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Synergistic and Cytotoxic Effects of the Combination of Inerolysin and Vaginolysin Toxins on VK2 and HeLa Cells Isolated from an Iraqi Woman who had an Abortion

Saja Ayad Najim*, Suzan Saadi Hussain, Raghad Abdulatef Abdulrazaq

Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.
*Correspondence to: Saja Ayad Najim (E-mail: sajaayad.ph.d.mic.2020@uomustansiriyah.edu.iq)
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Abstract

Objective: The goal of this study is to purify inerolysin produced by *Lactobacillus iners* and vaginolysin produced by *Gardnerella vaginalis*, and to investigate the cytotoxic effects of the combination of these purified toxins on VK2 and HeLa cells.

Methods: Out of 592 specimens from Baghdad teaching hospitals, 101 isolates were identified as *L. iners* and 165 isolates as *G. vaginalis*. The toxins were purified using ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. The cytotoxic effects of the combined inerolysin and vaginolysin on VK2 and HeLa cells were studied.

Results: For inerolysin, the optimal precipitation saturation ratio was 70%, with ion exchange chromatography yielding a specific activity of 14 mg and gel filtration increasing the specific activity to 40 U/mg. For vaginolysin, the optimal precipitation saturation ratio was 50%, with ion exchange chromatography yielding a specific activity of 4 mg and gel filtration increasing the specific activity to 10 U/mg. The combination of toxins showed a viability ratio of 38% for VK2 cells and 31% for HeLa cells at higher toxin concentrations.

Conclusion: Inerolysin and vaginolysin cause lysis of vaginal and cervical cells, which may lead to abortion. The killing ratio increases with higher concentrations of the toxins.

Keywords: *Lactobacillus iners*, *Gardenella vaginalis*, inerolysin, vaginolysin, abortion

Introduction

Lactobacillus iners is a common bacterium species found in the vaginal microbiome. It was first reported in 1999.[1] Historically, *L. iners* was an anaerobic, rod-shaped, non-spore-forming, Gram-positive bacteria.[1] Some research suggests that *L. iners*, in contrast to other species of Lactobacillus, may have a cocobacter rather than a bacilli shape and may not always exhibit obvious Gram-positive staining.[2]

The cholesterol-dependent cytolysins (CDCs) are a family of pore-forming toxins generated by *L. iners*[3] similar inerolysin, a toxin that is produced by certain *L. iners* isolates. The pore-forming action of most of these poisons is finished, and they are secreted. The presence of cholesterol in host cell membranes is essential for their survival; INY forms huge oligomeric complexes with the cell membrane, which insert into the bilayer to create water pores.[4] Since *Lactobacillus iners* is unique among Lactobacillus species, its prevalence in BV can be used as a microbiological indication to forecast when BV will start or when it will be in an intermediate stage.[5]

Vaginal lactobacillus have antagonistic mechanism which aids in the prevention of bacterial vaginosis except Lactobacillus iners, the most commonly found bacterial species in the female genital tract, is associated with increased risk for bacterial vaginosis (BV) and other adverse health outcomes.[6]

An in vitro study found that *L. iners* may increase the adhesion of the BV-causing bacterium, *G. vaginalis*.[4] infections caused by anaerobes commonly are caused by combinations of bacteria that function in synergistic pathogenicity. Although studies of the pathogenesis of anaerobic infections have often focused on a single species, it is important to recognize that the anaerobic infections most often are caused by several species of anaerobes acting together to cause infection.[5]

L. iners significantly reduced the adhesion of *G. vaginalis* strains from a healthy woman, but markedly enhanced pathogenic *G. vaginalis* adhesion,[5] suggesting that *L. iners* can cohabitate with BV-associated *G. vaginalis* and may contribute to *G. vaginalis*-dominated biofilm formation.[7] It has been hypothesized that Gardnerella spp. initiate biofilm formation, which supports the attachment of other BV-associated bacteria (BVAB) to the vaginal epithelium, further enhancing the biofilm thickness, additional BVABs are protected from antibiotics because Gardnerella biofilms act as a barrier.[6] The creation of biofilms, which shield the bacteria from antibiotic therapy and even supply a reservoir for pathogen renewal, is widely believed to be the cause of the high BV recurrence rate.[7]

L. iners significantly reduced the adhesion of *G. vaginalis* strains from a healthy woman, but markedly enhanced pathogenic *G. vaginalis* adhesion,[5] suggesting that *L. iners* can cohabitate with BV-associated *G. vaginalis* and may contribute to *G. vaginalis*-dominated biofilm formation.[7]

