

Ketamine used in the therapy of depressive disorders impacts protein profile, proliferation rate and phagocytosis resistance of enterococci

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Abstract

Background: Ketamine is known to cause rapid anti-depressive effect. Additionally, it has been also proved that at high concentrations ketamine inhibits bacterial growth. It is also widely known that even sub-inhibitory concentration of chemicals, as concentration of ketamine used in therapy of depression, may change bacterial properties, including their virulence. The knowledge about possible influence of ketamine on bacterial commensals seems to be essential, as the mechanism of ketamine's action in depression is believed to result also from its' anti-inflammatory activity. In the current study we aimed to evaluate the *in vitro* influence of ketamine on proliferation rate, phagocytosis resistance and toxicity of enterococci.

Results: The studied enterococcal strains were isolated as etiological agents of infection and collected in the Department of Medical Microbiology, Medical University of Gdansk. To measure metabolic activity of *Enterococcus faecalis* 10 μ M of CFDA-SE was added to bacterial suspension. The number of bacterial cells and fluorescence of particles were determined using FACSVerse flow cytometer. Additionally, for the determination of phagocytosis resistance, THP-1 human monocytes cell line from ATCC was used. Suspension of monocytes which engulfed bacteria was then stained with propidium iodide to determine cells' membrane permeability and to evaluate cytotoxicity of enterococci.

The result of the study proved diverse influence of therapeutic concentration of ketamine on Enterococci. In 23.1% of strains both proliferation rate and metabolism activity were inhibited. This group of strains was more susceptible to phagocytosis and had lower cytotoxicity than in culture without ketamine. Different response of isolates to ketamine was also visible in changes of protein profile determined by MALDI TOF.

Conclusions: The analysis of bacteria at early stage of growth curve demonstrate the bacterial diversity in response to ketamine and let us set the hypothesis that microbiome susceptibility to ketamine may be one of the elements which should be taken into consideration when planning the successful pharmacotherapy of depression.

Background

Ketamine's usage as a medicine has a long, over 50-years old, history. At first it was used as an anaesthetic and analgesic agent, but later on its' ability to block NMDA (glutamate N-methyl-D-aspartate calcium channels) receptors was discovered and the usage of the drug in psychiatry was also approved [1]. At first in case of hyperalgesia [2] and schizophrenia [3] but later on also in case of the treatment-resistant unipolar and bipolar depressions [4–6]. Its' rapid action, effectiveness in combating the symptoms, benign and transient adverse effects stay behind its' common, nowadays usage [7].

If we add the quick and effective anti-depressive action of ketamine to its well-proved immunomodulatory [8, 9] and antimicrobial potential [10–12] we need to get into consideration a possible influence of ketamine on the gut bacteria. Even though the dosage of ketamine used in anaesthesiology and anti-

depressive therapy is well below its' minimal inhibitory concentration (MIC) for majority of bacteria [10], the possible influence of ketamine on bacterial growth cannot be ignored. It is widely known that even sub-inhibitory concentration of chemicals, such as antibiotics, can affect many properties of bacteria, e.g. virulence factors' expression or the cells' morphology [13–17]. So far there are only few published results about the potential modulation of antibacterial properties of ketamine used in concentration range specific for therapy of depression. The knowledge about possible influence of ketamine seems to be essential, as mechanism of action of this drug in depression is believed to result from, amongst others, its' anti-inflammatory activity [18]. What is more interesting, some data even suggest that this anti-inflammatory activity is, at least partially the result of interaction between the drug and gut bacteria [19]. Changes of bacteria' virulence, apart from expression of virulence genes and formation of biofilm, include also changes in proliferation rate and the development of so called small colony variants (SCVs). SCVs are considered especially important in case of chronic infections, promoting chronic low-grade inflammation, which is also commonly linked with depressive disorders [20, 21]. In the current study we aimed to evaluate the *in vitro* ketamine's impact on the proliferation, cytotoxicity and phagocytosis resistance of *Enterococcus* when exposed to ketamine in concentrations used in depressive disorders therapy.

