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ORIGINAL

EFFECT OF METRONIDAZOLE ON THE GROWTH OF VAGINAL LACTOBACILLI IN FEMALE ATHLETES

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ABSTRACT

Methods: Lactobacillus strains isolated from female athletes were tested. MRS broth was supplemented with a range of Metronidazole concentrations from 128 to 2000 μg/ml. The growth of these strains was monitored by measuring optical density at 3-hour intervals over 24 hours. **Results:** It was observed that at Metronidazole concentrations up to 128 μg/ml, there was no significant impact on the growth of the Lactobacillus strains. However, at concentrations above 512 μg/ml, Metronidazole significantly inhibited their growth. The response to Metronidazole varied among different Lactobacillus strains. For instance, at a concentration of 256 μg/ml, Lactobacillus delbrueckii, Lactobacillus jensenii, and Lactobacillus vaginalis showed notable inhibition, whereas Lactobacillus crispatus, Lactobacillus gasseri, and Lactobacillus fermentum were not significantly affected. **Discussion:** High concentrations of Metronidazole were found to inhibit the growth of the six Lactobacillus strains isolated from female athletes. Lower concentrations had negligible effects. The differential response of Lactobacillus strains to varying concentrations of Metronidazole (between 128 μg/ml and 512 μg/ml) highlights the need for careful consideration of Metronidazole use in managing vaginal microbiota health in female athletes.

KEY WORDS: Female Athletes; Metronidazole, vaginal lactobacilli, in vivo, in vitro, Chinese women

1. INTRODUCTION

Lactobacillus in vagina is Lactobacillus, some important dominant bacteria in vaginal microenvironment of athletic women of reproductive age, which plays an important role in the sustainable balance of microecology. The predominant colonizing bacteria in the vagina of most healthy athletic women is the main factor in maintaining the balance of vaginal flora (Ullah, 2017). Estrogen in athletic women of reproductive age can stimulate vaginal epithelial cells and promote their proliferation, and then lead to high expression of glycogen related indicators. Glycogen is metabolized by Lactobacillus, and when a woman's vaginal levels of lactate and other organic acids increase, her vaginal pH decreases to 3.7 to 4.5 (Ser et al., 2015).

Decreased estrogen levels in postmenopausal athletic women lead to a decrease in Lactobacillus inductor, which is an important factor in the mechanism by which athletic women increase the risk of urogenital tract infection (UTI) (Freitas et al., 2017). The vaginal microbiota in a balanced state of normal vaginal microbiota may prevent the proliferation of pathogenic microorganisms, including those responsible for bacterial vaginosis (BV) (Yeruva, Rajkumar, & Donugama, 2017)(McMillan et al., 2018), yeast infections , urinary tract infections (Bertuccini, Russo, Iosi, & Superti, 2017)(Jayaram et al., 2014) and sexually transmitted diseases(STD) (Foschi et al., 2017). Therefore, clinical treatment of vaginal infection should not only focus on the effective inhibition of pathogens, but also avoid adverse effects on the normal synthesis of Lactobacillus during treatment.

Metronidazole is a kind of nitroimidazole drugs, which can achieve effective antibacterial. After passive diffusion into cells, the cell can exert cytotoxic effect by producing free radicals, and then exert its killing effect effectively (Sobel & Sobel, 2015). Activation is achieved by reduction of its nitro group promoting the formation of Metronidazole radicals which interact with nucleic acids eventually causing bacterial cell death. Metronidazole possesses direct trichomonial and amebicidal activity, which has been used to treat bacterial vaginosis (Ye et al., 2015) and trichomonial vaginitis (Hawkins, Carne, Sonnex, & Carmichael, 2015)(Aggarwal, Bhattar, Sahani, & Bhalla, 2016) as first-line regimen. Metronidazole is effective against most obligate anaerobic bacteria in vitro, but seems to have no obvious effect on facultative anaerobic bacteria or obligate aerobic bacteria. Theoretically, the application of metronidazole has no destructive effect on Lactobacillus (Bezzerra, Reis, & Prichoa, 2019; Hernandez-Flores, Rodriguez, Gastelum Arellanez, Alvarez-Morales, & Avila, 2015). However, some studies have shown that oral or vaginal use of metronidazole in the treatment of BV athletic women will lead to excessive proliferation of lactobacillus [13] , and some studies suggest that metronidazole may inhibit the growth of lactic acid bacteria (Valenti et al., 2018). This study was conducted to assess the in vitro effect of Metronidazole

on the growth of lactobacilli isolated from vaginas of healthy Chinese athletic women.

