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ORIGINAL ARTICLE

In vitro antiviral activity of *Lactobacillus casei* and *Bifidobacterium adolescentis* against rotavirus infection monitored by NSP4 protein production

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Keywords

antiviral effect, *Bifidobacterium adolescentis*, *Lactobacillus casei*, NSP4 protein, probiotic metabolites, rotavirus.

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Abstract

Aims: The aim of this study was to determine the antiviral activity of four probiotic metabolites (*Lactobacillus* and *Bifidobacetrium* species) against rotavirus *in vitro* infection monitored by the NSP4 protein production and Ca^{2+} release.

Methods and Results: The antiviral effect of the metabolites was performed due a comparison between a blocking model and an intracelullar model on MA104 cells, with the response of NSP4 production and Ca^{2+} liberation measured by flow cytometry. Significant results were obtained with the metabolites of *Lactobacillus casei,* and *Bifidobacterium adolescentis* in the reduction of the protein production ($P = 0{\cdot}04$ and $P = 0{\cdot}014$) and Ca^{2+} liberation ($P = 0{\cdot}094$ and $P = 0{\cdot}020$) in the intracellular model, which suggests a successful antiviral activity against RV infection.

Conclusions: This study demonstrates that probiotic metabolites were able to interfere with the final amount of intracellular NSP4 protein and a successful Ca^{2+} regulation, which suggests a new approach to the mechanism exerted by probiotics against the rotavirus infection.

Significance and Impact of the Study: A novel anti-rotaviral effect exerted by probiotic metabolites monitored by the NSP4 protein during the RV *in vitro* infection and the effect on the Ca^{2+} release is reported; suggesting a reduction on the impact of the infection by decreasing the damage of the cells preventing the electrolyte loss.

Introduction

Rotavirus (RV) is still one of the most important agents related to acute diarrhoea disease and continues to be a worldwide public health concern. RV infection is considered the second leading cause of death in children under the age of five in developing countries (Tate *et al.* 2012; Yen *et al.* 2014). As a consequence of the viral infection, diarrhoea and vomiting give place to severe and rapid dehydration in patients, especially children. The illness is associated with high rates of hospitalization among different populations (Dennehy 2013). Essentially, two biological pathways develop diarrhoea as a consequence of the infection. The first one is directly associated with cell death of mature enterocytes by the lytic effect of the RV infection; while the second one is due to the NSP4 production, first viral enterotoxin protein reported (Ball *et al.* 2005; Pachon del Amo *et al.* 2006; Greenberg and Estes 2009). It was discovered by Ball and co-workers in 1995, is codified by the tenth segment of the RV genome, and is essential to accomplish the viral cycle due to the fact that is one of the main proteins responsible for the final maturation of the new viral progeny (Trask *et al.* 2012). This protein increases diarrhoea through different

mechanisms, from which one of the most important is the loss of electrolyte homeostasis, by means of removing Ca^{2+} from cellular storages mainly from the endoplasmic reticulum (ER), followed by chloride ions elimination (Tian *et al.* 1994; Díaz *et al.* 2008; Zambrano *et al.* 2008). The Ca^{2+} release could be induced by the activation of different pathways in the infected cells as well as in neighbouring healthy cells; some of the principal mechanisms include: interactions of the NSP4 protein with β-integrins of uninfected enterocytes, activation of the enteric nervous system (ENS), signalling cascades through phospholipase C (PLC) and inositol 3-phosphate (I3P) pathway, regular production of NSP4 during the viral cycle; independently of the pathway used, the Ca^{2+} released leads to cell unbalance (Tian *et al.* 1994; Dong *et al.* 1997; Greenberg and Estes 2009).

Nowadays no specific antiviral treatments exist against RV infection; in general, the treatment is focused towards the symptomatology of the illness but not directly to the virus (Dennehy 2013). Contributing with the search of treatment and prevention alternatives of this pathology, the use of probiotic micro-organisms has gained a lot of influence in this field (Guandalini 2011; Liévin-Le Moal and Servin 2014; Papadimitriou *et al.* 2015). Probiotics are yeasts or bacteria which provide a beneficial effect against multiple pathological agents (Colbère-Garapin *et al.* 2007; Binns and Lee 2010; Thomas and Greer 2010). Several mechanisms of action have been suggested, some of which act by interfering with transcriptional processes, and the regulation of protein expression of ionic transport channels (Kopp-Hoolihan 2001; Borthakur *et al.* 2008; Oelschlaeger 2010; Raheja *et al.* 2010; Salminen *et al.* 2010; Russell *et al.* 2011; Saad *et al.* 2013).