Result

Ketamine impact proliferation of enterococcal cells .

Enterococcus faecalis strains shows two opposite resections when it comes to the response to ketamine. Among tested strains, there were those responding by inhibiting their metabolism as well as the strains which in the presence of ketamine boosted their metabolism considerably. In 42.3% of strains the inhibition of their metabolism, measured as CFDA-SE hydrolysis was observed. Within this group, in 23.1% of strains also proliferation rate was lower than in culture without ketamine and susceptibility to phagocytosis mediated by THP-1 cells increased up to 1.51 times while the median cytotoxicity was usually lower.

In contrast to our expectations, the majority of strains (57.7%) at tested concentration of ketamine, increased both proliferation and cellular metabolism. The increase of proliferation varied from 1.23 up to 4.5 times (median 2,45) while increase of metabolism varied from 1.24 up to 5.90 times (Median 2,75) when compared to bacteria not exposed to ketamine. Correlation between induction of proliferation and metabolism rate measured by CFDA-SE hydrolysis has not been proved.

Ketamine affect the proteins' profile in the bacterial strains.

The diverse response of strains to ketamine was also visible in protein profile analysed by MALDI-TOF (Fig. 1).

In detailed analysis, in the spectral profiles of studied enterococci 13 peaks were identified with m/z value of 3420, 3670, 4440, 4780, 5360 6080, 6230, 6400, 6840, 7330, 7570, 8910 and 9540. In both groups of

strains (inhibiting and intensifying the proliferation rate) relative intensity of the peak was the highest at m/z 4440 and this value remained unchanged after incubation with ketamine.

In the strains responding to the exposure to ketamine by increasing the proliferation, most of the proteins increased in amount, decreased peaks intensity were noticed at m/z 5360 and 6400. In the group of strains with inhibited proliferation the intensity of peaks were lower after incubation with ketamine with the exception of m/z value at 4439, 5360 and 9540 (Fig. 2).

Not only proliferation rate and protein profile were different in both groups. Susceptibility to phagocytosis was lower within “boosted” strains. The ANOVA analysis proved decreased cytotoxicity and increased phagocytosis rates in the group of strains inhibited by ketamine. The changes of cytotoxicity and phagocytosis were not as evident in case of strains with increased proliferation (Fig. 3).

Discussion

It is widely known that ketamine inhibits bacterial growth [10, 11], but MIC value usually is above even the concentration used for anesthetic purposes while in the therapy of depression are many times lower. However, in our study, we have identified the strains with unexpectedly heterogeneous response to subinhibitory concentration of ketamine. Bacterial response to ketamine was visible in all observed aspects – proliferation, metabolism rate, and protein profile and was shown to affect cytotoxicity, and the phagocytosis resistance of strains as well. That in itself is not surprising when we take into consideration bacterial (not only *E. faecalis*) diversity in response to many drugs and other chemical agents. When analyzing those diverse responses, we can outline some hypothesis though.

Inhibition of proliferation and metabolism together with increased susceptibility to phagocytosis can be considered as related to the reduced risk of chronic infection. For one, all those factors will affect the effective removal of bacteria from the host body by the immune cells, for others, the persistence of bacterial agents is considered an important factor affecting the development of depression [24]. So such observation may also be treated as an additional, possible explanation of the ketamine’s anti-depressive mode of action. Apart of course from the evident inhibition of NMDA (N-methyl-D-aspartate) receptors [25]. From the other hand significant part of the strains seems to be resistant to ketamine and react in the opposite way, which may be one of possible explanations behind the diverse response to ketamine when used long-term, among the patients with treatment-resistant depression. Observed diversity when it comes to the response to ketamine, could be also explained by the changes in proteins’ profiles. At the moment, it is believed, that the proteins’ profiles reflect bacterial metabolism [26]. The highest intensity peak was observed at m/z 4440 – in both groups of bacteria – inhibited and stimulated by the ketamine. What is interesting – the other groups also reported such occurrence [27].