In addition, research has revealed that *L. iners* can increase adherence by producing inerolysin, a pore-forming protein often seen in harmful bacteria.[8] These findings suggest that the particular adhesive action of *L. liner* mitigates the protective impact of beneficial vaginal microbiota against harmful bacteria.[9]

Materials and Methods

Identification of Lactobacillus iners and Gardnella vaginalis

Every bacterial isolate was examined under a microscope, and the morphological traits of the bacterial cells such as their form, organization, and Gram staining—were investigated. The spaceman is cultured in MRS agar and Colombian agar under anaerobic condition (anaerobic jar) for 24–48 hrs.[10] The appearance of colonies on solid media was studied for shape,

color, size, opacity, and margin. In addition, the biochemical test (oxidase and catalase).[11]

Confirm Identification of Lactobacillus iners and Gardnella vaginalis

The 16s rRNA confirmatory test for identifying the *L iners* isolates F (5′-GTC TGC CTT GAA GAT CGG-3′) and R (5′-ACA GTT GAT AGG CAT CAT C-3′) are the primers used in this investigation[12] the manufacturer's recommendations, DNA was extracted using the QiAMP Mini DNA extraction kit (Qiagen, Australia) by adding an enzymatic lysis step beforehand and eluting the result in 50 µL of sterile water.[13]

While the confemetery test for G vaginalis is done by VITEK test.[14]

Preparation of the Bacteria to Produce the Toxin

The *L. iners* strain was cultured in Man De Rogosa broth (MRS broth), which was enhanced with 2% human blood plasma. The cultures were regularly subcultured every 36 hours and kept at 37°C in an anaerobic jar. I 150 ml flasks with 125 ml of MRS broth and nonionic detergent (Tween 80) were inoculated with 10^7 bacteria. After that, the infected medium was harvested after being incubated anaerobically for 36 hours at 37°C. By using the dilution procedure and measuring the CFU in MRS agar with 5% blood base, the number of bacteria was determined. As previously mentioned, cultures were incubated under anaerobic conditions at 37°C.[15]

For *Gardnella vaginalis* the preparation of vaginolysin toxin crude is done by Giandomenico et al.[16]

Hemolytic Assay of Both Toxines

The assay combination included the necessary volume of sample, 50 ul of a 1% suspension of group 0 human erythrocytes, and 1 ml final volume of phosphate buffer (pH 7.4). For 30 minutes, the tubes were incubated at 37°C. spectrometer was used to measure the A415 of the supernatant fluid, which contained the hemoglobin that had been liberated from the lysed erythrocytes, following centrifugation (1,200 x g for 3 min). The erythrocyte concentration was changed in each trial so that, after full hemolysis.[17]

Precipitation of Toxins by Ammonium Sulfate

The crude enzyme was combined with varying weights of ammonium sulfate at graduated (%) saturation ratios of 50, 60, 70, and 80%. The mixture was gently swirled on a magnetic stirrer while cooling for a duration of 20 minutes. Following dissolved in an appropriate volume of 0.5M Tris-HCl buffer at pH 8, and the toxin activity, protein concentration, and specific activity of the toxin solution were measured. It was then centrifuged at 10,000 rpm for 10 min. at 4°C, while the supernatant was separated and mixed with ammonium sulfate to obtain the next saturation ratio.[17] Slice A dialysis clip was used to seal one end of the dialysis tubing after a 10-cm section of the bag (3KDa) was cut off and washed with distilled water. Since the volume expanded during dialysis, some room had to be left in the dialysis tube after the purified inerolysin was added.

The solution was gathered and centrifuged for 15 minutes at 4°C at 10,000 rpm.[18]

Purification of Inerolysin and Vaginolysin by Ion Exchange Chromatography

Twenty-five grams of DEAE-cellulose were suspended in one thousand milliliters of distilled water and packed onto a column according to Whitaker's (1972) procedure. After removing the floating flakes from the solution until the top layer was clear, a vacuum pump was used to filter the mixture. 250 ml of a solution containing 0.25 M NaCl and 0.25 M NaOH was used to suspend the precipitate and then cleaned once more with 250 milliliters of 0.25 M HCl. The column was sized (2.5 x 20 cm), the linked proteins were gradually eluted using varying sodium chloride concentrations (0.1–1 M). A UV-VIS spectrophotometer was used to measure each fraction's absorbance at 280 nm while maintaining a 30 ml/h flow rate throughout the column. Every fraction's level of toxin activity was measured.[14]