Keeping in mind that there are some probiotic bacteria already reported to manage efficiently the acute diarrhoea disease caused by RV (Pant *et al.* 2007; Grandy *et al.* 2010; Guandalini 2011; Erdoğan *et al.* 2012; Ventola *et al.* 2012; Mizock 2015); and some others proved *in vitro* antiviral activity (Botić *et al.* 2007; Maragkoudakis *et al.* 2010; Muñoz *et al.* 2011; An *et al.* 2012; Cha *et al.* 2012; Lee *et al.* 2013a); where, for instance, Muñoz and coworkers reported specific anti-rotavirus activity but were unable to understand exactly how the antiviral activity was performed (Muñoz *et al.* 2011); in this study, it was expected to determine if the antiviral effect of four probiotic bacteria against RV infection could be achieved by monitoring the NSP4 production and Ca^{2+} liberation in the infected cells. To accomplish this aim, an experimental design with bacterial metabolites was developed, where the cell line was first infected with the virus and then was exposed to the metabolites, evaluating the antiviral effect looking for percentage of positive cells with NSP4 and Ca^{2+} as well as the protein and Ca^{2+} amounts within the cells by flow cytometry.

Materials and methods

Cell line and virus

MA104 cells obtained from *Rhesus* embrionary kidney were cultured in Advance Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS and L-glutamine. Biological experiments were performed with 150 000 cells per well cultured in 24 well plates (TPP®). Cells were incubated at 37°C, with 5% CO_2 per 24 h until an 80–90% confluence with approx. 500 000 cells per well were grown. The RRV strain was propagated in MA104 cells as previously reported (Pando *et al.* 2002). For antiviral experiments, the RRV strain (donated by Human Genetics Institute from Pontificia Universidad Javeriana) was used with a multiplicity of infection (MOI) of 5 starting from a viral titre of $3{\cdot}6 \times 10^6$ FFU ml^{-1}. Viral infections performed during the study (including viral kinetics and antiviral activity evaluations) were done with RRV previously activated with trypsin (10 μg ml^{-1}) per 1-h incubation at 37°C.

Probiotic bacteria and protein-based metabolites

Probiotic bacteria were supplied from the CMPUJ (Pontificia Universidad Javeriana Microorganisms Collection). Four probiotic bacteria were used: *Lactobacillus casei* – CMPUJ 415, *Lactobacillus fermentum* – CMPUJ 413, *Bifidobacterium bifidum* and *Bifidobacterium adolescentis* DSM – 20083. Probiotic metabolites were obtained from 250 ml of bacterial cultures during its logarithmic phase (8–10 h according to each strain) grown in MRS broth (Oxoid, Basingstoke, UK) with anaerobiosis conditions at 37°C. Microbial cultures were centrifuged at 1000 ***g*** for 15 min as previously described (Botić *et al.* 2007) and the supernatants were recovered and filtered with 0·22 μm membrane pores (TPP®) to remove residual bacteria.

With the objective of recovering protein-based compounds present in the filtered supernatants, precipitation with poly ethylene glycol (PEG) was chosen due to the fact that PEG is the regular polymer used for protein conjugation (Katre 1993; Zalipsky 1995). Free bacteria supernatants were precipitated with 10% PEG-8000 by stirring at 4°C overnight. Following precipitation, the culture supernatants were concentrated by consecutive centrifugations at 16 000 ***g*** for 30 min each in 1·5 ml conical tubes until concentrating the total supernatant volume. They were finally re-suspended in 2·5 ml of PBS 1×. The metabolites were stored at −20°C until use. Quantification of the metabolites was performed using

the commercial BCA Protein Assay Kit from Pierce (Rockford, IL), using Bovine Serum Albumin for the standard curve.

Cytotoxic effect of the metabolites was evaluated in the MA104 cell line by the MTT technique in 96 well plates. Atoxic concentrations of the metabolites were defined as the concentrations in which more of the 90% of the cells per well were alive. For both metabolites production and cytotoxicity assays, there was considered a free bacteria broth control, to compare viability with metabolites and protein concentrations obtained from cultured broths in MA104 cells.

NSP4 production and Ca^{2+} liberation kinetics in RV infection

In order to determine the maximum common point of NSP4 production and Ca^{2+} liberation during the *in vitro* infection on MA104 cells, kinetics of RRV strain infection were tested. These were performed in 24-well plates with previously grown MA104 cells. Kinetics were evaluated during a 10 h postinfection (hpi) period and independent wells were harvested at 0, 2, 4, 6, 8 and 10 hpi. MA104 cells without infection were considered as experimental negative control, where independent wells inoculated with free serum medium instead of virus inoculation were also harvested at 0, 2, 4, 6, 8 and 10 h postinoculation, with the aim of comparing the experimental behaviour of the NSP4 production and Ca^{2+} liberation during the evaluated period. After completing the 10 h of infection, cells were processed and labelled for flow cytometry detection.

NSP4 detection was performed with an anti-NSP4 monoclonal antibody (Clone B4-2/55, 1/3000 titre) (kindly donated by Dr Joseph Hyser from Baylor College of Medicine and Dr Harry Greenberg from Standford University), and with an anti-mouse IgG secondary antibody labelled with Alexa Fluor 488 (1/2500 titre) (Hyser *et al.* 2008). Intracellular Ca^{2+} was detected with a 3-μmol μl^{-1} Calcium Green (Invitrogen; Eugene, OR) solution, by staining of the cells before they were removed from plates from 30 min in darkness.