The fact that *Enterococcus faecalis* phagocytosis mediated by THP-1 cells was diverse when exposed to ketamine, suggest that the ketamine change properties of bacteria. In previous study, the influence of ketamine on phagocytosis was observed when concentration above 100 µg/mL was used [28]. At lower concentrations (but still much higher than those used in our experiments) ketamine inhibited

phagocytosis of *Escherichia coli* and *Staphylococcus aureus* [29]. Some data suggest that the decrease in phagocytic activity is caused by the stimulation of prostaglandin E₂ (PGE₂) production [30]. PGE₂ is one of the chemicals engaged in anti-inflammatory response – Son K-A et al. proved that PGE₂ release led to decreased production of pro-inflammatory cytokines, such as TNF- α , which in turn is responsible for superoxide generation needed for effective oxidative burst in phagocytosis [30].

Based on our results, we set the hypothesis that microbiome susceptibility to ketamine may be one of the elements essential for the successful pharmacotherapy of depression. Although presented results are preliminary it is also clear that mechanisms and consequences of changes observed are the promising target for future studies including influence of ketamine on biofilm formation and survival of bacteria within phagocytes. This study contributes to the evidence for interplay between pro- and anti-inflammatory response with microbiome dysfunction.

We have also proved that this diverse reaction of bacterial cells to ketamine may be noticed only at very early stage of growth curve, later on, the ketamine does not affect the bacteria in any specific way (data not showed).

Conclusions

The results of the study proved the influence of therapeutic concentration of ketamine on cytotoxicity, proliferation and phagocytosis resistance as well as the diversity in the response of enterococcal isolates to ketamine. Considering that fact that such properties of bacteria are related with the risk of chronic inflammation, abovementioned findings might be essential for individualization of the therapy of patients suffering from depressive disorders.

Materials And Methods

Determination of growth rate and metabolic activity changes.

The studied twenty seven enterococcal strains were isolated from various clinical samples as etiological agents of infection and collected in the Department of Medical Microbiology, Medical University of Gdansk. The isolates were identified to species level by strep ID test (BioMerieux, Poland) and classified as different strains of *Enterococcus faecalis* by biochemical and antibiotic resistance profiles.

The growth rate was studied in Brain Heart Infusion Broth (BHI, Oxoid, England). Medium was inoculated with bacterial strains and cultured at 37°C in aerobic conditions with shaking (200 rpm). After 24h, the fresh BHI was inoculated with 10 μ l of bacterial suspension. Each isolate was then incubated at 37°C for 3 hours in two variants: with or without ketamine. The concentration of ketamine used in the experiment was 200 ng/ml – the maximal plasma concentration obtained from patient undergoing anti-depression therapy 40 minutes after infusion [22].

The samples were then centrifuged and pellets were washed three times with saline. To measure metabolic activity of *E. faecalis*, 10µM of CFDA-SE (carboxyfluorescein diacetate succinimidyl ester, Sigma-Aldrich, USA) was added to 200 µl of bacteria suspension of each strain. Mixtures were then incubated for 45 min at 37°C and centrifuged. The pellets were collected and washed three times with PBS (phosphate-buffered saline, Sigma-Aldrich, USA). The number of bacterial cells and their fluorescence were determined using FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA). Effect was measured as the ratio of number of the cells and green fluorescence intensity in culture with ketamine versus culture without ketamine.

Determination of phagocytosis resistance and cytotoxicity of the strains

For the assessment of phagocytosis, THP-1 human monocytes cell line (TIB-202™, ATCC, American Type Culture Collection) was used. The cells were cultured in RPMI-1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% (vol/vol) heat-inactivated foetal bovine serum (FBS) (all from Sigma-Aldrich, USA). For each phagocytosis assay, the enterococcal culture stained with CFDA-SE was used.