Purification of Inerolysin and Vaginolysin by Gel Filtration Chromatography

Certain amount of Sephadex G-100 was suspended in 0.05 M phosphate buffer pH7. After that, it was degassed, packed, and allowed to equilibrate using the same buffer. To achieve elution, the column was fed the concentrated sample from the previous stage using the same equilibration buffer at a flow rate of 30 mL/hr. Each fraction's absorbance was determined at 280 nm. In addition, we measured the amounts of toxins in each proportion.[19]

Determination of Toxins Concentration

The concentration of the toxin was examined using a bovine serum albumin standard curve, as described in Bradford's (1979) approach.[16]

The Cytotoxic Effect of Combination Vaginolysin and Inerolysin Toxins on Hella Cell and VK2 Cell

Preparation of culture media use in cytotoxicity of the combination of inerolysin and vaginolysin, equal amount of inerolysin and vaginolysin toxin (50:50) are prepared after final purification.

RPMI-1640 Medium

The Media RPMI-1640 was prepared and sterilized according to the manufacturing company's recommendation using a Nalgene filter with a 0.22 filter unit.[20]

Using the hemocytometer to count the cells and then applied the following formula to get the cell concentration.

MTT Protocol

Using an MTT ready-to-use kit, the cytotoxic effect of Cuprizone 50 mM in the presence of various levetircatam doses was assessed. Around ($1x10^4$ to cells/ml) of HeLa or VK2 cells were grown in 96 wells culture plates containing 200l/well growth culture medium. After gently shaking the plate coated in sterile parafilm, it was incubated for 24 hours at the ideal conditions for cell line culture (37°C and 5% CO_2). On the next day, Levetiracetam was serially diluted twice (200, 100, 50, 10, 2.5, and 0.5 M/ml) and added to the wells after the culture media was withdrawn. For every concentration, three duplicate wells

were employed, along with the controls, which were cells treated with serum-free media. For four hours, plates were incubated at the ideal temperature and CO_2 concentration for mammalian cell line culture: 37°C. At the end of the incubation period, 50 M/ml of cuprizone was add to each well for 24 hr, after which, 10 l of the MTT solution was added and the mixtures were further incubated at optimum culture conditions for another 4hr. Medium from each well was carefully removed and 100l/well of solubilization solution was added and incubated at room temperature for 5 min till complete solubilization. Optical densities of different wells were measured at 575 nm and statistically analyzed to calculate the concentration of toxin that causes 50% reduction in VK2 or HeLa cells viabilities, using the following equation: Y = D + A-D / 1 + 10 (x-logC)B.[21]

Result

Isolation and Identification of Lactobacillus iners and Gardnella vaginalis from Pregnant Women have Abortion

In the current study, five handrad ninety two specimen were collected from pregnant woman gave vaginal infection and at least have one abortion, from Baghdad teaching Hospital,. Only 154 confirmed isolate belong to *Gardnella vaginalis* and 101 confirmed isolate belong to *lactobacillus iners*. whereas on Colombia agar light grey-transparent colonies small, round, with regular edges as show on figure, the bacteria cultured on Colombia agar with 5% blood, 15% nalidixic acid and 10% collistin.[22]

The cotton swabs were cultured on MRS Agar (man de -ragosa agar), blood agar medium, under aerobic conditions at 37°C and incubated for 24–48 hours.[12]

The isolates grew well on the medium and produced white, smooth, round colonies as shown in (Figure 1).[21] However, MRS agar is a selective media for *Lactobacillus iners* because of its low pH, the presence of tween 80, and the presence of acetate.

Detaction of Lactobacillus iners and Gardnella vaginalis

There is no conformation test like vitak2 test for confirm the *lactobacillus iners* isolation but to use of 16 s rRNA.

