Journal of Education for Pure Science- University of Thi-Qar
Vol.10, No.1 (March., 2020)
Website: jceps.utq.edu.iq Email: jceps@eps.utq.edu.iq

DOI: http://doi.org/10.32792/utq.jceps.10.01.023

The effect of metabolic products of *Enterococcus faecalis* strains on the cell toxicity

Ahmed Khassaf Al Atya[1], Faez Khalaf Abdulmuhsen[2], Eenayh[1] Lecture Lecture Assist.prof

[1]Dept. Biology-Coll. Science-University of Thi-Qar
[2]Coll. Medicine -University of Thi-Qar

Received 29/10/2019 Accepted 31/10/2019 Published 20/1/2020

Abstract:
Enterococcus faecalis strains are common commensal of the intestines of humans and other animals**.** Production of different metabolic substance from Enterococci has highest effect on the microorganism and cytotoxicity. The present study highlights the effect of metabolic productions on the cell toxicity by used VERO cells. The results also showed that the metabolic productions of *E. faecalis* A1 and *E. faecalis* A2 were toxic untile dilution factor 12 and had cytoxicity effect on the VERO cells, While the *E. faecalis* A3 was less toxicity at dilution 1/8.

Keywords: LAB, Cytotoxicity, VERO cells, pathogen, hemolycin

Introduction:

The genus of Enterococcus, gram positive facultative anaerobic bacteria found in the environment, they are found in a wide variety of environments such as water, soil, sewage, plants, animals and the most of this type of bacteria abundant commensal flora of human and animal gut microbiomes (Goto & Yan, 2011; Layton et al., 2010). The lactic acid is the end product of enterococci and glucose fermentation, without production of gas (Klein, 2003). Reports have focused on the identification of the *Enterococcus* species, by using biochemical analysis and molecular identification techniques. Based on 16 rRNA gene similarities, there are 37 species of *Enterococcus*, some data reported that the species of *E. faecalis* and *E. faecium* may be more abundant in the feces of human than other species of enterococci, while *E. casseliflavus* and *E. mundtii* also found in the gut of human. Many strains of enterococci colonize the human body, especially the GIT, vagina, skin, oral cavity, the upper respiratory tract as "normal" commensals (Hayashie al., 2005; Jamet et al., 2012).Enterococci are also considered as a part of the natural gastrointestinal microbes of healthy human (Ben Said et al., 2015). Sometimes these bacteria can be involved in the nosocomial infections (Zi et al., 2017). Several studies carried out metabolic production such as enterocin showed bactericidal activity against different pathogens such as *Listeria monocytogenes*, *S. aureus*, *Clostridium sp*, *Bacillus cereus*, *Vibrio cholera*, and *E. coli* (Alvarez-Cisneros & Espuñes, 2011; Nishie et al., 2012). Many strains of Enterococci such as *Enterococcus faecalis* were associated with water contamination (Ahmed et al., 2019). However, Enterococci often been reported

that Pathogenic traits of *E. faecalis* strains often contain many antibiotic resistances, especially to vancomycin (Henning, Gautam, & Muriana, 2015).Enterococci have recently emerged as a prevalent multidrug-resistant nosocomical pathogen and the metabolic substance referred as a hemolysin is the most thoroughly characterized Enterococcal lantibiotic; it has the ability to inhibit and lethal for a broad range of prokaryotic and eukaryotic cells, such as erythrocytes from various animals, (Coburn & Gilmore, 2003). The aim of this study to elevate and determine the effect of some metabolic products of Enterococci stains on the viability of VERO cells.

Methods:

Culturing and Identification:

Atotal 123 of gram positive bacteria and isolated colonies were selected and picked for the fesses samples. Briefly, the fesses were collected from children were visited lille Hospital, france, serial diluted and 1 ml of each dilution was spread onto de Man-Rogosa-Sharpe (MRS) agar plates incubated at 37°C for 48h under a 5% CO2 condition. Morphological and biochemical preliminary tests permitted to select Gram positive bacteria, with a cocci shape, and without catalase activity. The isolates were then identified by ***VITEK 2*** system (bioMérieux, France). The cell free supernatants (CFS) of three strains used for the cytotoxicity effect on the VERO cells (E. *faecalis* A1, *E. faecalis* A2 and *E. faecalis* A3) measurement were obtained by centrifuging (9000 x *g*, 10 min,40C), overnight cultures of *E. faecalis* grown at 37°C for 18 to 24 h, on MRS broth. Neutralized cell-free supernatant (NCFS) (pH6.5).