Each phagocytic mixture contained 0.4ml of stained enterococci (8×10^7) and 0.4 ml of monocytes (2.5×10^7). The mixture was incubated for 45 min at 37°C. Suspension of monocytes was then stained with propidium iodide (PI, Sigma Aldrich, USA) at room temperature to determine cells' membrane permeability and to evaluate cytotoxicity of enterococci. Emitted fluorescence was determined using a FACSVerse flow cytometer. Differences in phagocytosis effectiveness between strains treated with ketamine and without ketamine were measured as the changes in green fluorescence (for CFDA-SE) and the cytotoxicity of bacterial cells (treated with ketamine vs. not treated with ketamine) was measured as the changes in red fluorescence (for PI).

Determination of protein profile in MALDI-TOFF analysis

To analyze protein profile, BHI medium was inoculated with bacterial strains and cultured at 37°C in aerobic conditions with shaking (200 rpm). After 24h, the fresh BHI was inoculated with 10µl of bacterial suspension. Each isolate was then incubated at 37°C for 18 hours in two variants: with or without ketamine. The samples were centrifuged and pellets were washed three times with saline. The bacterial pellet was applied to a metal plate, and dried at room temperature. Then, 1µl of an α-cyano-4-hydroxycinnamic acid matrix solution (HCCA, Bruker Daltonics) was applied and the sample was left to dry at room temperature. Measurement of the spectrum and comparative analysis with reference spectra of bacteria were performed using MALDI-Biotyper 3.0 (Bruker Daltonics, USA). Mass spectra were collected using FlexControl software (Bruker Daltonics, USA). Then, the spectra were baseline analysed using **mMass** Software, version 5.5.0. [23].

Statistical analysis

The ratio of the number of bacteria and the intensity of fluorescence, expressed as mean fluorescence per particle (green fluorescence/FSC; red fluorescence/FSC), were subjected to the analysis of variance (ANOVA) with Statistica™10 software (StatSoft, Poland). The normality of distribution was verified by χ^2 test and the data were normalized by Box–Cox transformation.

Declarations

- **Ethics approval and consent to participate** : Not applicable
- **Consent for publication**: Not applicable
- **Availability of data and materials**: the datasets used and analysed during the current study are available from the corresponding author on reasonable request.
- **Competing interests**

Tomasz Jarzembowski: none. Agnieszka Daca: none Wiesław J.Cubała M.D., Ph.D. has received research support from Acadia, Actavis, Alkermes, Allergan, Apodemus, Auspex, Biogen, Bristol-Myers Squibb, Cephalon, Celon, Cortexyme, Eli Lilly, Ferrier, Forest Laboratories, Gedeon Richter, GW Pharmaceuticals, Janssen, KCR, Lundbeck, NIH, NeuroCog, Orion, Otsuka, Sanofi, and Servier; he has served on speakers bureaus for Adamed, Angelini, AstraZeneca, Bristol-Myers Squibb, Celon, GlaxoSmithKline, Janssen, Krka, Lekam, Lundbeck, Novartis, Orion, Pfizer, PolfaTarchomin, Sanofi, Servier, and Zentiva; and he has served as a consultant for GW Pharmaceuticals, Janssen, KCR, Quintiles, and Roche. Marek Bronk: none, Łukasz Naumiuk: none

- **Founding**: Department of Psychiatry, Faculty of Medicine, Medical University of Gdansk, Gdansk, Poland
- **Authors contribution**

Tomasz Jarzembowski has designed the conception of the work; analysed and interpreted data; has drafted the work. Agnieszka Daca has acquired, analysed and interpreted data; have drafted the work or substantively revised it. Wiesław J. Cubała has designed the conception of the work; analysed patient data . Marek Bronk has acquired, analysed and interpreted data. Łukasz Naumiuk has acquired, analysed and interpreted data