2. MATERIALS AND METHODS

2.1 Study population

The study was approved by the Ethics Committee of Peking University First Hospital. Healthy volunteer Chinese athletic women were recruited for this study. Athletic Women without any symptoms of vaginal infections were included in the study. Exclusion criteria were the same as what was described in the study (Zhang et al., 2012). After exclusions, the study population consisted of 12 fertile athletic women between 25 and 40 years old, ranges of body mass index were 18–24 (mean 22±3.4). Each woman had one sex partner.

2.2 Sample collection and isolation of Lactobacillus strains

Two sterile cotton swabs were used to sample the midvaginal wall of each woman. One of the swabs was smeared to assess the ecology of the vagina according to the Nugent scoring system. The images made should be used to detect the presence of fungal mycelia (Candida) and trichomonas in the human body; The subjects were also analyzed for vaginitis, syphilis, Neisseria gonorrhoeae, Chlamydia trachomatis, mycoplasma, HPV, HIV and other diseases. Another swab was inoculated with MRS broth, after which the sample was incubated anaerobically overnight at 37℃. After the above procedure, the sample was diluted to 0.5 (McFarland, MCF). MRS AGAR anaerobic bacteria were diluted in 100ul at 0.5 MCF overnight at 37℃. The secondary colonies were seeded on new MRS AGAR plates and incubated anaerobically overnight at 37℃.

2.3 Identification of Lactobacillus strains

16SR RNA genes of vaginal strains from athletic women were amplified by colony PCR with bacterial universal primers. The primers were: forward primer 5 '-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 5 '-TacgyTACCTTGTTACT-3'. Amplification was performed in 25 ml reaction mixture including 16 PCR Master Mix (Promega). 1 ml of bacterial colonies was used as template, and 1 ml was used as forward and reverse primers. The annealing temperature was 55℃ and the amplicon size was 1500 base pairs. After amplification, the PCR products were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under UV light. PCR products were purified and sequenced using Big Dye Termination Chemistry (AppliedBiosystems) and ABI 3730 DNA analyzer (AppliedBiosystems). Using the standard nucleotide BLAST program (BLASTN, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) will these sequences with

ribosomes database project (RDP, http://rdp.cme.msu.edu/index.jsp) and 16 srrna gene sequences in GenBank database Make a comparison. Sequences have been identified to the species level. Sequences with more than 98% nucleotide sequence similarity were considered to belong to the same bacterial species.

2.4 Effect of Metronidazole on Lactobacillus strains

Metronidazole powder (Sigma-Aldrich) was obtained and Metronidazole dilution was prepared to a concentration of 20 mg/ml in MRS broth. Fresh subculture of each Lactobacillus strain, including Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii, Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus vaginalis, was used after overnight growth on an MRS agar anaerobically. The inoculum was prepared by suspending the colonies in sterile MRS broth to achieve a turbidity of 10 McF determined by turbidity meter (BioMérieux Corporate).

This resulted in a suspension containing approximate 1×10^8 CFU/ml～2×10^8 CFU/ml. Bacteria suspensions were further diluted with MRS broth to obtain a final inoculum suspension of 1×10^7 CFU/ml～2×10^7 CFU/ml. They were then dispensed to MRS broth prepared with different concentrations of Metronidazole. After the addition of Lactobacillus suspension, the final range of Metronidazole concentration was 128μg/ml～2000μg/ml. The suspension was incubated anaerobically at 37°C for 24hours. The optical density of each tested sample was measured at 3-hour interval.

2.5 Statistical analysis

The difference of growth of lactobacilli between Metronidazole groups and control was analyzed by repeated measures analysis of variance. $P<0.05$ was accepted as significant.

3. RESULTS

In the present study, 22 different Lactobacillus strains were isolated and identified from the vaginas of 12 healthy fertile Chinese athletic women. The 22 strains were included in 6 different Lactobacillus species (Table 1,Figure 1). One strain of each species was selected to evaluate the effect of Metronidazole.

Table 1. The isolated Lactobacillus strains

NAME OF LACTOBACILLUS SPECIES	STRAINS
L. crispatus	9
L. gasseri	6
L. jensenii	3
L. vaginalis	2
L. fermentum	1
L. delbrueckii	1

