In vitro evaluation of the antiviral activity and detection of the enterocin coding genes of the probiotic bacterium Enterococcus faecium NM213

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ABSTRACT
There is a growing concern globally due to the increasing spread of the resistance to current antiviral drugs which lead to innovative and discovery novel alternative antiviral means. Probiotics in particular Lactic acid bacteria (LAB) have been recognized recently as antiviral candidates via direct interaction with the virus, producing inhibitor substances or through modulation of the immune system. Here we represent, the antiviral activity of our probiotic Enterococcus faecium NM213 against four major human enteroviruses; Coxsackie B4, Hepatitis A Virus (HAV) Strain HM175, rotavirus Wa strain, and herpes simplex virus type-1 (HSV-1). The assessment was done in vitro by means of four different kidney and liver cell lines. In addition, the NM213 strain was screened for the presence of the enterocin encoding genes by PCR and sequencing. The results showed the significant ability of the cell-free supernatant (CFS) of NM213 in reduction of (HSV-1), HAV HM 175, Coxsackie (B4), and rotavirus (Wa) by 70%, 60%, 53.3%, and 50% respectively while its cell extract (CE) showed a fewer reduction to the four viruses by 20%, 10 %, 10%, and 20% in turn while neither PBS buffer nor MRS broth showed any antiviral activity or cytotoxicity effect. Screen the presence of enterocin by PCR and sequencing the amplified fragments revealed the presence of enterocin A and B which encoded by ent A and ent B genes. Further molecular characterization for such valuable enterococci NM213 will be conducted in particular achieving a complete genome sequence to present it for commercial and applied purposes.

Keywords: Coxsackie virus, Enterococcus, Herpes simplex virus, Probiotics, Rotavirus

List of Abbreviations:
CFS, Cell free supernatant; CE, Cell extract; DMEM, Dulbecco’s Modified Eagle Medium; ent A, Enterocins A; ent B, Enterocins B; E. faecium, Enterococcus faecium

1. Introduction
The genus Enterococcus is a candidate of lactic acid bacteria (LAB) group and forms a significant portion of human normal microbiota especially the gastrointestinal tract (GIT) in addition to other niches in mouth and on skin. These microbes are found also globally in an enormous diversity of vegetables, meats, dairy products and processed food (Devriese et al., 1995; Pesavento et al., 2014; Gomes et al., 2008). Although Enterococcus are not generally regarded as safe (GRAS) by Food Drug Administration FDA but many strains are measured as non-pathogenic and have been implemented as...
probiotic products for children for controlling diarrhea resulted from antibiotic treatment (Franz et al., 2011; Surawicz, 2003). Thus there is lots of research offer has been done for selective and distinguish between the food grade and pathogenic strains of enterococci. Research group indicate the smaller genome size of the enterococci probiotics compared with pathogenic enterococci (Bonacina et al., 2017).

Among Enterococcus the E. faecium strains have been used as cheese starters or probiotics in human and animal health promotion to improve the intestinal microbial balance (Franz et al., 2011; Yang et al., 2014). Actually, some E. faecium strains are currently in use as therapeutic treatments marketed as Cylactins (Hoffmann-La Roche, Basel, Switzerland), Fargo 688 s (Quest International, Naarden, the Netherlands), and ECOFLOR (Walthers Health Care, Den Haag, the Netherlands) designed for relieving the signs of irritable bowel disease. These products had been tested clinically and approved their effectiveness in several digestive, inflammatory, and neurodegenerative sicknesses (Suvorov et al., 2003; Lo Skiavo et al., 2013; Suvorov, 2013; Baryshnikova et al., 2015; Rho et al., 2017; Holzapfel et al., 2018; Suvorov et al., 2019). Furthermore, enterococci have known for bacteriocins with wide spectrum antimicrobial activity towards both the Gram-positive and negative microbes (Laukova et al., 2017). In addition to their ability to prevent sporulating bacteria such as C. botulinum and B. cereus and in some case they may inhibit endospores (Grande Burgos et al., 2014). Furthermore, some enteroccoccus bacteriocins (often named “enterocins”) retain antifungal and/or antiviral action (Hanchi et al., 2018). Their antiviral could be due via direct interaction with the virus and/or by activation of the immune system. Wang et al. (2013) reported the inhibition ability of E. faecium NCIMB 10415 strain against influenza viruses via direct contact.

Prevention and alternative treatment are needed to face the emergence of new infectious viruses and increased resistance against the available antiviral drugs.