Cell toxicity:

Effects of the culture supernatant on cell proliferation were measured utilizing Dojindo's Cell Counting Kit-8 (CCK-8), a highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium monosodium salt] (Dojindo Molecular Technologies, USA) producing a water-soluble formazan dye upon reduction in the presence of an electron mediator. WST-8 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. For toxicity tests, the VERO cells, a fibroblast-like kidney cell line from African green monkey (Sigma-Aldrich Chemie S.a.r.l., France), were transferred into a 96-well microtitre plate at a density of 6000 cells by well in 100 µl of medium (DMEM, 4.5 g l^{-1} glucose supplemented with 10% of foetal calf serum, 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} of streptomycin) and in the presence or in the absence of samples at different dilutions. After 48 h at 37 °C, 5% CO_2 atmosphere, cells were incubated in the presence of CCK-8 regent for 4 h. Absorbance (450nm) in each well was measured in a microplate reader spectrophotometer (SAFAS, Monaco). Results were expressed as percentages of basal growth activity.

Statistical Analysis:

Data are expressed as a mean _ standard error (SE) calculated over three independent experiments performed in triplicate.

Results:

Isolation and identification:

This study was carried out 123 strains of Enterococci from different feces samples of children. These isolates were obtained by a cultured-based method in MRS medium. Gut cocci were phenotypically identified through typical Enterococci shape, Gram-positive staining, and lack of catalase activity. According to the ***VITEK 2*** system the all isolates were identified as *Enterococcus faecalis*.

The toxicity of bacterial isolates:

The effect of the samples *E. faecalis* A1 neutralized was tested at different dilutions of the culture supernatants (1:2; 1:4; 1:8; 1:12) on the VERO cell growth (Table 1). The Absorbance reader showed that the metabolic productions of *E. faecalis* A1 were toxic untile dilution factor 12 and had cytoxicity effect on the VERO cells. The percentage of viability cells at dilution 1/4, 1/6, 1/8 and 1/12 were 5.4%, 47.5%, 67.1 % and 89.8% respectively (figure 1). The investigation of cell viability (VERO cells) when used the free cells supernatant of *E. faecalis* A2 (Table 2) showed also the all dilution untile 1/12 were toxic. The percentage of viability cells at dilution 1/4, 1/6, 1/8 and 1/12 were 3.09%, 46.2%, 83.4 % and 96.55 % sequentially (figure 2). Results shown that the sample of *E. faecalis* A3 (Table 3) neutralized had no effect on VERO cell growth with a dilution at 1/8 and dilution factor of neutralized exerted a positive effect (11 % increase) at 1/12 dilution. The percentage of viability cells at dilution 1/4, 1/6, 1/8 and 1/12 were 3.9%, 55.1%, 88.2 % and 99.4% respectively (figure 3).

Discussion:

Enterococci represent the major bacteria that colonized in the gastrointestinal tract of human and animals and also found in variety animal origin such as chesses and other foods (Jamet et al., 2012) **.** There are many types of Enterococci species such as *Enterococcus faecalis* isolated from healthy individuals (Tanaka et al., 2019). Moreover, different species of enterococci isolated from different source such as water waste (Ben Said et al., 2015). In addition, the high antibiotic resistance patterns of these bacteria isolated from GIT of clinical cases (Klein, 2003). Recent study reported that *Enterococcus faecium* clinically multi drug resistance pathogen and produce membrane vesicles (Kim et al., 2019). The results of toxicity of these three culture medium supernatant were assayed and demonstrated that sample *E. faecalis* A3 neutralized exerted less cytotoxicity on VERO cell growth, whereas *E. faecalis* A1 and *E. faecalis* A2 highly inhibited cell proliferation at a 1:8 dilutions. During the primary metabolic fermentation processes of LAB like Enterococci produce different number of minor metabolites such as acetic acid, acetaldehyde, ethanol, acetoin, acetate and lactate dehydrogenase (Bang et al ., 2001; Mehmeti et al., 2011). The increasing biological activity of enterocins such as antimicrobial substance produce by *Enterococcus faecalis* MRR 10 (Martín-Platero et al., 2006). This different effect could be correlated with the different strains samples, since *E. faecalis* A3 have been neutralized and less toxicity. In this regard, several studies have been showed that the another productions from this bacteria such as Enterocin (Franz et al., 2007; Huang et al., 2013; Izquierdo., 2009). On the other hand, Zommiti et al, (2018) found that the absences of cytotoxicity on the Caco-2/TC7 Culture by used cell free supernatant of *Enterococcus faecium* strain. Our result suggested that others compounds present in these complex culture supernatants could also be responsible of the toxicity on the VERO cells.