In vitro effect of protein-based metabolites evaluated against NSP4 production and Ca^{2+} liberation

For the experimental stage, MA104 cells previously grown on plates were first infected with the trypsin-activated virus at a MOI of five, trying to resemble natural conditions of infection in the intestine where a greater amount of virus would be found in comparison with the amount of enterocytes. Cells were exposed to the virus for 1 h and then were washed to remove the excess of virus. After this stage, cells were treated with the protein-based metabolites, in order to determine the intracellular effect of the treatment. The cells were incubated with the atoxic concentration of the metabolites of each bacterium for another hour and then cells were washed to remove excess of metabolites. Experiments were incubated until 8 hpi, time at which intracellular NSP4 and intracellular Ca^{2+} were measured.

Experimental controls were determined as follows: (i) non–treated MA104 cells (Mock of infection), (ii) MA104 exposed only to the metabolites (treatment control) and (iii) Infected MA104 cells (positive control). In addition, MA104 cells, first exposed to the metabolites and then infected with the virus, were considered in order to distinguish the intracellular effect of the metabolites with the blocking effect in the developed experimental design.

For intracellular Ca^{2+} detection, a release of Ca^{2+} storage in the ER was induced by a Thapsigargin (TG) solution—10-μmol μl^{-1} (ENZO – Life Science; Farmingdale, NY), for 15 min in culture conditions at the end of the 8 hpi, before the cells were labelled. TG releases Ca^{2+} into the cytoplasm by inhibiting SERCA pump from endoplasmic reticulum and makes possible the detection of intracellular Ca^{2+} (Treiman *et al.* 1998). After TG induction, cells were labelled with a 3-μmol μl^{-1} Calcium Green solution incubated and placed in the darkness for 30 min. NSP4 detection was done by labelling the cells with specific antibodies (α-NSP4 B4-2/55 – 1/3000 and α-mouse IgG Alexa Fluor 700 – 1/8000) (Hyser *et al.* 2008; Didsbury *et al.* 2011). Experiments were measured by flow cytometry using a FACS-Aria cytometer (Becton Dickinson; San José, CA) and analysed with FLOWJO software (ver. 9.3.2). Two parameters of analysis were taking into account for both the intracellular protein and Ca^{+2}: (i) Percentage of positive cells of treatments compared with the percentage of positive controls for both conditions independently, to get an approach of the global behaviour of the analysed population (approx. 10 000 cells of each well were considered) and (ii) the geometric mean of fluorescence intensity (MFI), which is associated directly with the amount of intracellular NSP4 protein and Ca^{+2} concentrations, thus, when greater concentrations of calcium or protein, greater fluorescence signals were obtained. All experimental assays were performed by three independent assays and duplicate each.

Statistical analysis

Results were analysed using an ANOVA parametric technique, considering treatments as fixed effects; followed by multiple comparisons tests (*post hoc* tests) including Tukey, least significant difference (LSD) and Dunnet's test. The P value of each test was counteracted with a significant level of $\alpha = 10\%$. Figures are presented as error

bars diagrams with their corresponding confidence interval of 90%. Significant results were considered when 2 of 3 *post hoc* tests obtained a P value less than 0·1.

Results

Standardized conditions to ensure the quality of the model: cell line and virus, bacteria metabolites and cytotoxic assays

NSP4 production and Ca^{2+} liberation kinetics performed by flow cytometry showed that a common point of maximum NSP4 production and Ca^{2+} liberation was at 8 hpi. Increased protein production and Ca^{2+} release was observed in parallel starting at 4 hpi. It was found that NSP4 production was stabilized at 10 hpi, while Ca^{2+} release began to decrease at this point. Negative control (MA104 cells without infection) did not show NSP4 production as expected and Ca^{2+} levels remained in their basal state during the 10 h of observation (Fig. 1a,b).

Metabolites obtained from probiotics cultures were quantified with BCA technique in which proteic concentrations were determined according to the BSA standard curve. Although precipitation of the cultured supernatants was performed using PEG, with the objective of recovering protein-based compounds, it could be possible that other molecules with different nature were trapped in the polymer matrix, which were not determined during the study.

Metabolites concentrations oscillated between 700 and 1700 μg ml^{-1}. Consecutive dilutions of the metabolites were added to MA104 cells in the cytotoxic assays. The atoxic concentration of the metabolites for each bacterium in the MA104 cell line varied between 10 and 90 μg ml^{-1} (data not shown).

Intracellular effect of protein-based metabolites against NSP4 production and Ca^{2+} release

The four metabolites used in intracellular experiments, showed significant results related to the amount of the protein inside the infected cells (MFI), suggesting an effect of the metabolites during the viral cycle that affects the protein production. However, when looking the behaviour in the whole population of each condition (percentage of positive cells), significant results for the NSP4 protein were only obtained with *B. adolescentis* metabolites, with a reduction of 20% of the infected cells compared with the positive control (62%) (Fig. 2a,b).