E. faecium NM213 (accession no. KC878685) is a potential probiotic strain which isolated previously in our laboratory from healthy Egyptian infants according to its immounodolatory properties by in vitro analysis (Mansout et al., 2014). E. faecium NM213 showed ability for stimulation the human immune cells by inducing IL-6 and TNF in mononuclear cells isolated from healthy human donors. Furthermore, it activated the human mono dendritic cells by the release of four diverse cytokines; the IL-1b, the chemotactic CXCL-8, IL-6, which has both pro- and anti-inflammatory actions and the strong TH1 polarizer IL-12. Also it showed strong activation to Toll-like receptor 2. Furthermore, it showed an acceptable safety pattern by expressing sensitive to vancomycin, chloramphenicol, tetracycline and erythromycin in addition to the absence of the major enterococcal virulence genes from its genomic DNA.

This study tests the ability of our strain E. faecium NM213 as antiviral against four type of human viruses; Rotavirus, Hepatitis A Virus (HAV), Coxsackie B4 Virus and herpes simplex virus type-1 (HSV-1). Furthermore, detection and identification of the enterococin genes within its genomic DNA has been done by PCR and sequencing analysis.

2. Materials and Methods

2.1 Preparation of bacterial cell-free supernatant (CFS) and bacterial cell extracts (CE)

E. faecium NM213 was grown in 30 mL MRS broth for 24 h at 37 °C. The culture contain 10⁶ CFU/ml was centrifuged at 3,000 g for 15 min. The supernatant was collected and adjusted to pH 7.0 using 1 M sodium hydroxide solution then filter-sterilized (0.22-µm pore size, Merck Millipore, Burlington, MA) and marked as (CFS). The CE was prepared by washing the pellet of bacteria obtained above twice with sterile Phosphate-buffered saline (PBS) to remove excess MRS followed by centrifugation at 3,000 g for 15 min. The washed pellet was re-suspended in 10 mL of PBS. The colony forming units (CFUs)/mL were determined using the plate count technique on MRS agar plates.

2.2 Cytotoxicity test

The Cytotoxicity test for CFS and CE of E. facieumNM213 was carried by means of cell morphology assessment via inverted light microscope (Simões et al., 1999) and cell viability investigation through trypan blue dye exclusion technique then viable cells were counted by the phase contrast microscope (Walum et al., 1990). The MA104, BGM, FRHK4, and Vero cell lines were provided by vacsersa (the Holding Company for Biological Products & Vaccines VACCSERA, Egypt) and the non-toxic dose of the tested samples on the cell lines was evaluated.
2.3 Determination of rotavirus Wa strain, HAV HM175, Coxsackievirus B4, and herpes simplex virus type 1 titers using plaque assay

Non-toxic dilutions were mixed (100μl) with 100μl of different doses of rotavirus Wa strain, HAV HM175, Coxsackievirus B4 and Herpes simplex virus type 1 (1X10^5, 1X10^6, 1X10^7). The infectivity of the rotavirus stocks were activated with 10 μg/ml trypsin for 30 min at 37°C. The mixture was incubated for 1/2 hr in 37°C. The inoculation of (100μl) 10 fold dilutions of treated and untreated rotavirus Wa strain, HAV HM175, Coxsackievirus B4 and Herpes simplex virus type 1 was carried out separately into MA104, FRHK4, and BGM cell lines for rotavirus Wa strain, HAV HM175, Coxsackievirus B4, and Herpes simplex virus type 1 respectively in 12 multi well plates. The plates were incubated 1 hr at 37°C for adsorption in a 5% CO₂ water vapor atmosphere with occasionally rocking to avoid drying the cells. After adsorption, 1 mL of 2X media (Dulbecco’s Modified Eagle Medium, Gibco-BRL (DMEM) plus 1ml 1% agarose was added to each well, 0.5 μg/ml was added to the media-agarose mixture in the case of rotavirus Wa strain and the plates were incubated at 37°C in a 5% CO2-water vapor atmosphere. Then the cells were fixed with formaline and stained with 0.4% crystal violet, in addition the number of plaques counted. The viral titers were then determined, and stated as plaque-forming units per milliliter (pfu/mL) (Schmidtke et al., 1998).

2.4 DNA extraction and PCR

DNA was extracted from bacterial cultures using the AxyPrep bacterial genomic DNA miniprep kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer’s instructions.

PCR was carried out on a thermal cycler system BioRad T100 (Bio Rad, Hercules, CA, USA) using PCR Master Mix (Fermentas Life Sciences, Vilnius, Lithuania), and primers were obtained from (Bioserve, Cairo, Egypt). For purification, a QIAquick PCR purification kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s instructions.