The Qiagen DNA easy tissue kit (Qiagen, Valencia, CA, USA) was used to extract DNA from pure cultures. The procedure was carried out using logarithmic growth phase cells in Todd Hewitt broth, as instructed by the manufacturer (THB, Difco).[12,13]

One solitary colony was transferred, well-fixed, and stained with Gram stain on a microscope slide. After observing the Gram reaction, the cell's form, and organization, bacterial colonies were moved to blood agar and Colombian agar in an aerobic environment (anaerobic jar) for routine microbiological culture studies. The shape, color, size, opacity, and border of colonies on solid media were examined.[10] Additionally, the VITEK system performs the biochemical test (oxidase, catalase, and whiff test) at the final conference identification.[15]

Purification of Inerolysin

Preparation of crude

After incubating the *L. iners* isolate in 100 ml of MRS broth under anaerobic conditions for 24 hours at 37°C, the toxin was extracted. The bacteria was then washed with 0.05M sodium phosphate buffer (pH 7.0), which broke down the cell walls of the bacteria. Centrifugation was then used to separate the cells for 15 minutes at 10,000 rpm, and the toxin activity in the crude supernatant was calculated. According to Table 1, the specific activity was 1.56 U/mg protein and the enzyme activity was 0.75 U/ml.[16]

While the result for vaginolysin, the toxin activity in crude supernatant was estimated. The toxin activity was 0.3 U/ml, and specific activity 0.75 U/mg toxin, as shown in (Table 2).[16]

Ammonium sulfate precipitation

The results showed that the best range for toxin precipitation was at the saturation ratio 70%.[21] When ammonium sulfate was used at various saturation rates (40, 50, 60, 70, 80, and 90%). According to the results in Table 1, inerolysin specific

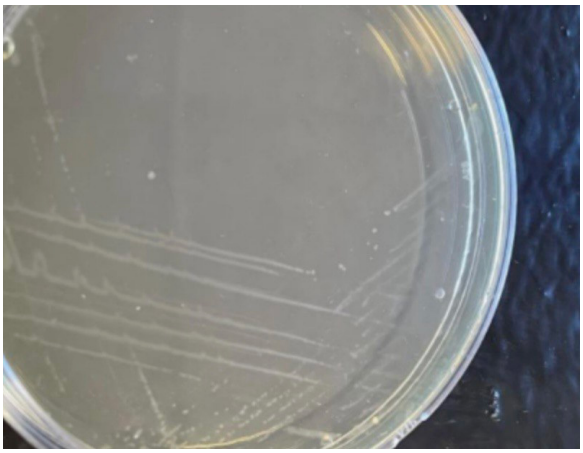

Fig. 1 ***Lactobacillus iners* growth on MRS agar, the colonies look like white, round, smooth shape under anaerobic condition.**

Table 1. **Purification steps for inerolysin**

Purification step	Volume (ml)	Toxin activity (U/ml)	Toxin concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	75	0.75	0.48	1.56	56.2	1	100
Ammonium sulphate precipitation 70%	15	2	0.21	9.52	30	6.1	53.3
DEAE-cellulose	21	1.4	0.1	14	29.4	8.9	52.3
Sephadex-G 100	18	1.6	0.04	40	28.8	25.6	51.2

Table 2. **Purification steps for vaginolysin produced by *G vaginalis***

Purification step	Volume (ml)	Toxin activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	75	0.3	0.4	0.75	22.5	1	100
Ammonium sulphate precipitation 50 %	13	0.9	0.5	1.8	11.7	2.4	52
DEAE-cellulose	24	0.4	0.1	4	9.6	5.3	42.6
Sephadex-G150	18	0.5	0.05	10	9	13.3	40

activity was determined to be 9.52 IU/mg toxin, and its purification fold reached to 6.1 with a 53.3% overall yield.

The optimal range for vaginolysin precipitation was at the saturation ratio of 50%. According to the results displayed in Table 2, the toxin's specific activity was found to be 1.8 IU/mg of protein, and it had a 52% overall yield and a purification fold of 2.4. the obtained ammonium sulphate precipitate in solution was kept in the refrigerator at 4°C for further purification and then added to a dialysis tube for dialysis against 1 ml of phosphate buffer.[23]

Ion exchange chromatography (DEAE-Cellulose.)

The eluted fractions' absorbance reached the line of zero (the line base), the absorbance of the sodium phosphate buffer was measured at 280 nm. Subsequently, the same buffer was used with NaCl gradient chloride ranging from 0.1 to 1 M. When the absorbance of each fraction was examined at 280 nm, three protein peaks were apparent, corresponding to the results in Figure 2, and one peak at the washed fractions, where the majority of the inerolysin activity was detected in tubes 46–60, with only one peak among the gradient elution peaks representing with 0.5 M of NaCl. This result indicated that inerolysin produced by *L. iners* has a negative net charge, since it bound with the anionic ion exchange DEAE-cellulose.[24]

Toxin activity tasted, specific activity of 14 mg (protein), and that its purification fold was 8.9 with a 52.3% overall yield as shown in Table 1. and Figure 2 using a DEAE-cellulose exchanger purification fold.

