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Inhibition of coxsackievirus B4 by *Lactobacillus plantarum*

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ABSTRACT

The enterovirus Coxsackievirus B4 (CV-B4) can infect different human tissues and provoke abnormal function or destruction of various organs and cells. Moreover, its infections have been linked to the onset of type 1 diabetes. Coxsackievirus B4 is classified as a "challenging virus", due to the intense yet vain efforts to find effective prevention and therapeutic agents, especially within biological compounds. *Lactobacillus plantarum* is a lactic acid bacterium that is endowed with probiotic properties, and holds great potential for applications in medical and food industry sectors. Several compounds produced by this microorganism have been associated with various benefits including antimicrobial activity. In this work, we investigated the possible antiviral abilities of two *Lb. plantarum* strains and their derivatives against CV-B4. The different assays carried out (e.g. pre-incubation, competition and post-infection, using HEp-2 cells as human cell model) suggest that the tested microorganisms and their derivatives have an *in vitro* inhibiting activity against CV-B4. This is the first report showing the anti-CVB4 activity of *Lb. plantarum* strains and their derivatives.

1. Introduction

Coxsackieviruses B (CV-B) are enteroviruses belonging to the family of *Picornaviridae.* (Liu et al., 2013). The genus *enterovirus* comprises 12 species (enterovirus A–D and rhinovirus A–C), whose classification is based on genomic identification. Over 63 serotypes belong to *Enterovirus* B (EV-B), including CV-B 1-6 and echoviruses (Alidjinou et al., 2015; Weng et al., 2017).

CV-B cause spastic paralysis and can infect many different tissues, such as the central nervous system, liver, endocrine and exocrine pancreas, brown fat, and striated muscle in human (Chehadeh et al., 2000). The infection of murine pancreas by CV-B4 leads to massive cellular infiltrates, including natural killer cells, T cells, and macrophages, which causes the dysfunction or destruction of pancreatic β-cells (Mena et al., 2000; Sane et al., 2013a). Several studies have inferred a relationship between CV-B4 and diabetes, suggesting that virus infection could increase the risk of type 1 diabetes by inducing autoimmune antibodies directed against β-cells and/or accelerating β-cell destruction before the onset of diabetes (Hober and Sauter, 2010; Jaïdane et al., 2010). CV-B4 is involved in various diseases, including myocarditis, pancreatitis, and hepatitis (Liu et al., 2013). Remarkably, neither efficacious vaccines, nor robust therapeutic agents are currently available for the prevention or treatment of CV-B4 infections (Rotbart, 2002). Although the research is focusing on virus replication inhibitors, their effectiveness *in vivo* is often disappointing (Thibaut et al., 2011). Ribavirin, a synthetic guanosine analog, has been shown to counteract several enteroviruses, by stopping the synthesis of viral RNA, e.g. resulting in decreased inflammation and CV-B3 titer (Heim et al., 1997). However, ribavirin treatment can induce drug resistance (Feigelstock et al., 2011) and hemolytic anemia (Nomura et al., 2004). Emodin, i.e. a resin extracted from the plant *Polygonum cuspidatum,* seems to contrast RNA and DNA viruses (Hsiang and Ho, 2008; Schwarz et al., 2011), including CV-B4 (Liu et al., 2013). Recently, fluoxetine, a selective serotonine reuptake inhibitor (SSRI), has been reported to inhibit *in vitro* CV-B4 replication (Alidjinou et al., 2015). However, the need for an effective anti-CV-B4 agent remains of major importance. Thus, attempts to find natural products-based alternative treatments have been undertaken, and some recent studies provide insights into the antiviral potential of lactic acid bacteria (LAB) and/or their metabolites (Al Kassaa et al., 2014a; Seo et al., 2012; Lakshmi et al., 2013). The antiviral activity of probiotic LAB, mainly documented as strain-dependent, can be due to a close probiotic-virus interaction, to the production of antiviral bacterial compounds, and/or to the probiotic-driven modulation of the immune system (Al Kassaa et al., 2014b). The

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close interaction between bacterial cell and virus is the principal, plausibly not the exclusive, mechanism through which viruses are absorbed and/or trapped, and thus inhibited, by bacteria (Wang et al., 2013). Moreover, probiotics are well documented to exert immune modulation activity that can enhance the immune defenses against virus, such as induction of interleukins, natural killer cells, macrophages, immunoglobulins, T-helper cells action (Maragkoudakis et al., 2010; Cha et al., 2012). LAB can also produce several molecules presenting antiviral and/or, antagonistic activity including hydrogen peroxide (H_2O_2), lactic acid, bacteriocins and bacteriocin-like substances, short-chain fatty acids and polysaccharides (Conti et al., 2009; Martin et al., 2010). All of these compounds are secreted into culture medium, thus the bacterial cell free supernatant (CFS) may contain such products. Indeed, CFSs are frequently analysed to preliminarily characterize the potential antagonistic aptitude of bacteria (Wang et al., 2014; Arena et al., 2016).

Lactobacillus plantarum is a widespread LAB that fulfills probiotic properties and holds a great potential in both medical and food industry applications (Bove et al., 2012; Arena et al., 2014a, 2016). Like other LAB, *Lb. plantarum* can colonize the mucosal surfaces of mammals, including that of the human gastro-intestinal tract, thereby providing health benefits to the host (Arena et al., 2017).

This study aimed at investigating the antiviral potential of two *Lb. plantarum* strains from wine origin, against CV-B4. The impact of live and heat-treated bacterial cells, as well as their cell-free-supernatant (CFSs) on the *in vitro* infection of HEp-2 cells by CV-B4 was determined.