2.5 Detection the presence of the E. faecium NM213 enterocin encoding genes

Genomic DNA of E. faecium NM213 was screened for enterocin A, B, P, and L50A genes by PCR using enterocin-specific primers (du Toit et al., 2000; De Vuyst et al., 2003; Cintas et al., 1997; Cintas et al., 1998). The presence and molecular size of the PCR products were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Purified PCR products were sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA) as directed in the manufacturer's protocol. The resulting sequence data were analyzed using Molecular Evolutionary Genetic Analysis version 7 (MEGA7) (Kumar et al., 2016; Altschul et al., 1990).

3. Results

3.1. A cytotoxicity test

The cytotoxicity test showed no toxic effect of E. faecium NM213 CFS or CE at dilutions up to 40% on MA104, Hep2, BGM, and Vero cell lines at concentrations of 10^5 to 10^7 CFU/ml (data not shown). Their effect onto the viability of MA104, Hep2, BGM, Vero cell lines was evaluated at a concentration of 10^5, 10^6, and 10^7 CFU/ml, the integrity of monolayers was unaltered (data not shown). Moreover, MTT assay indicated no reduction of cell proliferation compared to control (data not shown).

3.2 in vitro antiviral activity of E. faecium NM213 (CFS) and (CE)

The neutralized CFS and CE from overnight culture E. faecium NM213 (10⁹ CFU/ml) were tested for their antiviral activity against the four proposed viruses the experiments have done in triplets using 10% (v/v) dilution from each. CFS and CE showed reduction of the four viruses replication compared to two controls (virus-infected cells with PBS buffer) and (virus-infected cells with MRS). CFS showed 53.3 % inhibition of virus replication Coxsackie B4 while CE showed 10 % as presented in Figure 1, on Hepatitis A Virus (HAV) Strain HM175 CFS and CE showed 60% and 10 % reduction respectively in Figure 2, while they showed reduction rotavirus Wa to 50 % by CFS and 20% by CE in Figure 3. The Data shown in Figure 4 display the reduction of herpes simplex virus type-1 (HSV-1) to 70% by CFS and 20% by CE.
Fig. 1: In vitro antiviral activity of *E. faecium* NM213 (Culture free supernatant CFS and Cell-free extract CE) against Coxsackievirus B4 compared to the controls; MRS and PBS. Viral titers were analyzed by titration in BGM cell lines. The Blue column shows the initial viral titer and the Red column shows the final viral titer.

Fig. 2: In vitro antiviral activity of *E. faecium* NM213 (Culture free supernatant CFS and Cell-free extract CE) against Rotavirus Wa compared to the controls; MRS and PBS. Viral titers were analyzed by titration in MA104 cell lines. The blue column shows the initial viral titer and the red column shows the final viral titer.
**Fig. 3:** *In vitro* antiviral activity of *E. faecium* NM213 (Culture free supernatant CFS and Cell-free extract CE) against Hepatitis A virus (HM 175) compared to the controls; MRS and PBS. S stands for CFS while C stands for CS. Viral titers were analyzed by titration in FRHK4 cell lines. The blue column shows the initial viral titer and the red column shows the final viral titer.

**Fig. 4:** *In vitro* antiviral activity of *E. faecium* NM213 (Culture free supernatant CFS and Cell free extract CS) against Herpes simplex virus type 1 compared to the controls; MRS and PBS. S stands for CFS while C stands for CS. Viral titers were analyzed by titration in Vero cell lines. The blue column shows the initial viral titer and the red column shows the final viral titer.
3.3 Detection of the enterocin encoding genes within the genome of *E. faecium* NM213

The detection of the possible enterocins genes in the NM213 strain was done by PCR amplification using specific primers for the presence of the known enterocin genes entA, entB, entP, L50A as presented in Table (1). Amplifications revealed only two bands with entA and entB genes with size 475 and 200 bp respectively and no bands obtained for the L50 gene.

The analysis sequencing of the two fragments using blast search at NCBI server revealed the 100% identical with the enterocin genes encoding by entA and entB from many *Enterococcus faecium* strains which available at the NCBI data base.

**Table 1:** Primers sequence for detecting the enterocin genes

| Primer name | Primer sequence 5-3 | References |
|-------------|---------------------|------------|
| EntA F      | AAATATTATGGAAATGGAGTGTAT | du Toit et al., (2000) |
| EntA R      | CTCGTTAAGGTCCCTTCACG  |            |
| EntB F      | CAAAATGTAAAGAATATGACG  | De Vuyst et al., (2003) |
| EntB R      | AGAGTATACATTTGCTAACCC  |            |
| EntP F      | ATGAGAAAAAAATTATTTAGTTT | Cintas et al., (1997) |
| EntP R      | TTAATGTCCCTAATCTGCCAAACC |            |
| L50AF       | ATG GGA GCA ATCGCAAAA TTA | Cintas et al., (1998) |
| L50AR       | TTT GTT ATG TGCCA TCC TTC |            |