For vaginolysin the fractions were combined and assessed activity using a DEAE-cellulose exchanger purification fold; the results showed a specific activity of 4 mg and a purification fold of 5.3 with an overall yield of 42.6%, as seen in Figure 3 and Table 2.

Gel filtration chromatography

One protein peak emerged from the fractionation process, found in fractionation tubes 13–18, which were used to evaluate a particular inerolysin activity. The purified enzyme's specific activity (40 U/mg) has increased, according to Table 1's results, which also reveal a purification fold of 25.6 and an inerolysin yield of 51.2% as seen in Figure 4.

To obtain a purified toxin with a specific activity of 366 U/mg, a fold of 6.6%, and a yield of 1.5, the third step of the process involves ammonium sulfate precipitation and DEAE-cellulose.

The vaginolsin obtained from *G. vaginalis* was further purified and characterized using the Sephadex G-100 chromatography system. This was the third step in the process, following the precipitation of ammonium sulfate and the DEAE-cellulose, to produce a purified toxin with a specific

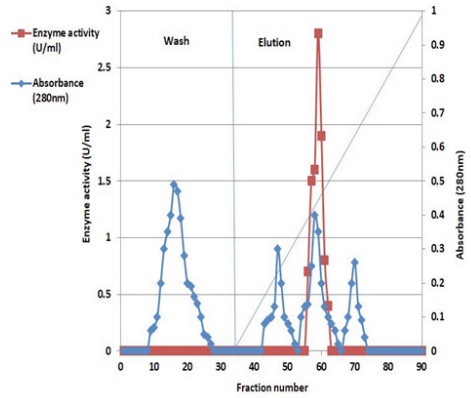

Fig. 2 **Ion exchange chromatography of inerolysin toxin.**

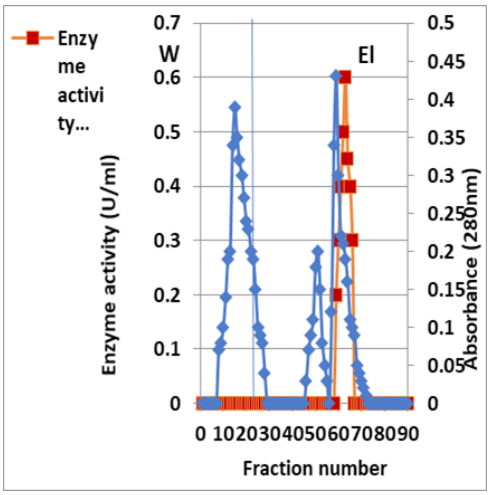

Fig. 3 **Ion exchange chromatography of vaginolysin.**

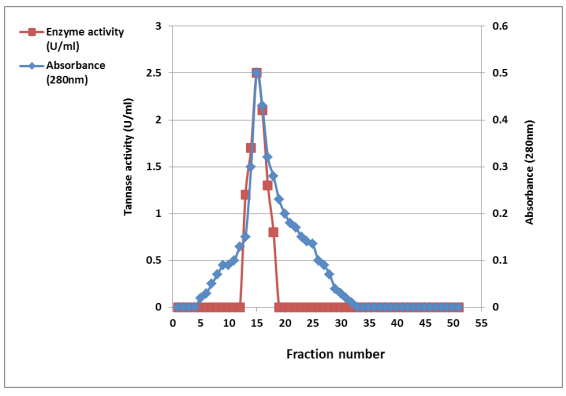

Fig. 4 **Gel filtration chromatography of inerolysin.**

activity of 1.457 U/mg and a fold 22%, as shown in Figure 5 and Table 2.

Cytotoxic effect of combination of purified inerolysin and vaginolysin

The results indicated that the cytotoxicity of the toxins combination with VK2 cells increased with concentration as well as with HeLa cells, the result of the combination of inerolysin and vaginolysin for VK2 cell we observed that 38%, 41.78%, 50.34%, 54.5%, 69% and 76.5% of cell death occurred when 400, 200, 100, 50, 25 and, 12.5 µg/ml respectively show Figure 6 and Table 3, while the result for HeLa cell is 31%, 32.2%, 38.29%, 49%, 56.5% and 76.9% of cell death occurred when 400, 200, 100, 50, 25 and, 12.5 µg/ml, as shown in Figure 7 and Table 3.