All authors read and approved the final manuscript

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Figures

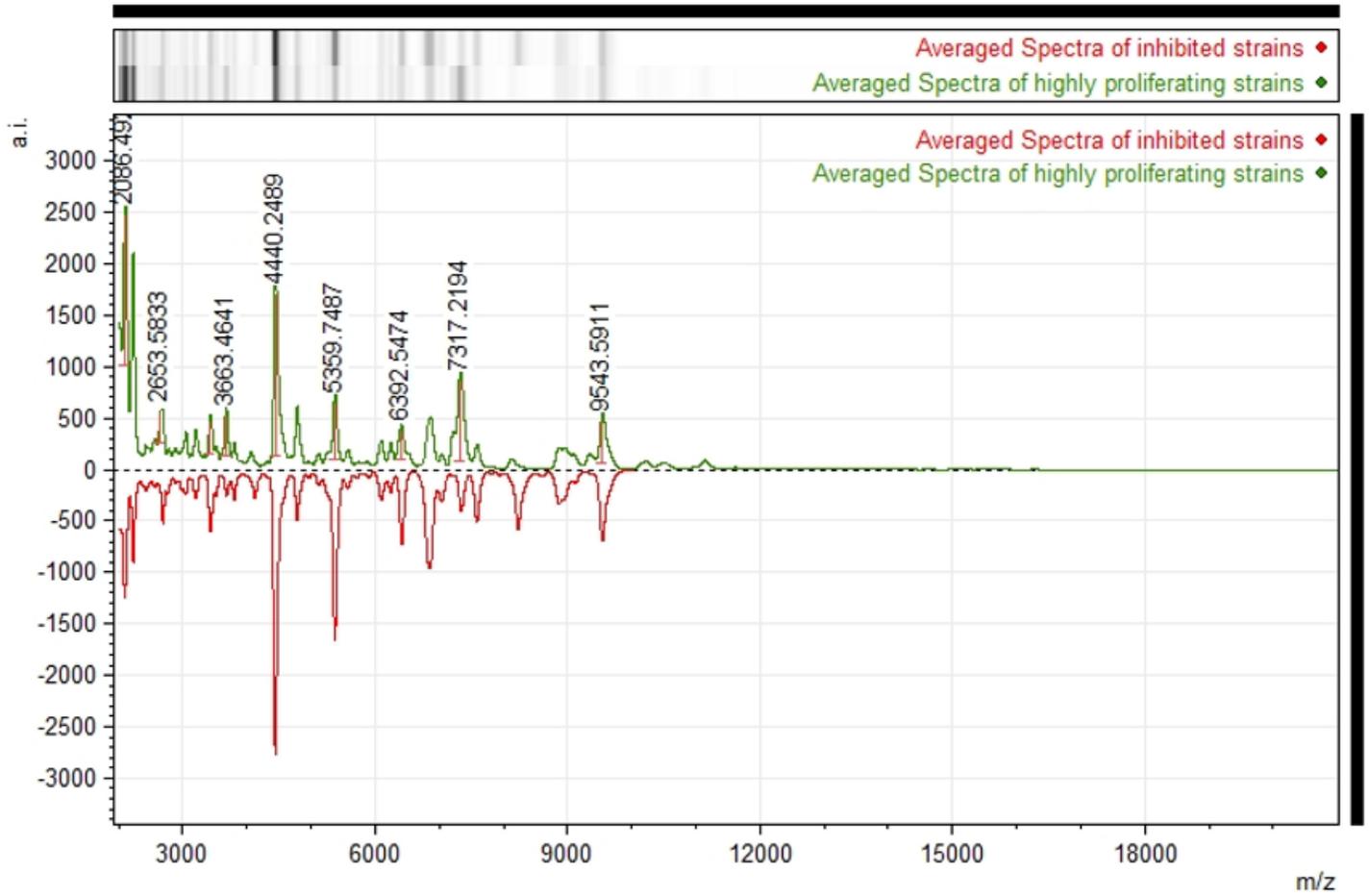


Figure 1

Comparison of the general protein spectra of strains with inhibited and boosted proliferation after addition of ketamine. The peaks are mean values for every strain exposed to ketamine which responded with increase or decrease in the proliferation rate.