4. Discussion

There is a great interest about the vital role of the human microbiome in maintaining its health and discovering the interactions between microbiome and invading microbes. Actually, a healthy microbiome in addition to its metabolites products is crucial for protecting the host from a diversity of pathogenic contagions via direct exclusion and indirect suppression (Piewngam et al., 2018; Kim et al., 2017; Sassone-Corsi et al., 2016; Schuitj et al., 2016). Viruses are one of the most common invaders which interact with the commensal microbes of the hosts throughout their infection routes. There is an evidence has been shown that the microbiome regulates the viruses and is regulated by viruses through several mechanisms which ended by destructive or beneficial effects for the host (Karst, 2016; Pfeiffer & Virgin, 2016; Berger & Mainou, 2018). Microbiome shows critical roles in the regulation the viral infection, in addition to utilize substantial inhibitory effects. Reviews describing the role of microbiome in regulation viral infections are available (Karst, 2016; Pfeiffer & Virgin, 2016; Robinson & Pfeiffer, 2014; Sullender & Baldrige, 2018).

The probiotic bacteria have been suggested as alternative means of antiviral treatment (Chai et al., 2013; Kwak et al., 2013; Lee et al., 2013; Kim et al., 2014; Lehtoranta et al., 2014). Their action could be through neutralizing viruses (Rolfe, 2000; Minocha, 2009; Preidis et al., 2011; Zhang et al., 2013) [43-46] or promote the innate immune response and stimulate cellular multiplication of epithelial cells (Preidis et al., 2011; Sur et al., 2011). Wang et al. (2013) confirmed the antiviral effect of *E. faecium* strain *in vitro* experiments using porcine H1N1- and H3N2-influenza virus in MDBK- and D4/21 cells.

The probiotic bacterium *E. faecium* NM213 was isolated from Egyptian infant stool in our laboratory and demonstrated remarkable immunomodulatory abilities *in vitro* by releasing of the cytokines IL-1b, CXCL-8, IL-6 and IL12 which suggested it as potential for preventing atopic diseases (Mansour et al., 2014), here we aimed to investigate its ability against four viruses.

When we evaluated the antiviral ability of the cell free supernatant of *E. faecium* NM213 against the four viruses Rotavirus, Hepatitis A Virus (HAV), Coxsackie B4 Virus and herpes simplex virus type-1 (HSV-1) using relevant cell lines as stated at the Methods section the results confirmed its ability to reduce the viruses: (HSV-1), HAV HM 175, Coxsackie (B4), and rotavirus (Wa) by 70%, 60%, 53.3%, and 50% respectively.

Furthermore, studies have shown that enterocins secreted by enterococcus has antimicrobial properties against Gram positive and Gram negative bacteria (Hanchi et al., 2018). A few studies have stated the antiviral effect of enterocins through the production of antimicrobial molecules such as Enterocin CRL35 which produced by *Enterococcus faecium*CRL35 and expressed antiviral activity against thymidine-kinase positive and deficient strains of herpes simplex (HSV) type 1 and 2 in and BHK-21 cells(Wachsman et al., 1999), *Ent. faecium* ST5Ha secretes a pediocin-like bacteriocin showed high activity against HSV-1 virus in addition to other pathogens (Todorov et al., 2010). However,
screened the genome of the E. faecium NM213 for the presence of the antimicrobial by PCR targeting common enterocin genes, the results showed that presence of only entA and entB genes. Taking together, antiviral activity, the immunomodulatory properties in addition to detection two enterocin genes make this strain is valuable and looking for further analysis to introducing it for industrial purpose.

**Conclusion**

In conclusion, here we present the in vitro antiviral activity of E. faecium NM213 against the main human viruses; Rotavirus, Hepatitis A Virus (HAV), Coxsackie B4 Virus and herpes simplex virus type-1 (HSV-1). Furthermore, the detection of enterocins revealed the presence of two enterocins genes encoding entA and entB within its genome. Thus more attention should be given for characterization for probiotic purposes. We are working to translate these *in vitro* findings to *in vivo* experiment on mice. In addition, doing complete genome sequence and molecular characterization of the bacterium to be satisfied with it is safe.

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**Declarations**

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'Not applicable'

**Conflicts of interest**

The authors declare that there is no conflict of interests

**Availability of data and material**

All relevant data are included within the paper

**Code availability (software application or custom code)**

'Not applicable'

**Authors' contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nahla M Mansour, Hayam Mansour, Ahmed I Abd El Naem, and Waled M Elsenousy. The first draft of the manuscript was written by Nahla M Mansour and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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