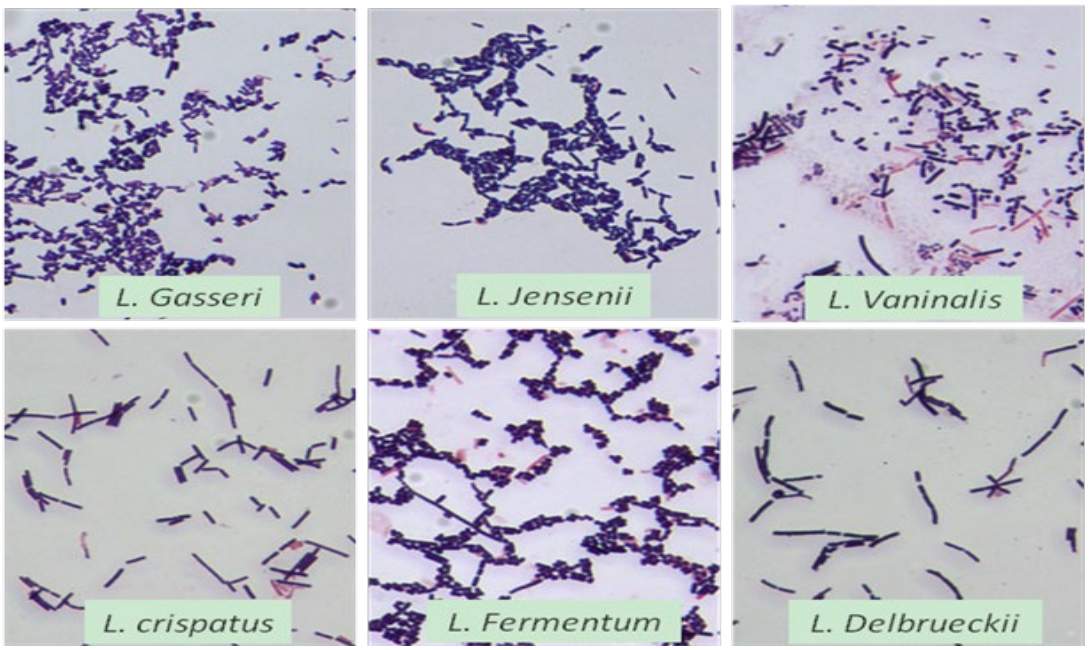

Figure 1: Lactobacillus strains isolated from healthy Chinese athletic women women (1000×)

As shown in Figure 2(2a, 2b, 2c, 2d, 2e, 2f), the six lactobacillus strains had a rapid growth before 12~15 hours after inoculation. The Exponential growth phase of these lactobacillus strains (including Lactobacillus crispatus, Lactobacillus delbrueckii, Lactobacillus jensenii, Lactobacillus vaginalis and Lactobacillus gasseri) was from 3 hours to 15 hours after inoculation. Whereas the Exponential growth phase of Lactobacillus fermentum was from 3 hours to 12 hours after inoculation.

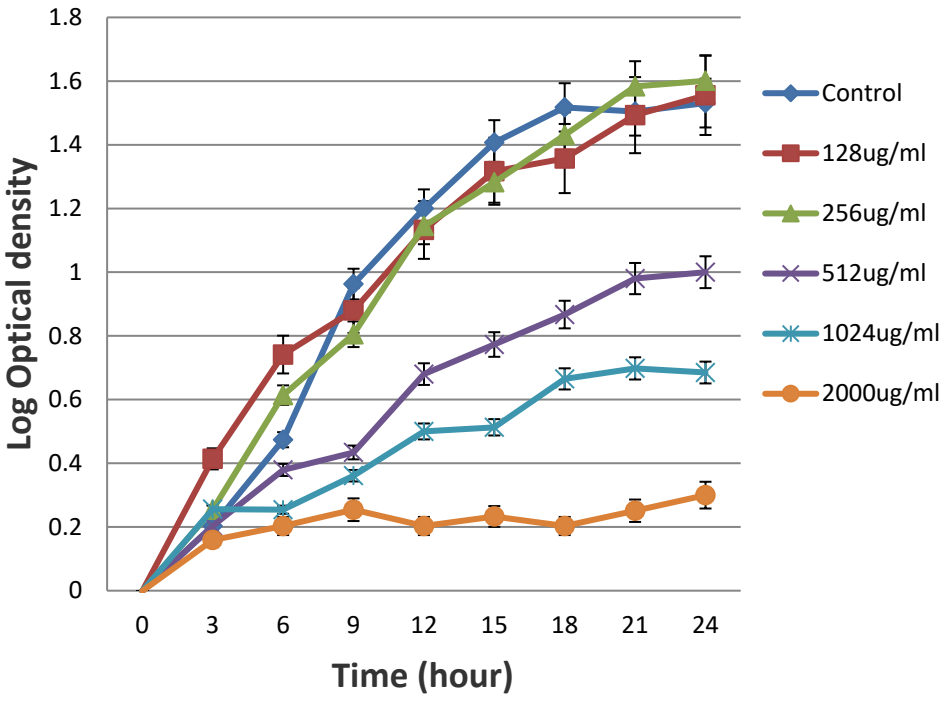

Figure 2a. Proliferation curve of L. Crispatus

Table 2a Comparison of L. Crispatus proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.52±0.03
128μg/ml	1.55±0.04
256μg/ml	1.60±0.05
512μg/ml	1.00±0.03*
1024μg/ml	0.68±0.02*
2000μg/ml	0.03±0.01*
F	4.031
P	0.026

Table Note: *Compared with the normal group,*$^{*}P<0.05$。

Significance between Control and 512μg/ml or 1024ug/ml or 2000ug/ml ($P<0.05$), no significance between Control and 128μg/ml or 256μg/ml ($P>0.05$).