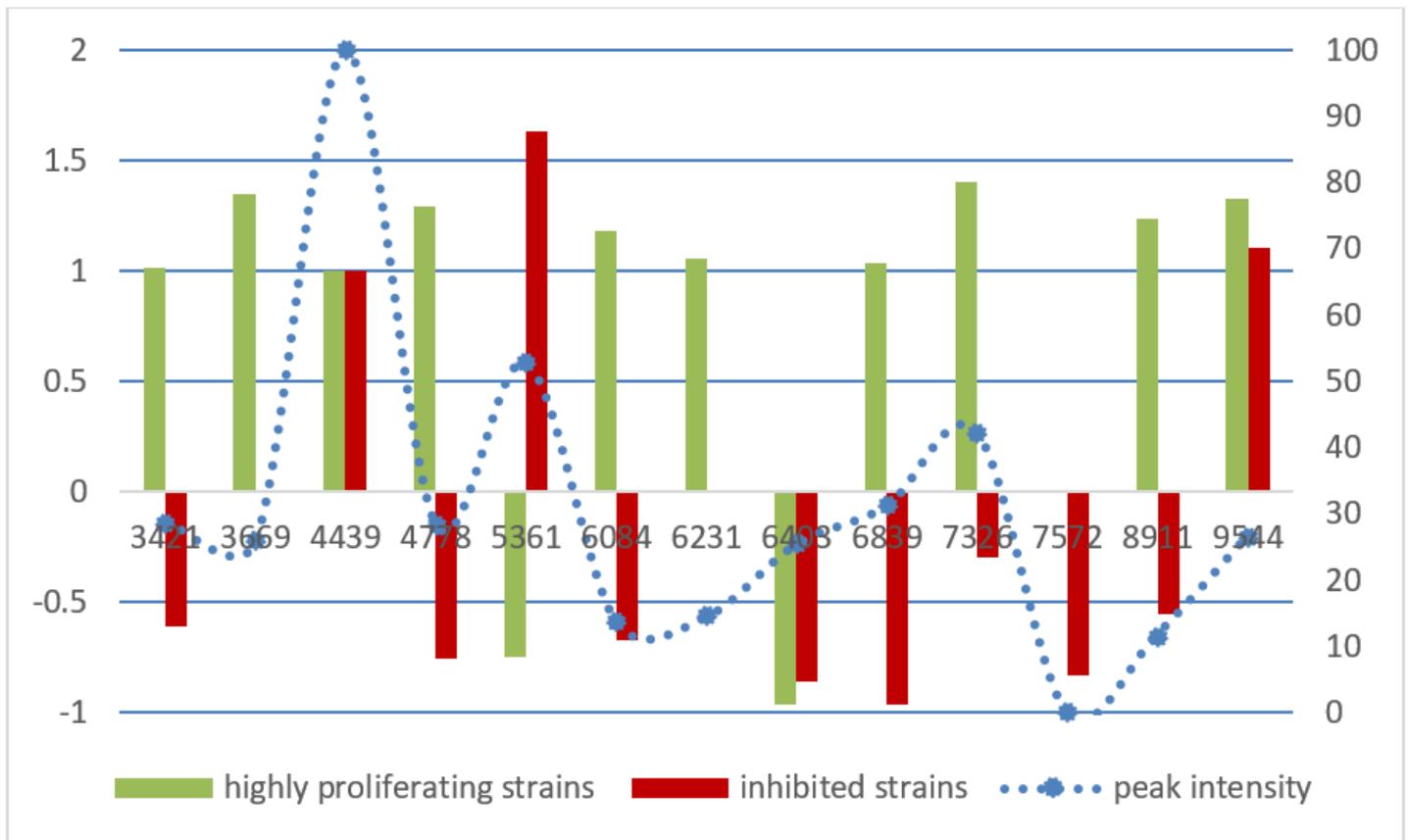


Figure 2

Relative changes of the peaks value after incubation of the particular strains with ketamine (within groups of strains identified by certain response in proliferation). The value 0.00 means no changes in peak intensity when compared with bacteria not treated with ketamine. Left axis shows the ratio of changes for the strains exposed to ketamine when compared with the same strains not exposed to ketamine. Right axis shows the relative amount of proteins in specific peaks (blue dots)