When intracellular Ca^{2+} was observed in the presence of TG, all analysed populations showed a percentage of positive cells close to the 100%, that was expected due to the fact that every single cell should have an intracellular Ca^{+2} concentration and an ER Ca^{+2} storage. Nevertheless, *B. bifidum* showed a reduction in the percentage of positive cells in the cell population. Now, regarding the amount of Ca^{2+} released in the presence of each probiotic metabolite, those of *L. casei* ($P = 0{\cdot}06$), *B. bifidum* ($P = 0{\cdot}02$) and *B. adolescentis* ($P = 0{\cdot}04$) presented a significant decrease in the quantity of intracellular Ca^{2+} according to the MFI with a P value less than 0·1 (Fig. 2c,d).

With the purpose of verifying that the decrease in NSP4 and intracellular Ca^{2+} was done by an intracellular process and was not related with a small amount of viral entrance into the cell, a blocking experiment was performed in which MA104 cells were first exposed to the metabolites and then infected with the virus. In this case, a significant decrease was found in the number of NSP4 producing cells as well as in the amount of NSP4 produced inside of them (Fig. 3a,b). Both observations were

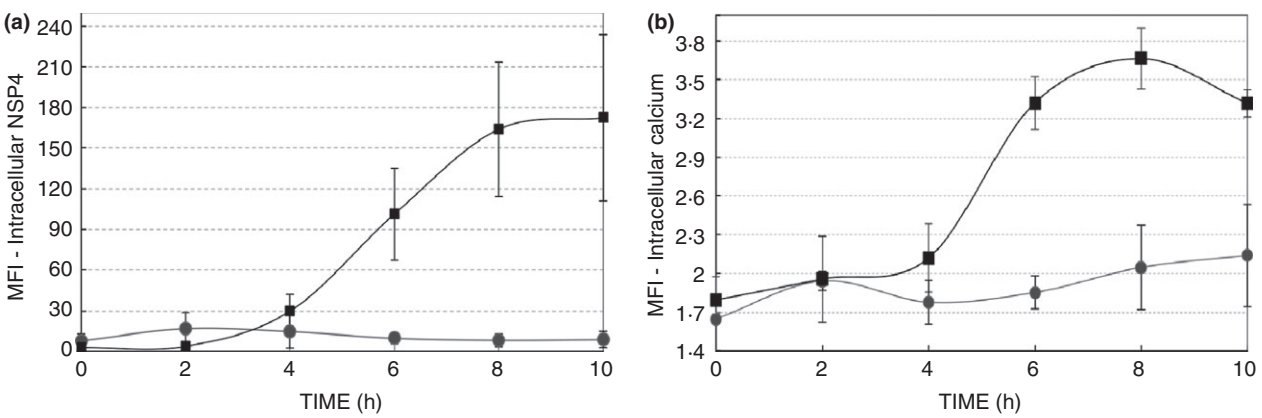
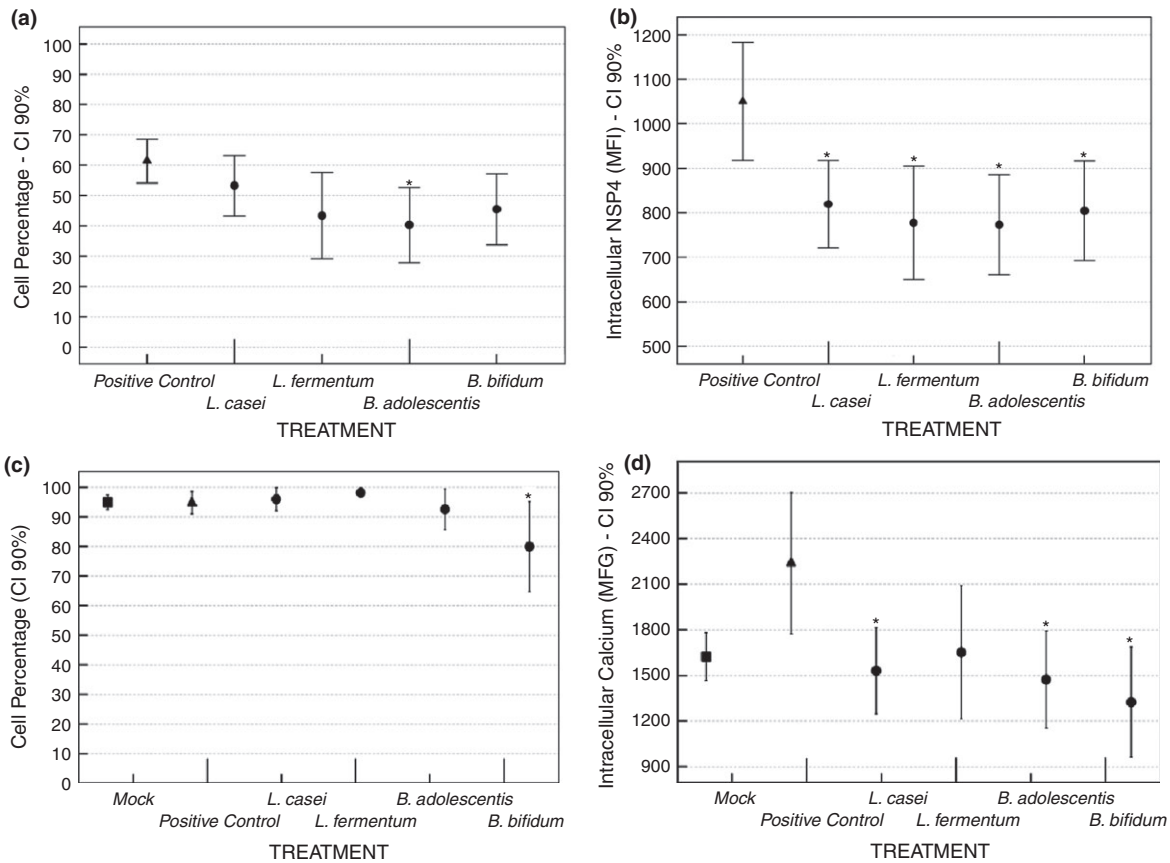
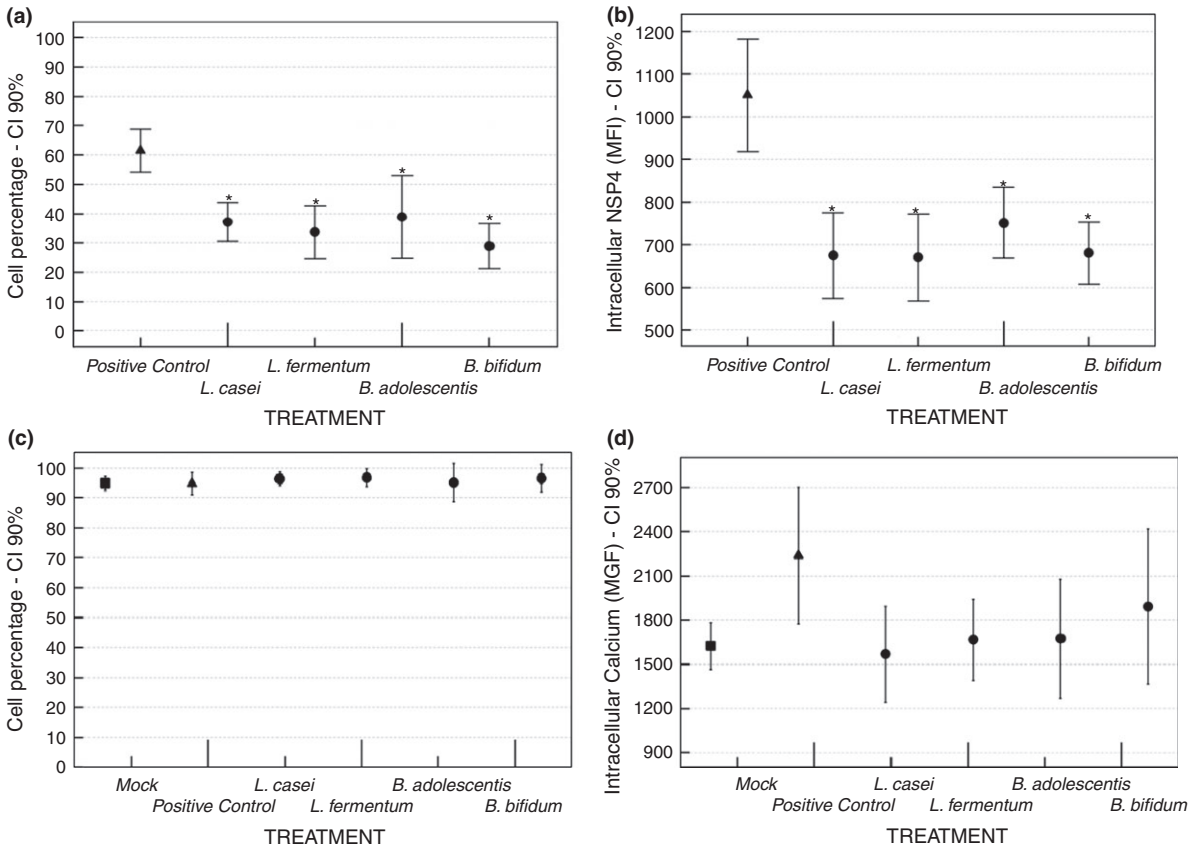


Figure 1 NSP4 Production and Ca^{2+} Liberation Kinetics. (a) NSP4 production in RRV infected cells (■) harvested at 0, 2, 4, 6, 8, 10 hpi compared with Mock of infection (MA104 cells without virus) (●). (b) Ca^{2+} liberation in RRV infected cells (■) harvested at 0, 2, 4, 6, 8, 10 hpi compared with Mock of infection (MA104 without virus) (●). *Notice maximum point of protein production and Ca^{2+} release at 8 hpi.