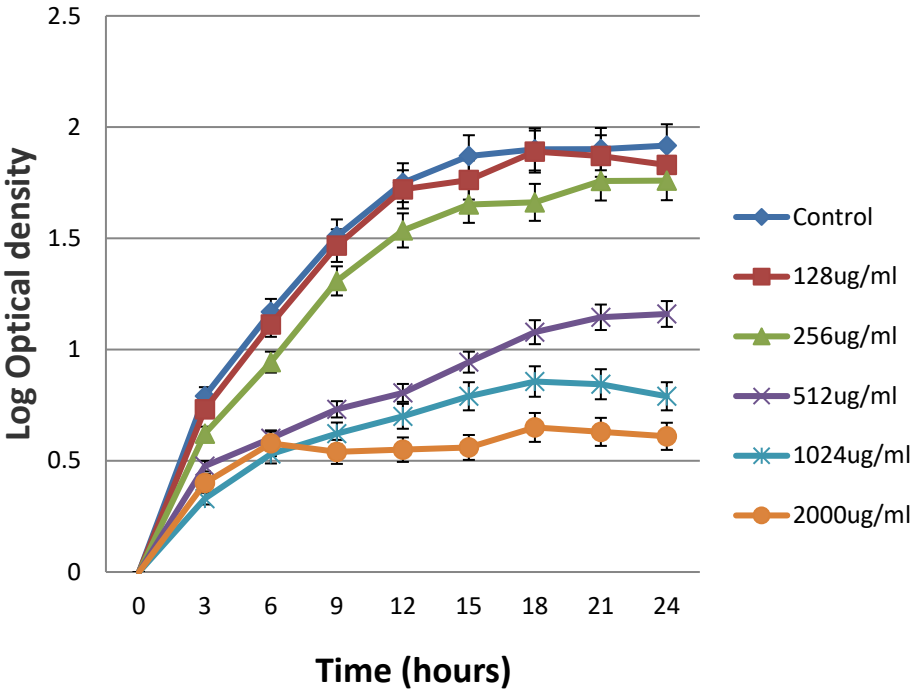

Figure 2b. Proliferation curve of L. Gasseri

Table 2b Comparison of L. Gasseri proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.91±0.05
128μg/ml	1.88±0.04
256μg/ml	1.80±0.04*
512μg/ml	1.23±0.03*
1024μg/ml	0.74±0.02*
2000μg/ml	0.57±0.02*
F	5.012
P	0.003

Table Note: *Compared with the normal group,* $^{*}P<0.05$。

No significance between Control and 128μg/ml($P>0.05$), Significance between Control and other groups ($P<0.05$).