Lambda Wilksa=,55468, F(2, 18)=7,2257, p=,00497

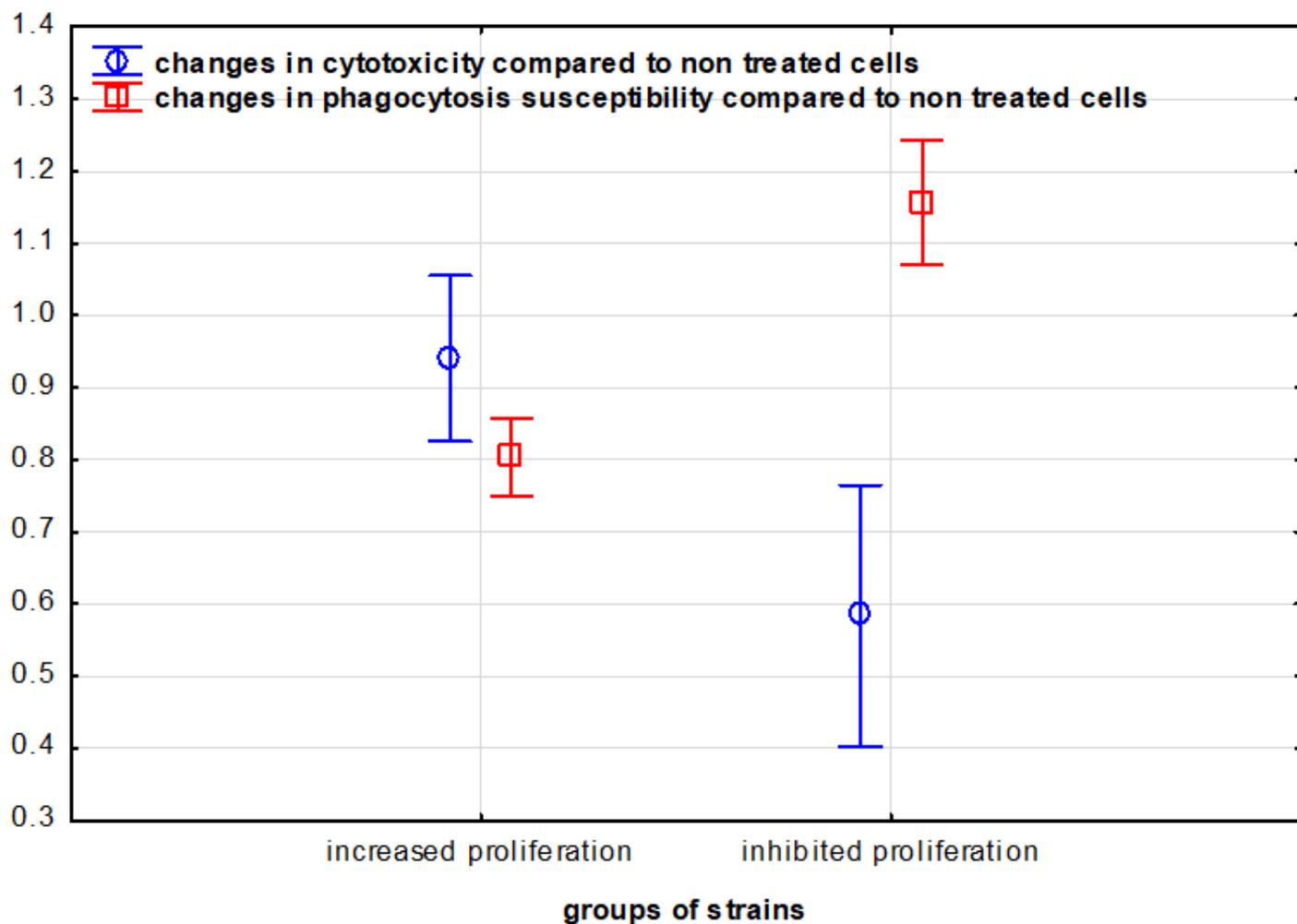


Figure 3

Changes in cytotoxicity and phagocytosis in two groups of *Enterococcus faecalis* strains. The value 1.00 means no changes when compared with bacteria not treated with ketamine.