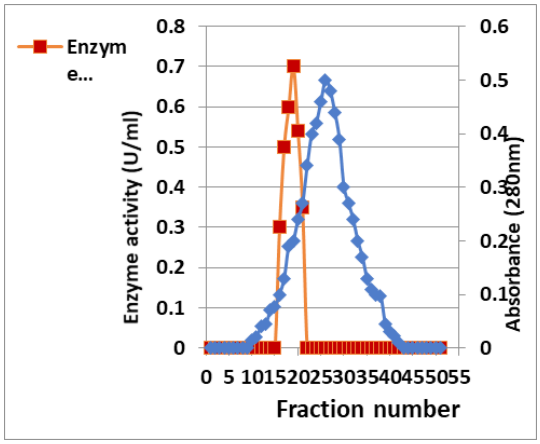

Fig. 5 **Gel filtration chromatography of vaginolysin.**

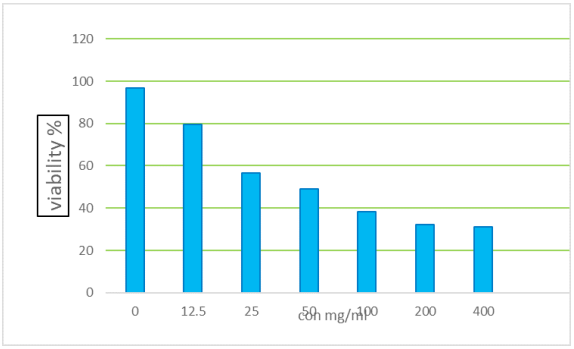

Fig. 6 **Cytotoxic activity of combination of purified inerolysin and vaginolysin on VK2 cell.**

Discussion

Many people wonder if *Lactobacillus iners* is a harmful or typical flora. *L. iners* has more complex nutritional requirements and a Gram-variable morphology than other Lactobacillus species. It can be found in normal settings as well as during vaginal dysbiosis, such as bacterial vaginosis. The pore-forming toxin inerolysin, which is linked to *Gardnerella vaginalis*' vaginolysin, is also encoded in the genomes of *L. iners* strains. It's possible that this organism contains clonal variants that, depending on the situation, either support a healthy vagina or are linked to dysbiosis and illness.[25]

The findings of this study demonstrate the ideal conditions for toxin production. I found that, while the bacteria produce toxins at 37°C and an acidic pH, inerolysin extracted from *L. iners* and vaginolysin extracted from *G. vaginalis*[26] exhibit similarities. However, the two toxins differ when grown in *L. iners* culture media, which is best in MRS broth for 72 hours.[27]

Ammonium sulfate is a cheap salt that is highly soluble, inexpensive, non-toxic to most enzymes, and has a stabilizing effect on several toxins when used in the purifying process of neurolysin. To decrease volume and concentrate poison, it is also helpful to precipitate proteins or toxin during the initial purification stage.[21] The best range for toxin precipitation was at the saturation ratio 70% when ammonium sulfate was used at various saturation rates (40, 50, 60, 70, 80, and 90%).

According to the study by Ryan et al. (2011),[15] INY was active at neutral pH and much less active at pH 4.5. This decrease in ILY activity at acidic pH was in line with what was reported before.[26] INY, on the other hand, worked better at pH levels between 4.5 and 6.0 and less well at pH 7.4.

The cytotoxic result of combination toxins on VK2 cell we observed that 38%, in higher toxin concentration 400 µg/ml, while the result for HeLa cell is 31% in higher toxin concentration 400 µg/ml.

Based on Ragaliauskas et al (2019)[8] most of the arciform structures made by VLY and INY are inserted into the lipid bilayer and create a water-filled pore. *L. iners* cytolysins are thought to be virulence factors, similar to other CDCs, but no one knows what role they play in living things.

In study done by Ryan et al, (2011)[15] at high doses, INY and VLY causes epithelial cell lysis; at low amounts, it

Table 3. **Cytotoxic activity of combination of purified inerolysin and vaginplysin on VK2 and HeLa cell s**

	VK2		HeLa	
Conc.(µg/ml)	**Mean**	**SD**	**Mean**	**SD**
400	38	4.6	31	6.98
200	41.78	14.2	32.20	10.8
100	50.34	4.1	38.29	4.52
50	54.5	2.28	49	7.40
25	69	1.64	56.50	6.88
12.5	76.5	2.02	79.10	2.3
0	96.8	0.53	97.2	0.3