Conclusion:

This work allowed us to observe the effect the metabolic product of three strains of *E. faecalis* isolated from children gut on the specific cells that used for testing the cell cytotoxicity.

Table1: The Absorbance of microtiter plate reader of supernatant culture of *E. faecalis* A1 on the VERO cells

(microg.mL-1)	Abs1	Abs2	Abs3	Abs4	Abs5	Abs6
Control	0.422	0.42	0.413	0.412	0.401	0.392
	1.341	1.24	1.417	1.691	1.578	1.039
D 1/4	0.889	0.898	0.895	0.893	0.87	0.843
	0.941	0.906	0.917	0.938	0.889	0.878
D 1/6	0.723	0.71	0.706	0.72	0.701	0.684
	1.066	1.492	1.04	1.297	1.11	0.941
D 1/8	0.632	0.624	0.525	0.589	0.549	0.643
	1.498	1.387	1.485	1.522	1.549	0.998
D 1/12	0.545	0.157	0.321	0.503	0.524	0.539
	1.500	1.806	1.823	1.754	1.634	1.205

Abs: Absorbance

D: Dilution

Table2: The Absorbance of microtiter plate reader of supernatant culture of *E. faecalis* A2 on the VERO cells

(microg.mL-1)	Abs1	Abs2	Abs3	Abs4	Abs5	Abs6
Control	0.375	0.376	0.376	0.374	0.363	0.355
	1.452	1.552	1.581	1.502	1.369	1.336
D 1/4	0.781	0.786	0.788	0.76	0.767	0.739
	0.812	0.82	0.849	0.849	0.841	0.809
D 1/6	0.620	0.621	0.624	0.626	0.621	0.617
	1.265	1.212	1.321	1.119	1.131	0.806
D 1/8	0.530	0.529	0.519	0.52	0.527	0.514
	1.066	1.548	1.485	1.222	0.904	1.332
D 1/12	0.431	0.438	0.435	0.431	0.434	0.437
	1.266	1.596	1.489	1.433	1.401	1.329

Abs: Absorbance

D: Dilution

Table3: The Absorbance of microtiter plate reader of supernatant culture of *E. faecalis* A3 on the VERO cells

(microg.mL-1)	Abs1	Abs2	Abs3	Abs4	Abs5	Abs6
Control	0.386	0.382	0.384	0.385	0.377	0.376
	1.185	1.307	1.331	1.461	1.199	0.866
D 1/4	0.776	0.777	0.792	0.81	0.785	0.793
	0.835	0.816	0.826	0.819	0.827	0.809
D 1/6	0.621	0.624	0.627	0.626	0.614	0.608
	0.962	1.232	0.959	1.149	1.152	1.054
D 1/8	0.538	0.544	0.549	0.535	0.529	0.533
	1.295	1.202	1.293	1.434	1.395	1.072
D 1/12	0.431	0.433	0.436	0.438	0.437	0.439
	1.437	1.387	1.459	1.36	1.317	1.287

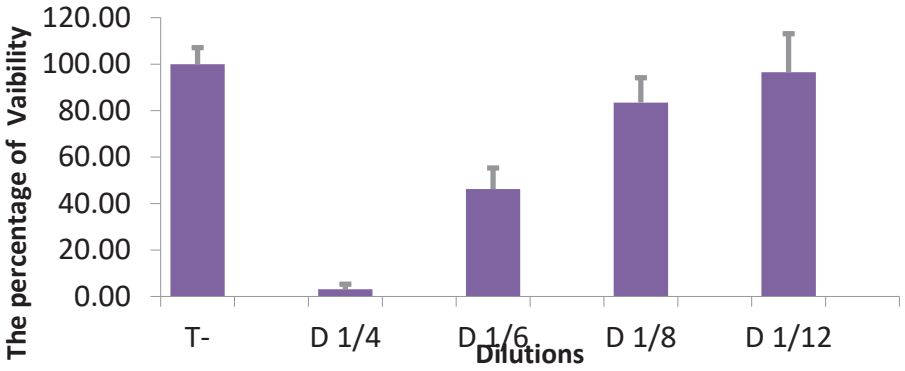

Figure1: The cytotoxicity of supernatant culture *E. faecalis* A1 on VERO cell growth