Intracellular Effect					
Conditions		Intracellular NSP4		Intracellular Calcium	
Post-Hoc Test	Metabolites	% Positive Cells (Fig 2a)	MFI (Fig 2b)	% Positive Cells (Fig 2c)	MFI (Fig 2d)
DHS, Tukey	*L. casei*	0.8770	0.0897	0.9995	0.164
	L. fermentum	0.2300	0.0340	0.9810	0.385
	B. bifidum	0.1120	0.0302	0.9958	0.114
	B. adolescentis	0.3720	0.0804	0.1107	0.040
DMS	*L. casei*	0.3470	0.0128	0.8354	0.027
	L. fermentum	0.0390	0.0044	0.5862	0.080
	B. bifidum	0.0170	0.0038	0.7138	0.018
	B. adolescentis	0.0740	0.0113	0.0170	0.005
Dunnet test (bilateral)	*L. casei*	0.7590	0.0444	0.9989	0.094
	L. fermentum	0.1270	0.0158	0.9602	0.254
	B. bifidum	0.0570	0.0139	0.9905	0.062
	B. adolescentis	0.2250	0.0394	0.0604	0.020

Figure 2 Intracellular effect of probiotic metabolites. Comparison between Mock of Infection (■), Positive control (▲) and Treatments (●). (a) Percentage of positive cells labeled for intracellular NSP4. (b) Geometric mean fluorescence intensity corresponding to intracellular NSP4 produced. (c) Percentage of positive cells labeled for intracellular Ca^{2+}. (d) Geometric mean fluorescence intensity corresponding to intracellular Ca^{2+} released from ER.



Blocking Effect					
Conditions		Intracellular NSP4		Intracellular Calcium	
Post-Hoc Test	Metabolites	% Positive Cells (Fig 3a)	MFI (Fig 3b)	% Positive Cells (Fig 3c)	MFI (Fig 3d)
DHS, Tukey	*L. casei*	0.009	0.000	0.989	0.245
	L. fermentum	0.002	0.000	0.976	0.395
	B. bifidum	0.018	0.003	1.000	0.408
	B. adolescentis	0.000	0.000	0.985	0.813
DMS	*L. casei*	0.001	0.000	0.641	0.044
	L. fermentum	0.000	0.000	0.562	0.083
	B. bifidum	0.002	0.000	0.908	0.087
	B. adolescentis	0.000	0.000	0.607	0.287
Dunnet test (bilateral)	*L. casei*	0.004	0.000	0.977	0.147
	L. fermentum	0.001	0.000	0.950	0.261
	B. bifidum	0.008	0.001	1.000	0.271
	B. adolescentis	0.000	0.000	0.967	0.693

Figure 3 Blocking effect of probiotic metabolites. Comparison between Mock of Infection (■), Positive control (▲) and Treatments (●). (a) Percentage of positive cells labeled for intracellular NSP4. (b) Geometric mean fluorescence intensity corresponding to intracellular NSP4 produced. (c) Percentage of positive cells labeled for intracellular Ca^{2+}. (d) Geometric mean fluorescence intensity corresponding to intracellular Ca^{2+} released from ER.

obtained from the comparison of the values in each treatment of the infection with the metabolites, against those of the positive controls (only infected cells). Moreover, intracellular Ca^{2+} was not affected in the presence of the metabolites neither on the cell percentage nor the Ca^{2+} amount in the blocking model (Fig. 3c,d).

Discussion

In the probiotics field, different mechanisms of action associated with the antiviral effect exerted by probiotics or their metabolic products have been reported (Rodríguez-Díaz and Monedero 2013). A frequent mechanism proposed, is the one exerted by a blocking effect, where is suggested that the probiotics are able to attach to cell surfaces and affect the first stages of the viral infection, by blocking the viral binding to the cell receptors and giving as a consequence the reduction of the entrance of the virus to the cell by the barrier formed by the probiotics (Botić *et al.* 2007; Bermudez-Brito *et al.* 2012; Varyukhina *et al.* 2012). Another mechanism, is related with a direct effect of the probiotic or metabolites against the viral particle, in this case, authors refer to a 'virucidal' effect of the probiotic where a neutralizing effect of the viral particle is suggested, and as a result, a decrease in the viral infection is observed (Zabihollahi *et al.* 2012; Torres *et al.* 2013). Furthermore, an intracellular mechanism has been proposed by other authors, which suggest that probiotics may interfere in steps of the viral cycle inside the cells, unfortunately this proposal has not been elucidated at all, many questions still remain to be solved to explain exactly which is the intracellular process that is affected such as transcription, translation, morphogenesis, and some others (Botić *et al.* 2007; An *et al.* 2012; Lee *et al.* 2013a, 2015; Kim *et al.* 2014).