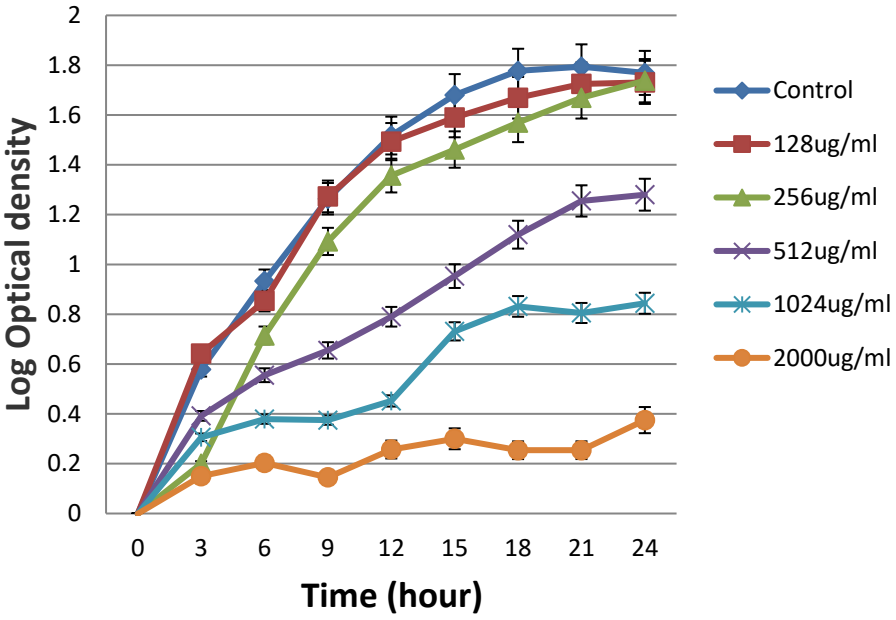

Figure 2c. Proliferation curve of L. Delbrueckii

Table 2c Comparison of L. Delbrueckii proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.82±0.03
128μg/ml	1.78±0.04
256μg/ml	1.70±0.04*
512μg/ml	1.33±0.03*
1024μg/ml	0.81±0.03*
2000μg/ml	0.40±0.02*
F	5.332
P	＜0.001

Table Note: *Compared with the normal group, *P＜0.05。*

No significance between Control and 128μg/ml(P＞0.05), Significance between Control and other groups(P＜0.05).

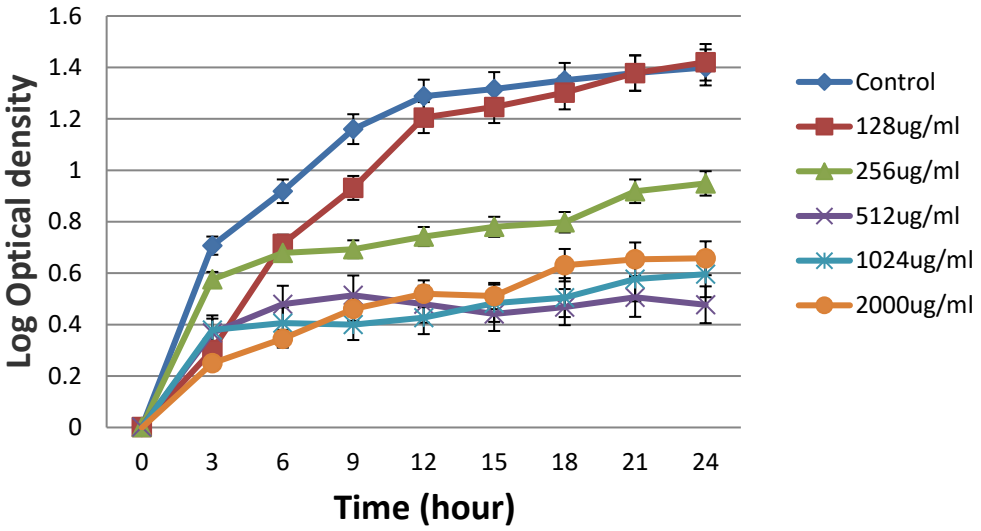

Figure 2d. Proliferation curve of L. Jensenii

Table 2d Comparison of L. Jensenii proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.46±0.05
128μg/ml	1.44±0.05
256μg/ml	0.93±0.04*
512μg/ml	0.49±0.02*
1024μg/ml	0.58±0.03*
2000μg/ml	0.69±0.03*
F	5.115
P	＜0.001

Table Note: *Compared with the normal group, *P＜0.05。*

No significance between Control and 128μg/ml(*P*＞0.05), Significance between Control and other groups(*P*＜0.05).