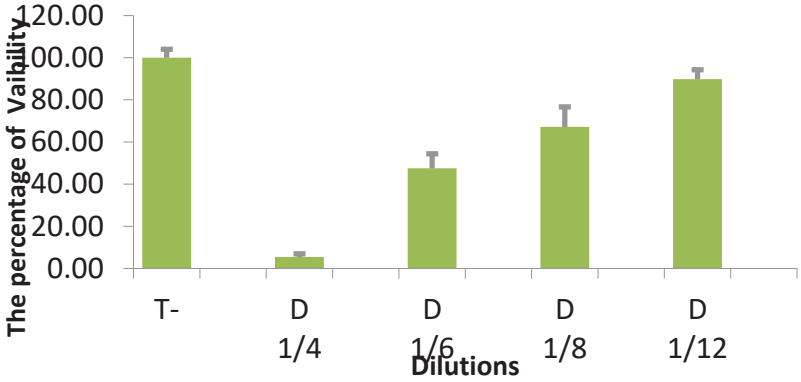

Figure2: The cytotoxicity of supernatant culture *E. faecalis* A2 on VERO cell growth

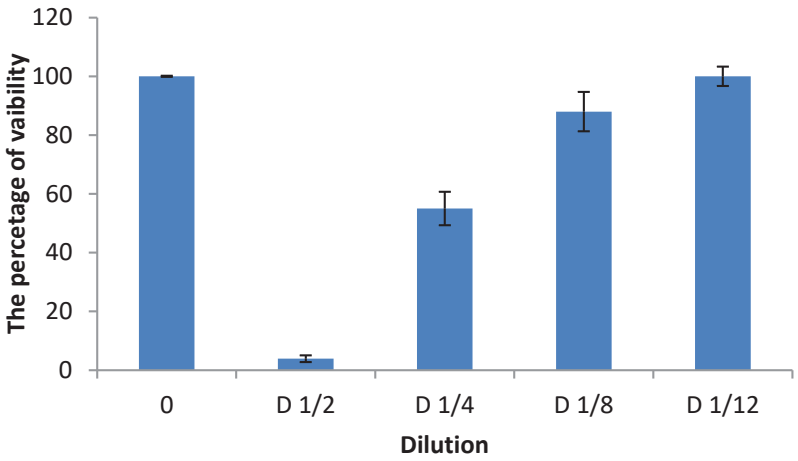

Figure3: The cytotoxicity of supernatant culture *E. faecalis* A3 on VERO cell growth

References:

Ahmed, W., Payyappat, S., Cassidy, M., & Besley, C. (2019). Enhanced insights from human and animal host-associated molecular marker genes in a freshwater lake receiving wet weather overflows, (April), 1–13. https://doi.org/10.1038/s41598-019-48682-4

Alvarez-Cisneros, Y., & Espuñes, T. (2011). Enterocins: Bacteriocins with applications in the food industry. *Formatex.Info*, 1330–1341. http://www.formatex.info/microbiology3/book/1330-1341.pdf

Bang, N., Jensen, S., Melchiorsen, C. R. I. X., Jokumsen, K. V., & Villadsen, J. (2001). Metabolic Behavior of Lactococcus lactis MG1363 in Microaerobic Continuous Cultivation at a Low Dilution Rate, *67*(6), 2677–2682. https://doi.org/10.1128/AEM.67.6.2677

Ben Said, L., Klibi, N., Lozano, C., Dziri, R., Ben Slama, K., Boudabous, A., & Torres, C. (2015). Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia. *Science of The Total Environment*, *530–531*, 11–17. https://doi.org/10.1016/j.scitotenv.2015.05.091

Coburn, P. S., & Gilmore, M. S. (2003). The Enterococcus faecalis cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cellular Microbiology*, *5*(10), 661–669. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12969372