Based on the intracellular model proposed by other authors, and remembering that the NSP4 protein in a RV infection, is produced in a strict intracellular way (Ball *et al.* 2005; López *et al.* 2005; Yang and McCrae 2012), this research was allowed to formulate the hypothesis that probiotic metabolites might alter the production of viral proteins, specifically the amount of intracellular NSP4 in an intracellular way. In our model, with the aim of getting an approach of this mechanism, two experimental conditions were considered: (i) Intracellular model, where the virus was able to enter to the cell without any restriction followed by cell exposure to the metabolites and (ii) as a control, intracellular model was compared with a blocking effect that was already reported in the *in vitro* rotavirus infection (Muñoz *et al.* 2011; Varyukhina *et al.* 2012; Lee *et al.* 2015), in order to support our proposal suggesting an intracellular effect and to be sure that the antiviral effect obtained in our experiments was not influenced by the blocking effect. Even though, in both cases an antiviral activity of the tested probiotics was obtained reducing the NSP4 protein in the model studied.

The results obtained in the second model where a blocking effect was evaluated, suggested that the four metabolites were able to decrease viral entrance as long as the amount of positive NSP4 cells was reduced, as well as the quantity of intracellular NSP4 (Fig. 3a,b). When compared to other studies which suggest a blocking effect of the virus entrance with different probiotic strains (Botić *et al.* 2007; Muñoz *et al.* 2011; Chai *et al.* 2013; Rodríguez-Díaz and Monedero 2013), in this case, a decrease in the virus entrance in the cell population could be seen. However, our results suggests that the viruses able to enter the cells were enough to induce the Ca^{2+} release as supported by no significant differences in

Table 1 Metabolites impact over NSP4 protein and intracellular Ca^{+2} compared with positive control

		Probiotics metabolites			
Experimental design	Condition	*Lactobacillus casei*	*Lactobacillus fermentum*	*Bifidobacterium adolescentis*	*Bifidobacterium bifidum*
Intracellular effect	NSP4 – % Cells	NS	NS	S	NS
	NSP4 – MFI	S	S	S	S
	Ca^{2+} – % Cells	NS	NS	NS	S
	Ca^{2+} – MFI	S	NS	S	S
Blocking effect	NSP4 – % Cells	S	S	S	S
	NSP4 – MFI	S	S	S	S
	Ca^{2+} – % Cells	NS	NS	NS	NS
	Ca^{2+} – MFI	NS	NS	NS	NS

S, significant decrease compared to positive control; NS, nonsignificant result—same behaviour as positive control; % Cells, percentage of positive cells: corresponds to the amount of positive cells for NSP4 or Ca^{2+}; MFI, geometric mean fluorescence intensity—corresponds to the amount of intracellular protein or Ca^{2+}.

Ca^{2+} levels when compared to the positive control (Fig. 3c,d). This could be understood as either if the metabolites achieved interference in the virus entrance, the few amount of produced NSP4 induced Ca^{2+} liberation just as the positive control.

On the other hand, when evaluating the NSP4 protein and Ca^{2+} behaviour in the intracellular model; all four metabolites showed different behaviours. These results agree with suggestions of other authors who proposed that the effect of probiotics is species and strain specific, where in our case, a strain specific effect was evidenced (Salminen *et al.* 2010; Chapman *et al.* 2012; Lee *et al.* 2013b).

Supporting our hypothesis of the intracellular effect *vs* the blocking experiment, although during the study was not determined the exactly composition of the extracted metabolites, it can be suggested that treating the supernatants with PEG and facilitating the molecule—conjugations with the PEG matrix, increases the solubility of the compounds present in the whole supernatant, giving more opportunities to the compounds to go inside the cells and exert an intracellular effect, maybe related with the early steps of transcription and translation during the viral cycle.

Now, looking forward to provide more clarity to the interpretation of the results, Table 1 was made using the statistical analysis results obtained from the RV infection experiments. Table 1 shows the effect that each metabolite exerted to the protein and the intracellular Ca^{2+} depending on the significant ($P < 0{\cdot}1$) and nonsignificant ($P > 0{\cdot}1$) results.

When analysing the behaviour of *L. fermentum,* either if there is a significant effect over the decrease of NSP4 protein in the infected cells, this managed to induce Ca^{2+} release and behaved the same way as the positive control. This suggests that in spite of few NSP4 protein, it is still able to disturb the amount of Ca^{2+}, being this the only metabolite that despite lowering the protein, it does not manage to decrease the intracellular Ca^{2+}, what could take to electrolyte unbalance and a possible cellular death without showing an antiviral effect in the studied model.

In the case of *B. bifidum* metabolites, a significant reduction was obtained in the amount of the protein as well as in the Ca^{2+} amount in contrast with the positive control. This behaviour could be understood alike *L. casei,* based on the possible interference over the intracellular protein production. Hence, this metabolite was able to affect the amount of liberated Ca^{2+}, what should prevent the cell from losing electrolyte homeostasis, and therefore, preventing damage. These results suggest a positive antiviral effect with these metabolites that reduces the impact of the viral infection and probably the cell damage caused by the virus.