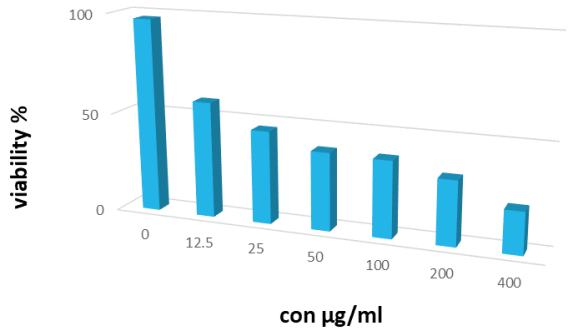

Fig. 7 **Cytotoxic activity of combination of purified inerolysin and vaginolysin on HeLa cell.**

triggers proinflammatory signaling. HeLa INY is most similar to the human-specific CD59-dependent toxins VLY and INY at the primary amino acid sequence level; however, it does not depend on the species or cell type it is attacking. INY has been linked to a putative CD59 binding site; however, VLY lacks this sequence. Consequently, a general foundation for CDC host specificity has not been established. INY is the closest cousin of the CDCs that rely on CD59, and it could be a helpful tool for more functional and evolutionary comparisons.

Conclusion

In this study I discovered that the vaginolysin caus lysis of vaginal cell may lead to abortion, the killing ratio increase by increasing the concentration of the toxin and the effect of the toxin on HeLa cell is more than on VK2 cell.

Conflict of Interest

None.