Goto, D. K., & Yan, T. (2011). Effects of Land Uses on Fecal Indicator Bacteria in the Water and Soil of a Tropical Watershed. *Microbes and Environments*, *26*(3), 254–260. https://doi.org/10.1264/jsme2.ME11115

Hayashi, H., Takahashi, R., Nishi, T., Sakamoto, M., & Benno, Y. (2005). Molecular analysis of jejunal, ileal, caecal and rectosigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *Journal of Medical Microbiology*, *54*(11), 1093–1101. https://doi.org/10.1099/jmm.0.45935-0

Henning, C., Gautam, D., & Muriana, P. (2015). Identification of Multiple Bacteriocins in Enterococcus spp. Using an Enterococcus-Specific Bacteriocin PCR Array. *Microorganisms*, *3*(1), 1–16. https://doi.org/10.3390/microorganisms3010001

Huang, E., Zhang, L., Chung, Y. K., Zheng, Z., & Yousef, A. E. (2013). Characterization and application of enterocin RM6, a bacteriocin from enterococcus faecalis. *BioMed Research International*, *2013*, 1–7. https://doi.org/10.1155/2013/206917

Izquierdo, E., Wagner, C., Marchioni, E., Aoude-werner, D., & Ennahar, S. (2009). Enterocin 96 , a Novel Class II Bacteriocin Produced by Enterococcus faecalis WHE 96 , Isolated from Munster Cheese □, *75*(13), 4273–4276. https://doi.org/10.1128/AEM.02772-08

Jamet, E., Akary, E., Poisson, M. A., Chamba, J. F., Bertrand, X., & Serror, P. (2012). Prevalence and characterization of antibiotic resistant Enterococcus faecalis in French cheeses. *Food Microbiology*, *31*(2), 191–198. https://doi.org/10.1016/j.fm.2012.03.009

Kim, M. H., Kim, S. Y., Son, J. H., Kim, S. Il, Lee, H., Kim, S., … Lee, J. C. (2019). Production of Membrane Vesicles by Enterococcus faecium Cultured With or Without Subinhibitory Concentrations of Antibiotics and Their Pathological Effects on Epithelial Cells, *9*(August), 1–11. https://doi.org/10.3389/fcimb.2019.00295

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiology*, *88*(2–3), 123–131. https://doi.org/10.1016/S0168-1605(03)00175-2

Layton, B. a., Walters, S. P., Lam, L. H., & Boehm, a. B. (2010). Enterococcus species distribution among human and animal hosts using multiplex PCR. *Journal of Applied Microbiology*, *109*(2), 539–547. https://doi.org/10.1111/j.1365-2672.2010.04675.x

Martín-Platero, A. M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M., & Martínez-Bueno, M. (2006). Characterization of antimicrobial substances produced by Enterococcus faecalis MRR 10-3, isolated from the uropygial gland of the hoopoe (Upupa epops). *Applied and Environmental Microbiology*, *72*(6), 4245–4249. https://doi.org/10.1128/AEM.02940-05

Mehmeti, I., Jo, M., Fergestad, E. M., Mathiesen, G., Nes, I. F., & Holo, H. (2011). Transcriptome , Proteome , and Metabolite Analyses of a Lactate Dehydrogenase-Negative Mutant of Enterococcus faecalis V583 □ †, *77*(7), 2406–2413. https://doi.org/10.1128/AEM.02485-10

Nishie, M., Nagao, J.-I., & Sonomoto, K. (2012). Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. *Biocontrol Science*, *17*(1), 1–16. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22451427

Tanaka, K., Watabe, T., Kato, K., Tsukimi, T., Sato, M. P., & Odamaki, T. (2019). crossm Draft Genome Sequences of Enterococcus faecalis Strains, (October), 17–19.

Zi, P., Gnat, S., & Nowakiewicz, A. (2017). Characterization of Multidrug Resistant E . faecalis Strains from Pigs of Local Origin by ADSRRS-Fingerprinting and MALDI -TOF MS ; Evaluation of the Compatibility of Methods Employed for Multidrug Resistance Analysis, 1–19. https://doi.org/10.1371/journal.pone.0171160

Zommiti, M., Cambronel, M., Maillot, O., & Barreau, M. (2018). Evaluation of Probiotic Properties and Safety of Enterococcus faecium Isolated From Artisanal Tunisian Meat " Dried Ossban ," *9*(August), 1–12. https://doi.org/10.3389/fmicb.2018.01685