An interesting result was that this metabolite was the only one capable to decrease the percentage of Ca^{2+} positive cells in infected cells. This result was not expected, due to the fact that TG, which was a reactant added to the evaluated models to induce maximum Ca^{2+} liberation from ER to the cytoplasm in the cell population, and close to the 100% of cell population should be labelled as a positive cell for Ca^{+2} detection. This finding may be interpreted as an atypical behaviour between the metabolite, the virus and TG. What cannot be assured is whether if the metabolite effect was exerted directly to the ER membrane, regulating the ionic flux and reducing the Ca^{2+} release to the cytoplasm; or if the effect was exerted right into TG or the virus itself. As a manner of fact, we had an extra control where we evaluated MA104 cells only with the metabolites without viral infection, to be sure that the system was not altered directly by the metabolites, and results were the same as negative controls (Mock of infection). This finding opens the path for more questions about the interaction of the metabolite with other agents and more complex systems, and in this case the antiviral effect of the probiotic could not be certainly outlined.

Lactobacillus casei metabolites behaviour showed that the percentage of positive NSP4 cells were the same as those of the positive control, implying that the virus was being replicated inside of the cells in the same proportion of the population of the positive control; regardless, NSP4 amount was significantly lower when compared with the positive control, suggesting that the metabolite might be interfering with any step of the viral cycle affecting the amount of intracellular NSP4 produced as previous authors report the effect of probiotics within viral proteins production or gene expression (An *et al.* 2012; Lee *et al.* 2013a; Kim *et al.* 2014). On the other hand, the percentage of Ca^{+2} positive cells was the same as the positive control, as it was expected; but the amount of intracellular Ca^{2+} was significantly decreased in comparison with the positive control; which agreed with the NSP4 behaviour (Table 1). These results suggest that *L. casei* metabolites have a successful antiviral effect when monitoring the behaviour of NSP4 protein and thus the reduction of Ca^{2+} release suggesting that cell will not reach the electrolyte imbalance caused by this pathway.

Finally, *B. adolescentis* metabolites showed a significant decrease in 3 out of 4 considered conditions. In this case, the percentage of Ca^{2+} positive cells did not decreased as expected due to the behaviour of TG. However, the amount of intracellular NSP4 protein, the percentage of NSP4 positive cells and the intracellular Ca^{2+} amounts were significantly reduced in contrast with the positive control (Table 1). This behaviour could explain that the

virus was able to enter the cells, but *B. adolescentis* metabolites probably interfered during intracellular stages of the viral cycle reflected in our study by the reduction of NSP4 production, influencing in the cells the capacity of Ca^{2+} release, where Ca^{2+} amount was also decreased. The reduction of the protein could be seen in the experimental population as well, taking into account that the percentage of NSP4 positive cells was also decreased compared with the positive control, suggesting an efficacious antiviral effect, carrying out an inhibitory effect during the viral cycle and cellular damage. Once again is being reported an antiviral activity of a *B. adolescentis* strain, that compared with previous reports, other strains of this micro-organism, already had antiviral activity against other viruses by different mechanisms of action (An *et al.* 2012; Cha *et al.* 2012; Lee *et al.* 2013a; Kim *et al.* 2014).

In a global manner, we are reporting two probiotic metabolites that suggest a successful anti-rotaviral activity, measured by the reduction of NSP4 protein and its consequent damage caused by the Ca^{+2} release. From the four metabolites tested in this research, the best antiviral effect against RV infection was the one obtained with *L. casei* and *B. adolescentis*. Our assays suggest that these metabolites were able to interfere with the final amount of intracellular NSP4 protein and a successful Ca^{2+} regulation, although the mechanism employed was not clear at all. In previously reported studies where probiotics were evaluated against rotavirus infection, the most common mechanism evaluated was due to a blocking effect (Muñoz *et al.* 2011; Varyukhina *et al.* 2012; Lee *et al.* 2015), in this case, our model also confirmed that probiotics may exert a blocking effect, reducing the amount of protein produced when exposed the cells first to the metabolites and then infected with the virus; but our interest was to analyse the intracellular effect exerted by the metabolites, where we obtained the relevant results.

Considering that one of the cytolytic mechanisms used by RV is disrupting the electrolyte balance in the cell related to the NSP4 production, and through the Ca^{2+} release from the ER; the results obtained in this study may suggest that *L. casei* and *B. adolescentis* complement their antiviral activity avoiding cellular lysis by dehydration when regulating the Ca^{+2} release. Thus, as a manner of fact that the NSP4 protein is in charge of a good part of the RV impact on the cells, this is the first report where the antiviral activity against RV was monitored by the amount of intracellular NSP4 protein.

This study reports other probiotic strains with antiviral effect and opens the possibility to generate new hypotheses related to the mechanisms employed by probiotics in the antiviral activity and how they maybe reduce the damage that viruses cause to culture cells. Damage may be similar to that exerted *in vivo* as well as the mechanism of probiotics due to the fact that they are part of the intestinal microbiota. Eventually, further studies are needed to clearly elucidate the probiotics mechanisms against viral agents.

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Conflict of Interest

The authors declare that they have no financial and nonfinancial conflict of interests.

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