2. Material and methods

2.1. Bacterial strains, growth conditions and cell-free-supernatant (CFS)

The bacteria used in this study were two strains of *Lb. plantarum*, i.e. *Lb. plantarum* UNIFG30 and *Lb. plantarum* UNIFG121 previously investigated for their antimicrobial properties against some pathogenic bacterial strains (Arena et al., 2016) and spoilage fungi (Russo et al., 2017). The strains were grown in de Man-Rogosa-Sharpe (MRS) (pH 6.5) (Sigma-Aldrich, St. Louis, MO, USA) at 30° C.

CFSs were obtained as recently described (Arena et al., 2016). Briefly, lactobacilli cultures were grown for 18 h and culture broth was centrifuged (8000 × *g* for 20 min, 4 °C). The pellet was kept (see below) and the supernatant was sterilized by filtration through Millex-GV 0.22 µm hydrophilic Durapore PVDF membrane (Millipore, Billerica, MA, USA). An aliquot of the filtered CFS was treated with 2 M NaOH reaching pH 6.5, in order to neutralize the organic acids.

Furthermore, the pellet obtained from 1 ml of bacterial culture was washed with phosphate-buffered saline (PBS), centrifuged (6000 *g* for 20 min), washed, and resuspended in 1 ml of Minimum Essential Media (MEM) (Gibco, USA) and heated at 65 °C for 30 min (Martin et al., 2010).

2.2. Human cell line and growth conditions

HEp-2 cells (from human laryngeal carcinoma) were obtained from BioWhittaker (Vervier, Belgium) and were grown in Minimum Essential Media (MEM) (Gibco, USA), supplemented with 10% foetal calf serum (Gibco, USA), and 1% l-glutamine (Gibco, USA), and incubated at 37 °C, 5% CO_2 in tissue culture flasks, until confluency. 2×10^4 cells/well were seeded in 96-well plates and incubated under the same conditions to 80% confluency (Sane et al., 2013a). HEp-2 is one of the most common cell lines used as a cellular model for several *in vitro* assays (Yin et al., 2014; Akram et al., 2018; Chieux et al., 2001). Moreover, HEp-2 line is permissive to CV-B4, therefore we chose it as model to study virus infection.

2.3. Virus

The diabetogenic strain CV-B4 E2 was provided by Ji-Won YOON (Julia McFarlane Diabetes Research Centre, Calgary, Alberta, Canada). The viral strain was propagated in HEp-2 cells and recovered by releasing from cells due to three freeze-thaw cycles (Sane et al., 2013b). Viral particles were harvested by centrifugation of the supernatant (2000 × *g* for 10 min at 4 °C) and the virus titration in Hep-2 cells was calculated by limiting dilution assay to 50% of tissue culture infection dose, according to the method of Reed-Muench (Sane et al., 2013a). Titrated virus stock was stored at −80 °C.

2.4. Cytotoxicity assay

The possible cytotoxic effect of the *Lb. plantarum* strains and their metabolites contained in their supernatants was tested measuring HEp-2 cell viability at 37 °C in controlled atmosphere with 5% of CO_2 after 24 and 48 h of incubation. Each sample was diluted in MEM. HEp-2 monolayers inoculated with live bacteria (10^8, 10^7, and 10^6 CFU/ml) or their derivatives heat-treated cells (issued from 10^8, 10^7, and 10^6 colony forming units (CFU)/ml), filtered CFS and neutralized CFS (used as 10, 20, 30, 40, 50, 60, 70 and 80% (v/v) respect to the final volume of the well). The integrity of cell monolayer and the possible cytopathic effect (CPE) were ascertained visually by inverted light microscopy. Moreover, cell viability was measured by the crystal violet method according to Strober (Strober, 2001). Briefly, the supernatant was eliminated from each well, HEp-2 cell monolayers were washed with PBS and, then, 250 µl of crystal violet 2 g/L (in ethanol:distilled water, 1:4) were added, and plates were incubated at 37 °C for 15 min. Then cells were rinsed, and 250 µl of SDS 1% were used to dissolve the cell monolayer and absorbance at 595 nm was measured.

Not infected HEp-2 cells and HEp-2 cells infected with CV-B4 E2 (MOI 0.01) served as controls.

2.5. Cell proliferation

To determine whether *Lactobacillus* strains and their derivatives could affect cell proliferation, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described by Khan and Kang (2017). In order to avoid that the growth inhibition could be influenced by cell-to-cell contact (Lucarelli et al., 2003), HEp-2 cells were seeded at a concentration of 2×10^4 cells/well in 96-well plates in order to obtain approximately 70% confluence. Briefly, pre-confluent HEp-2 cells were incubated with *Lactobacillus* strains and their derivatives for 1, 2, and 24 h at 37 °C and 5% CO_2, and, then, absorbance was measured at wavelength of 540 nm. The cell viability was calculated by the following formula: (Abs540 nm A/Abs540 nm B) * 100, where A and B indicated HEp-2 cells incubated with and without bacterial samples, respectively.

2.6. Antiviral activity

The possible antagonistic activity of *Lb. plantarum* against CV-B4 E2 was investigated by testing the antiviral capability of various samples including: i) live bacterial cells at the concentration of 10^6 CFU/ml (from *Lb. plantarum* UNIFG30 and UNIFG121), ii) filtered CFS (from *Lb. plantarum* UNIFG30 and UNIFG121), iii) neutralized CFS (from *Lb. plantarum* UNIFG30 and UNIFG121), and iv) heat-treated bacterial cells (from *Lb. plantarum* UNIFG30 and UNIFG121). Pure MRS and MEM were used as negative controls. HEp-2 cells infected with viral suspension without any potential antiviral sample were designated as positive control. All trials, including controls, were performed in three biological independent replicates and three technical repetitions were carried out. Three experimental conditions (pre-incubation, competition and post-infection assay) were used as described below (Wang et al., 2013).