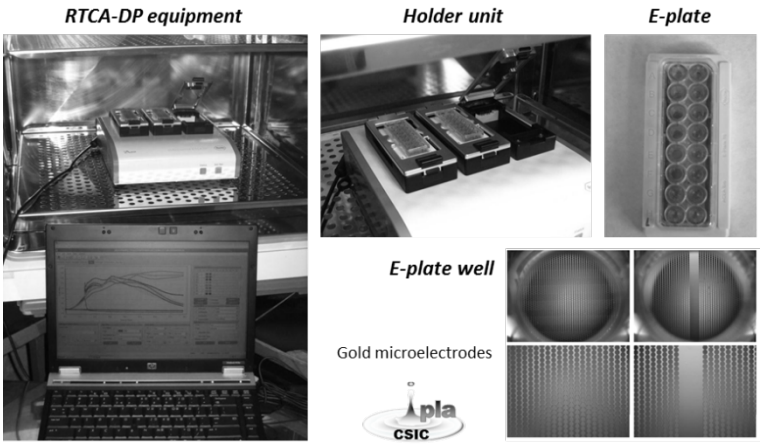
An *in vitro* biological model to study the effect of *Clostridium difficile* toxins in real time: an opportunity to select probiotics for elders. By L. Valdés, M. Gueimonde and P. Ruas-Madiedo, *Departamento de Microbiología y Bioquímica de Productos Lácteos, Instituto de Productos Lácteos de Asturias-Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Asturias, Spain* (ruas-madiedo@ipla.csic.es)

During recent years the incidence and mortality of *Clostridium difficile* infection (CDI) is increasing, affecting specially to the elderly population[1]. The CDI antibiotic treatment can be dissatisfying since protective members of intestinal microbiota are also removed and does not prevent recurrent episodes that could end in an antibiotic refractory disease[2]. Probiotics have been proposed as a way to restore the microbiota caused by antibiotic treatment[3], but it is difficult to choose the correct strain, or bacterial consortium, able to be efficient in intervention cases[4]. Thus, it is imperative the rational selection of good probiotic candidates to be used in patients; in this regard, there are few models to *in vitro* assess the capability of probiotic candidates to counteract the infection and toxicity of *C. difficile.*

The purpose of this study was to develop an *in vitro* real-time assay allowing the continuous monitoring of *C. difficile* toxicity upon human intestinal cells with the final aim to select probiotics able to counteract the effect of the pathogen on the host cells. To this end we have used the Real Time Cell Analyzer (RTCA xCELLigence, ACEA Bioscienes Inc., San Diego, CA, USA) equipment that continuously measures changes in the phenotype and behaviour of (adherent) cells using gold microelectrodes that record electrical impedance (Figure 1). After the addition of bioactives, cellular events such as growth, morphological variations, lysis or apoptosis, will be denoted by modification in the impedance signal.

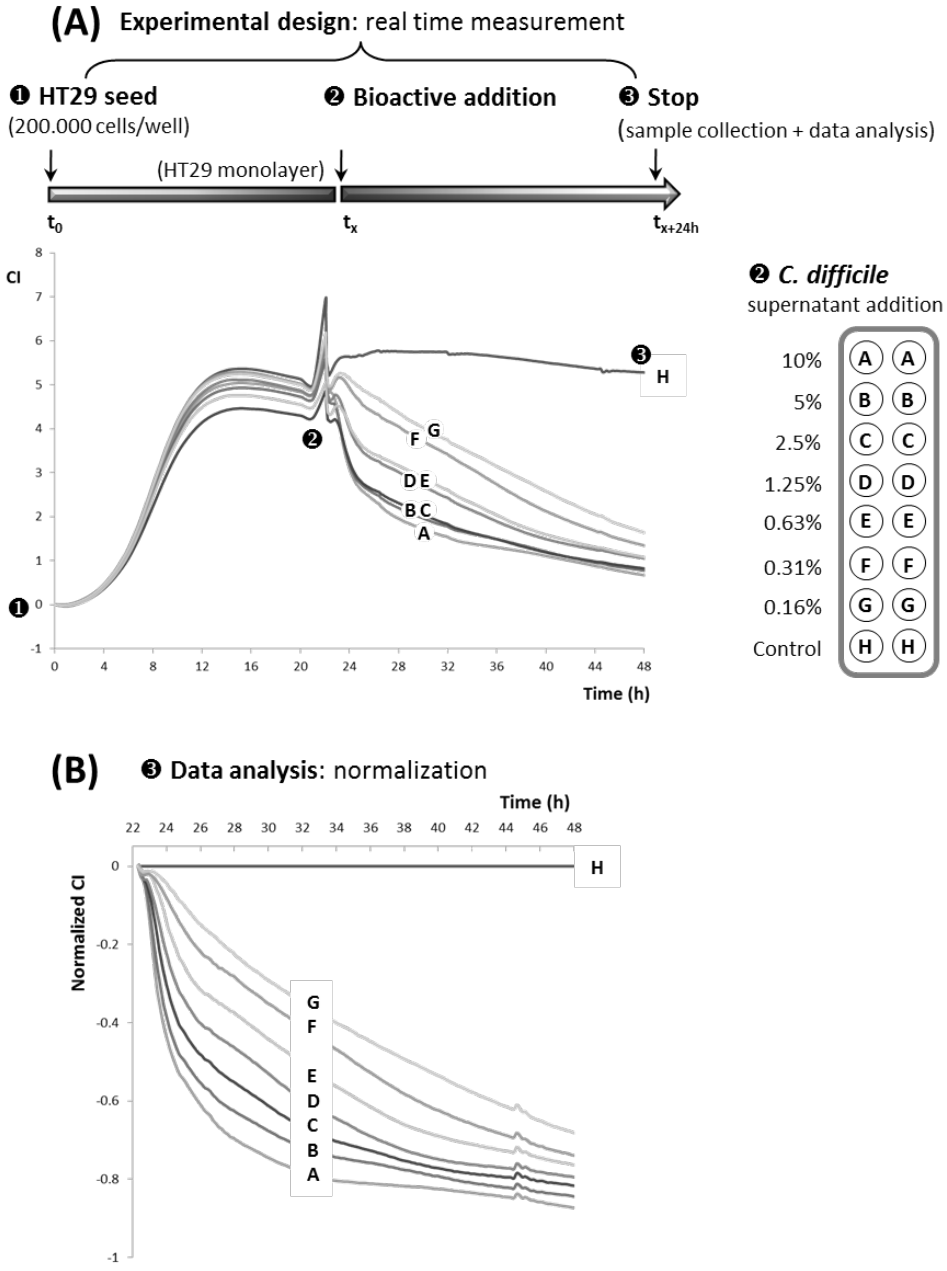
Figure 1 Real Time Cell Analyzer (RTCA), also known as Real Time Cell Electronic sensing (RT-CES), equipment located at IPLA-CSIC.