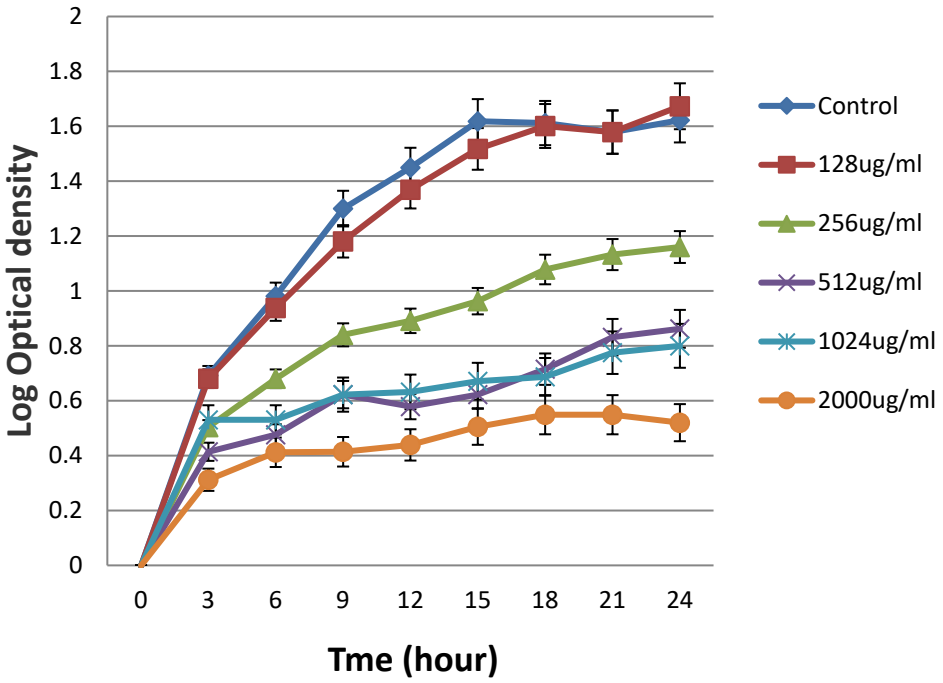

Figure 2e. Proliferation curve of L. Vaginalis

Table 2e Comparison of L. Vaginalis proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.61±0.11
128μg/ml	1.60±0.10
256μg/ml	1.14±0.07*
512μg/ml	0.83±0.05*
1024μg/ml	0.80±0.05*
2000μg/ml	0.56±0.03*
F	5.441
P	＜0.001

Table Note: *Compared with the normal group, *P＜0.05。*

No significance between Control and 128μg/ml(*P*＞0.05), Significance between Control and other groups(*P*＜0.05).

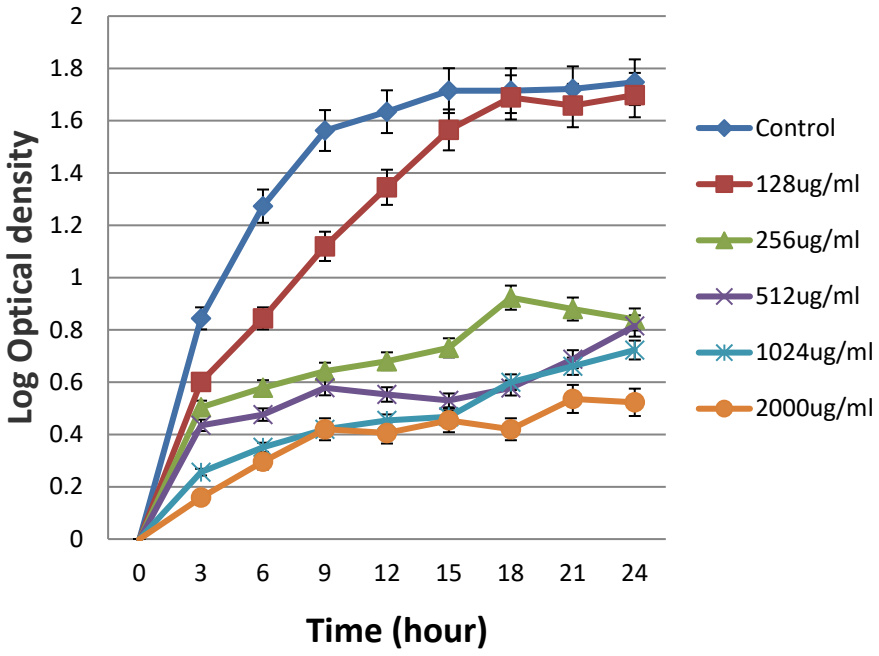

Figure 2 f. Proliferation curve of L. Fermentum

Table 2 f Comparison of L. Fermentum proliferation in different groups

GROUP	LOG OPTICAL DENSITY
control	1.77±0.12
128μg/ml	1.74±0.09
256μg/ml	0.84±0.06*
512μg/ml	0.80±0.05*
1024μg/ml	0.74±0.04*
2000μg/ml	0.52±0.03*
F	5.513
P	＜0.001

Table Note: *Compared with the normal group, *P＜0.05。*

No significance between Control and 128μg/ml(P＞0.05), Significance between Control and other groups(P＜0.05). When the concentration of Metronidazole was no more than 128ug/ml, no statistically significant differences were found between the control and Metronidazole group. When the concentration of Metronidazole was more than 512ug/ml, the difference of inhibition of Metronidazole on the growth of lactobacillus strains was found to be statistically significant between the control and the Metronidazole group.

In addition, various lactobacillus strains had different response to Metronidazole. When the Metronidazole concentrations were 256ug/ml, Lactobacillus delbrueckii, Lactobacillus jensenii and Lactobacillus vaginalis were inhibited apparently, however Lactobacillus cripatus, Lactobacillus gasseri and Lactobacillus fermentumi were not prohibited significantly.