References

1. Falsen, E., Pascual, C., Sjoden, B., Ohlen, M., Collins, M. D. (1999). Phenotypic and Phylogenetic Characterization of a Novel Lactobacillus Species From Human Sources: Description of Lactobacillus Iners Sp. Nov. Int. J. Syst. Bacteriol. 49 Pt 1, 217–221.
2. Lebeer, S., Vanderleyden, J., De Keersmaecker, S. C. (2008). Genes and Molecules of Lactobacilli Supporting Probiotic Action. Microbiol. Mol. Biol. Rev. 72 (4), 728–764.
3. Park, S. E., Jiang, S., Wessels, M. R. (2012). CsrRS and Environmental pH Regulate Group B Streptococcus Adherence to Human Epithelial Cells and Extracellular Matrix. Infect. Immun. 80 (11), 3975–3984.
4. Macklaim, J. M., Fernandes, A. D., Di Bella, J. M., Hammond, J. A., Reid, G., Gloor, G. B. (2013). Comparative Meta-RNA-Seq of the Vaginal Microbiota and Differential Expression by *Lactobacillus Iners* in Health and Dysbiosis. Microbiome 1 (1):12.
5. Muzny C. A., Taylor C. M., Swords W. E., Tamhane A., Chattopadhyay D., Cerca N., et al.. (2019). An Updated Conceptual Model on the Pathogenesis of Bacterial Vaginosis. J. Infect. Dis. 220 (9), 1399–1405.
6. Gustin A. T., Thurman A. R., Chandra N., Schifanella L., Alcaide M., Fichorova. (2021). Recurrent Bacterial Vaginosis Following Metronidazole Treatment is Associated With Microbiota Richness at Diagnosis. Am. J. Obstet. Gynecol. 226(2), 225.e1-225.e15.
7. Gottschick C., Deng Z. L., Vital M., Masur C., Abels C., Pieper D. H., . (2017). Treatment of Biofilms in Bacterial Vaginosis by an Amphoteric Tenside Pessary-Clinical Study and Microbiota Analysis. Microbiome 5 (1), 119.
8. Basavaprabhu, H. N., Sonu, K. S., Prabha, R. (2020). Mechanistic Insights Into the Action of Probiotics Against Bacterial Vaginosis and its Mediated Preterm Birth: An Overview. Microb. Pathog. 141, 104029.
9. Ragaliauskas, T., Pleckaityte, M., Jankunec, M., Labanauskas, L., Baranauskiene, L., Valincius, G. (2019). Inerolysin and Vaginolysin, the Cytolysins Implicated in Vaginal Dysbiosis, Differently Impair Molecular Integrity of Phospholipid Membranes. Sci Rep. 9(1), 10606.
10. Zozaya-Hinchliffe, M., Lillis, R., Martin, D. H., Ferris, M. J. (2010). Quantitative PCR Assessments of Bacterial Species in Women With and Without Bacterial Vaginosis. J. Clin. Microbiol. 48 (5), 1812–1819.
11. Tell Jakobsson Linköping (2008). *Lactobacillus iners* and the normal vaginal flora, University Medical Dissertations, Clinical Microbiology Department of Clinical and Experimental Medicine Faculty of Health Sciences Linköping University Sweden Linköping .
12. Prescott, L. M. Harley, J.P. and Klein, D.A. (2005). Microbiology. (6th Ed). Published by McGraw Hill. New York. 69(12), 1644–1651.
13. Mohammed A. Alqumber, Jeremy P. Burton, John R. Tagg, Celia Devenish, (2008). A species-specific PCR for *Lactobacillus iners* demonstrates a relative specificity of this species for vaginal colonization, Microbial Ecology in Health and Disease, 20(3), 135–139.
14. Karen G. Kelly (2008). Clinical Methods: The History, Physical, and, Laboratory, Examinations. 3rd edition. Chapter 179 Tests on Vaginal Discharge. Jessica L. O'Callaghan, Dana Willner, Melissa Buttini (2021). Limitations of 16S rRNA Gene Sequencing to Characterize Lactobacillus Species in the Upper Genital Tract. Molecular and Cellular Pathology, Volume 9–2021.
15. Ryan Rampersaud, Paul J Planet, Tara Randis, Ritwij Kulkarn (2014). Inerolysin, a Cholesterol-Dependent Cytolysin Produced by *Lactobacillus iners.* Journal of Bacteriology 193(5):1034–41.
16. G Rottini, A Dobrina, O Forgiarini, E Nardon, G A Amirante, P Patriarca. (1990). Identification and Partial Characterization of a Cytolytic Toxin Produced by *Gardnerella vaginalis*. Infection and Immunity, 58(11), 3751–3758.
17. Shahnazari, M. Bigdeli, R. Dashbolaghi, A. Ahangari, C. R. Shoari, A. Hosseini, H. Nouri Inanlou, D. and Asgary, V. (2022). Biochemical and Biological Evaluation of an L-Asparaginase from Isolated Escherichia coli MF-107 as an Anti-Tumor Enzyme on MCF7 Cell Line. Iran Biomed J. 1,26(4):279–90.
18. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dyebinding. Anal. Biochem. 72: 248–254.
19. Inas S. Mahmood, Rajwa H.Essa, Suzan H. Saadi and Khetam H. Rasool. 2022. Effect of Enterobacter cloacae toxin on immune cell isolated from Leukemia patients. Revistabionature, Volume 7/Issue 2/10.
20. Shari E Gelber, Jorge L Aguilar, Kanako L T Lewis, Adam J Ratner (2008). Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from Gardnerella vaginalis. J Bacteriol. 190(11):3896–903.
21. Suzan S., Hussin, Saadi M. D. Al-Nuzal and R,awaa Abdul-Jabbar (2013). The Susceptibilities of Gardnerella vagina,lis Isolates from Iraqi Hospitals Towards Various Antibiotics and New Ligand Complexes of 5,5-diphenyl-imidazolidine2,4-dione with transition metals(II). Al- Mustansiriyah J. Sci. Vol. 24, No 2.
22. AA Hussein, SI Albarazanchi, AF Alshanon. (2020).Evaluation of anticancer potential for L-glutaminase purified from *Bacillus subtilis*. Int. J. Pharm. Res. 12, 293–299.
23. Yuanhui, H. Xiaoxi, N. Ben W. Risu, N. Bingbing, X. and Huixia, Y. (2020). Evaluation of the Inhibitory Effects *of Lactobacillus gasseri* and *Lactobacillus crispatus* on the Adhesion of Seven Common Lower Genital Tract Infection-Causing Pathogens to Vaginal Epithelial Cells., Front. Med. 7:284.
24. Fathi, F. Kasra-Kermanshahi, R. Moosavi-Nejad, Z. and Qamsari, E.M. (2021). Partial purification, characterization and immobilization of a novel lipase from a native isolate of *Lactobacillus fermentum*. Iran J Microbiol.;13(6):871–877.
25. Nagamune, H., et al. 1996. Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. Infect. Immun. 64:3093–3100.
26. Mariya Petrova, Gregor Reid, Mario Vaneechoutte, Sarah Lebeer. 2017. *Lactobacillus iners*: Friend or Foe. Trends Microbiol. 25(3):182–191.
27. Zheng N, Guo R, Wang J, Zhou W, Ling Z (2021). Contribution of Lactobacillus iners to Vaginal Health and Diseases: A Systematic Review. Front Cell Infect Microbiol. 11: 792–787.