To develop this model the colonocyte-line intestinal cell line HT29 (ECACC 91072201, European Collection of Cell Cultures, Salisbury, UK) and the strain *Clostridium difficille* LMG21717 (Belgian Coordinated Collections of Microorganism, Ghent University Ghent, Belgium, ~ ATCC 9689), which produce both A and B toxins, were used. HT29 was routinely grown at 37°C, 5% CO_2 in supplemented McCoy´s medium (MM) as previously described[5]. After trypsin treatment, HT29 cells were seed at $2x10^6$ cells/ ml (100 μl per well) in 16-wells E-plates (ACEA Bioscienes Inc.) which were introduced in the holder unit of the RTCA-DP and incubated until reach the monolayer state. In this moment different percentages of a supernatant collected from *C. difficile* grown in GAM (Gifu anaerobic medium, Gentaur GmbH, Aachen, Germany) at 37°C in anaerobic chamber, were added. Supernatants were diluted at concentration ranging from 0.16% to 10% in final 200 μl MM medium; a control well (MM without C. *difficile* supernatant) was also included. Afterwards, the behaviour of HT29 monolayer was monitored in real-time to complete a total of 48 h follow up. The concentration of toxins A and B produced by *C. difficile* LMG21717 was determined by ELISA tests (tgcBIOMICS GmbH, Bingen, Germany).

A representative graphic obtained under these experimental conditions is showed in Figure 2A. The impedance signal, determined as the arbitrary unit "cell index" (CI), increased from the initial point (0 h, corresponding to cells seed) until reaching stable values around 14 h, which indicates that the proliferative state ended and a confluent (monolayer) state was reached. The ensure a proper cell differentiation and establishment of strong tight junctions in the monolayer, which would mimic the physiological state of the intestinal epithelium barrier, this confluent state was maintained for 8 additional hours before adding the bioactive to be tested. Thus 22 h after HT29 seeding, *C. difficile* supernatants were added at different concentrations. Two events were detected in the RTCA; in short term, immediately after supernatant addition, the CI signal abruptly increased in all wells due to the change of medium. Latter the impedance was recovered in the control sample (H) but in all samples containing *C. difficile* supernatants (from A to G) the CI decreased continuously to almost reach the value of the initial point (0 h). This behavior indicates that the monolayer integrity was compromised due to the detachment of HT29 cells, which was reflected in the loss of impedance signal. This event could be directly correlated with the presence of toxins in the supernatant of *C. difficile* LGM21771. Indeed, preliminary results showed that the concentration of toxin A and B was 36.97 ± 0.46 and 10.01 ± 1.12 ng/ml, respectively.

Figure 2 Experimental design to test the toxicity of *Clostridium difficille* LMG21717 supernatant added in McCoy´s medium at different percentages (in duplicated wells) upon the human colonocyte-like cell line HT29. The RTCA monitored the cell index (CI) from the initial cells seed to the monolayer formation, and after the addition of the supernatants (about 22 h) to the end of the experiment (about 48 h) **(A)**. For data analysis, CI index values of each well were normalized by the control sample (McCoy´s medium) and by the time of supernatant addition (~ 22 h) **(B)**.



After double normalization of the data (respect to the control sample and respect the point of supernatant addition) it was detected a doses dependent effect since the reduction in the CI, and therefore the toxic effect, was higher in the sample containing the highest supernatant concentration and this reduction proportionally diminished when the toxin percentages decreased (Figure 2B).

These results show that we have successfully developed a system to monitor in real time the toxic effect of *C. difficile* upon intestinal cell lines. Currently, we are using the method to check the capability of *Bifidobacterium* strains to counteract this cytotoxic effect and the preliminary observations suggest very promising results. The final goal will be the selection of a strain (or strains combinations) that could be tested in other complex *in vitro* models, such as fecal slurries harboring the intestinal microbiota of the target human population, before to accomplish expensive, time-consuming and Ethical-concerning *in vivo* experiments and intervention studies.

Reference list

1. Keller JM, Surawicz CM (2014) *Clin Geriatr Med* **30**, 79-93.
2. Taur Y, Pamer EG (2014) *Nature Medicine* **20**, 246-247.
3. Ley RE (2014) *Nature Medicine* **20**, 248-249.
4. Allen SJ, WarehamK, Wang D *et al*. (2013) *Lancet* **382**, 1249-1257.
5. Hidalgo-Cantabrana C, Kekkonen R, de los Reyes-Gavilán CG *et al*. (2014) *J Funct Foods* **6**, 348-355.