4. DISCUSSION

Unlike previous studies (Odhong' et al., 2019), the present study

prompted that Metronidazole could not stimulate the growth of lactobacilli. In the present study, a series of concentration of Metronidazole were used to make the evaluation, and we concluded that Metronidazole could either inhibit the growth of lactobacilli or have no effect on the growth of lactobacilli, which was similar to conclusions of others researches. However, no data in present study supported that Metronidazole could stimulate the growth of lactobacilli. The difference between present study and previous ones may be result from the following reasons. Firstly, the present study was conducted as a repeated measures design, and the data were analyzed by repeated measures analysis of variance.

Actually the minimum inhibitory concentration (MIC) of an antibiotic was often used as a clinical marker of its effect. However, the MIC is an arbitrary measurement since microorganism growth was determined at only one point in time after 18 hours to 24 hours exposure to a certain antimicrobial concentration. In contrast, microorganism time-kill curves after in vitro exposure to fluctuating antimicrobial drug concentrations more closely reflect the in vivo situation (Bayramov & Neff, 2017) (Trivedi, Lee, & Meibohm, 2013). And repeated measures analysis of variance was a better way to analyze the date from time-kill curve study. As an example, in Jose's study , the stimulation of Metronidazole on lactobacilli was observed when the concentration was between 128 mg/ml and 256 mg/ml. However, the stimulation was seen after 16 hours, while in the first 16 hours the Metronidazole may inhibit lactobacilli (Figure 1 in Jose's study). A repeated measures analysis of variance of the date may yield a better conclusion (Williamson, 2014).

Secondly, due to the fact that bacteria in exponential growth phase grew rapidly only if inhibitory intervention were exerted to them and bacteria in post-exponential phase may resistant to the various of drugs (Kasper et al., 2015), therefore, in present study, lactobacillus strains which were in exponential growth phase were used to make the assessment of Metronidazole in vitro. Thirdly, lactobacillus strains in present study were isolated from Chinese athletic women. Since the character of inheritance, foods, sanitary of different women were various; and the bacteria in these women may experience distinct selective pressure from antibiotics; and these bacteria may have different ability to disseminate antibiotic resistance (Bradshaw & Sobel, 2016).

Vaginal disease is a typical gynecological disease formed in the process of mutual dependence and restriction of the outer microecosystem, host and normal microbiota. The female body has the corresponding microcommunity and micropopulation dynamic change in different age and physiological state. It should be noted that a healthy ecological balance of vaginal flora is a necessary condition to maintain the physiological state of the vagina and prevent the invasion and colonization of numerous frequent

external environmental microorganisms. If this equilibrium state is broken, it will lead to the overgrowth of some microorganisms, the reduction of some microbial communities, and then the imbalance of vaginal microbial state, and lead to inflammation of vaginal mucosa, the appearance of abnormal quantity, color and quality of leucorrhea. In the past, due to the long-term application of broad-spectrum antibiotics and adrenocorticosteroids in clinical practice, mold infection can be greatly increased, which can lead to the imbalance of bacteria in the body, and change the mutual restriction relationship between microbes in the vagina, and the ability of anti-infection is decreased. Bacterial vaginitis is a mixed infection caused by dysregulation of normal bacteria in the vagina. It is caused by Gardella, various anaerobic bacteria and mycoplasma, among which anaerobic bacteria are in the majority.

Bacterial vaginosis is the most common vaginal infectious disease in athletic women of reproductive age. The change of vaginal ecological environment and PH is the cause of vaginal flora disorder. The incidence of bacterial vaginosis is related to the number of previous pregnancies and the number of sexual partners. Previous studies have found that Tibetan athletic women have a high incidence of trichomonad vaginitis, which is closely related to their environment, society, family, personal and psychological factors. Most Tibetan athletic women in China live on a plateau with an altitude of more than 3,000 meters, and their living conditions are poor. Medical conditions in plateau areas are relatively weak compared with urban areas, and athletic women of childbearing age have a low level of education. They know little about the prevention and health care of gynecological diseases, and do not pay enough attention to the health care knowledge of sexual life. Many athletic women have never had gynecological examination. Other reports have pointed out that athletic women of childbearing age have frequent sexual life, have no fixed husband, have weak self-protection consciousness, do not know or are unwilling to take safety protection measures, and have spontaneous abortion and induced abortion due to unmarried pregnancy and multiple pregnancies, resulting in vaginal infection and bacterial vaginitis caused by bacteria imbalance. Therefore, it is necessary to study lactic acid bacteria strains from different female populations.

Although results obtained from in vivo testing are often employed over in vitro because it is better suited for observing the overall effects of an experiment on a living subject, yet it was not easy to prove the mechanism of the overall effects of an in vivo experiment. For example, Metronidazole was used to treat patients with bacterial vaginosis so that to evaluate the effect of Metronidazole on lactobacilli in vivo in Agnew's study. The result showed that after the treatment, the oral and vaginal Metronidazole therapy groups had an increase of vaginal lactobacilli. This result did not mean that Metronidazole could stimulate the growth of vaginal lactobacilli. In Hillier SL's study, in group treated with Metronidazole, lactobacilli were recovered in 83% patients in the

first visit compared with 67% before therapy; while in placebo group lactobacilli were recovered from only 57% patients compared with 65% before therapy. This result prompted that Metronidazole could help to restore the microbiota dominated by lactobacilli. In Agnew's study and Hillier SL's study, the results that Metronidazole may be beneficial to the restoration of vaginal microbiota predominated by lactobacilli, did not mean that Metronidazole could stimulate the growth of lactobacilli. Actually, in the microbiota, the fact that the anaerobic constituents of the microbiota were suppressed by Metronidazole would lead to the increase of lactobacilli. Therefore, in vitro experiments were always needed to demonstrate the mechanism of results in vivo.

As expected, and confirming the results of previous studies, results in the present study showed that the growth of the six Lactobacillus strains in the presence of Metronidazole depended on the concentration of Metronidazole. In the present study, 128ug/ml was a critical point of Metronidazole concentration. When the concentration of Metronidazole was lower than 128ug/ml, it did not affect the growth of lactobacillus strains, while a concentration more than 128ug/ml would inhibit the growth of all the six lactobacillus strains. With the increasing of Metronidazole, inhibitory effect on growth of lactobacilli became more and more significant. Whereas in Jose A. Simoes' study, the critical point of Metronidazole concentration was 512ug/ml. The inhibition of Metronidazole on growth of lactobacilli was not detected when its concentration was lower than 512ug/ml. In Arnold S. Bayer's conclusion, the critical point of Metronidazole concentration was 320ug/ml. Metronidazole did not inhibit the growth of lactobacillus strains when its concentration of was lower than 320ug/ml. The difference of critical point of Metronidazole concentration among various studies may be caused by the fact that lactobacillus trains from athletic women may have diverse characteristics.

Metronidazole was used to treat bacterial vaginosis or trichomonial vaginitis in patients. The recommended regimens include Metronidazole 500mg orally, twice a day for 7 days, or Metronidazole gel 0.75%, 5g (containing 37.5mg of Metronidazole) intravaginally once a day for 5 days, or Metronidazole 2000mg orally, single dose. Oral administration of 500mg or 2000mg produced peak plasma concentration of 12ug/ml or 40ug/ml respectively, and the mean elimination half-life in serum was 14.4 hours after oral administration. However very little information on vaginal concentration after oral or vaginal Metronidazole dosing is available. In another study, 6 hours after a 2000mg oral dose, vaginal concentration of Metronidazole was 26ug/ml. After vaginal administration of 37.5 mg (a 5g applicator dose) of 0.75% Metronidazole gel, the maximal serum concentration was 0.2ug/ml, whereas vaginal concentrations may be 1000ug/ml. So it seems that vaginal administration of Metronidazole may inhibit the growth of lactobacillus, and then destruct the vaginal microbiota. And it may be better to treat bacterial vaginosis or trichomonial vaginitis by oral administration of Metronidazole than vaginal administration of Metronidazole gel. Because according to

recommended regimen, oral administration could not only ensure effective in treating patients, but also avoid the negative effect on lactobacillus and vaginal microbiota. In order to make a reliable extrapolation, our future studies will focus on in vivo experiments on Metronidazole which include the serum and vaginal concentration after oral or vaginal Metronidazole dosing, and testify the presumption that treating bacterial vaginosis or trichomonial vaginitis by oral administration of Metronidazole than vaginal administration of Metronidazole gel.

5. Conclusion:

In conclusion, the study highlights the potential impact of metronidazole treatment on the vaginal microbiome of female athletes. Metronidazole was found to disrupt the growth of vaginal lactobacilli, raising concerns about the implications for vaginal health in this population. Individual variability in the response to metronidazole treatment was evident, suggesting a need for personalized healthcare approaches for female athletes, particularly when antibiotics are prescribed. Proactive monitoring and potential probiotic interventions may help mitigate disruptions in the vaginal microbiome. This research underscores the importance of considering the holistic health of female athletes, including reproductive and vaginal health, alongside their physical fitness. Further studies are warranted to explore optimal antibiotic regimens and strategies to maintain vaginal health in female athletes, ultimately supporting their overall well-being as they pursue excellence in sports.

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Disclosure statement

The authors report no conflicts of interest.

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