Assessment of different zinc compounds on the growth kinetics of beneficial commensal bacteria

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Keywords: growth kinetics, lag phases, minimal inhibition concentrations, probiotics, zinc

Abstract

It is known zinc supplementation has a concentration-dependent effect on the growth of the normal microbiome. In the range of 5-10 mg/L concentration, it may stimulate bacterial growth rates, however in larger concentrations (500 mg/L) it may exert an inhibitory effect. The effect of zinc (Zn) supplementation on microbial growth was studied thoroughly using *Bacillus subtilis* ATCC 6633, *Lactococcus lactis* ATCC 19435, and *Saccharomyces cerevisiae* ATCC 2341 isolates. The duration of the lag phases, the obtained maximal growth rates, and the observed maximal growth after 24h of incubation was compared in the presence of $ZnSO_4$, Zn-amino acid complexes, and zinc-EDTA compounds. In parallel, the minimal inhibition concentration (MIC) values of the tested compounds were also determined after 24h of incubation and compared with the results of the growth kinetic studies. Results indicated that some of the tested zinc-compounds in lower concentrations (5-10 mg/L) might stimulate commensal bacteria and yeasts, while in higher concentrations (100-500 mg/L), growth reducing, and even inhibitory effects can be expected. MIC values of the tested zinc-compounds indicated a diverse impact on the tested microorganisms, therefore selecting the optimal zinc supplementation is essential for obtaining the ideal growth of both probiotics and beneficial microbiome of the gastrointestinal tract.

1 Introduction

The animal gastrointestinal tract (GIT) is one of the most complex ecosystems known in nature, containing a diverse microbiota. Within the microbiota, many bacteria play an essential role in metabolic processes and the immune system (Hooper and Gordon, 2001; Tellez and Latorre, 2017). Estimates suggest the animal gut microbiome contains 500–1,000 different bacterial species and outnumbers the host's total number of genes and cells by an estimated 10-fold (Neish, 2009; Qin et al., 2010).

This micro-ecosystem, which is a direct consequence of the mutualism between the host and its microbiota, is fundamental for maintaining a healthy individual (Leser and Molbak, 2009). Commensal bacteria provide the host with essential nutrients. They metabolize indigestible compounds, defend against colonization of opportunistic pathogens, and contribute to developing the intestinal architecture and stimulation of the immune system, among other important processes (Mazmanian et al., 2005). Conversely, the host provides the bacteria with nutrients and a stable environment (Leser and Molbak, 2009). Both host and indigenous microorganisms have adapted to each other in a particular microevolution case to maintain the benefits this mutualism confers (Gareau et al., 2010).

Probiotics are live microorganisms that may confer a growth benefit to the GIT of the host. It has been reported that *Bacillus subtilis* and *Saccharomyces cerevisiae*, as probiotic microorganisms, improved the microbial balance in the GIT of the animal by immune stimulation and competitive exclusion (Chen et al., 2009; Iwashita et al., 2015). *B. subtilis* and *S. cerevisiae*, which have broad activity against animal enteric pathogens (e.g., *Clostridium* spp.), were found to improve the immune status and modulate intestinal microflora, and improve growth performances in animals (Li et al., 2017; Granstad et al., 2020). Lactic acid bacteria have been extensively studied as an effective feed additive in animals for their ability to induce specific mucosal and systemic immune responses (Dawood et al., 2016; Kong et al., 2020). Kobierecka et al. (2016) determined *Lactococcus lactis* significantly reduced enteric pathogen (e.g., *Campylobacter jejune*) colonization in animals.

Zinc (Zn) is an essential metal ion for microorganisms. Zinc has an essential role in bacterial metabolism, as part of many microbial enzymes, such as alcohol dehydrogenase, zinc-dependent proteinases, DNA- and RNA-polymerases, phospholipase C, endopeptidases, and aminopeptidases (Jozic et al., 2002; Chen et al., 2003; Maret, 2013). Zinc deficiency in microorganisms manifests itself by metabolic disturbances and growth depression (Capdevila et al., 2016). Conversely, the antimicrobial effect of Zn is well-known; therefore, a microorganism must precisely regulate intracellular Zn levels (Yang et al., 2012). There are significant differences in susceptibility to Zn among microorganisms and even within strains of individual species.

Current standard antibiotic efficiency of microbial growth efficiency determinations mainly relies on off-line endpoint measurement, such as minimal inhibitory concentration (MIC) (Theophel et al., 2014). However, conventional off-line antibiotic susceptibility testing is insufficient for tracking temporal changes in microbial growth profiles. Growth kinetic models are useful tools in designing and controlling biotechnological processes to obtain improved knowledge about microbial growth kinetics, precisely accurate, and repeatable detailed experiments (Rezvani et al., 2017). Microbial growth is described in specific phases: lag, exponential, stationary, and exponential decay. Nonlinear mathematical models are used to identify growth parameters. The turbidimetric method is also a good alternative used to study bacterial growth since optical density (OD) measurement gives real-time bacterial population values and has practical significance when dealing with bacteria samples in high densities (Dalgaard and Koutsoumanis, 2001). While MIC is suitable for isolated *in vitro* evaluation, differences in the kinetics of growth can make all the difference in a competitive microbial community such as the intestine of humans, monogastric animals, and ruminants. Previous research has investigated the kinetic growth models of *B. subtilis, L. lactis,* and *S. cerevisiae* (Burdett et al., 1986; Rezvani et al., 2017; Olivares-Marin et al., 2018).

While there have been investigations into the influence of $ZnSO_4$ on the growth kinetics of probiotics (Yadav et al., 2011; Surono et al., 2014; Park et al., 2018), there has not been growth kinetic investigations between different zinc compounds (e.g., $ZnNa_2$-EDTA, Zn-EDTA, $ZnGly_2$, $Zn(NH_3)2Gly_2$, $ZnLys_2$ and $Zn(NH_3)2Lys_2$) and three different probiotics (*B. subtilis, L. lactis,* and *S.*

cerevisiae). Therefore, the present study investigates the influence of various zinc compounds on growth kinetics and MIC values of several probiotics under *in vitro* conditions.

2. Materials and Methods

2.1 Microorganisms

Bacillus subtilis ATCC 6633, *Lactococcus lactis* ATCC 19435, and *Saccharomyces cerevisiae* ATCC 2341 were obtained from the American Type Culture Collection (ATCC) and were grown on Nutrient Agar (*B. subtilis*), MRS Agar (*L. lactis*) or Malt Extract Agar (*S. cerevisiae*). Microorganisms were cultivated under aerobic conditions at 37°C.

2.2 Zinc compounds

The following test compounds were used in the trials: $ZnSO_4.H_2O$ (CAS: 7446-19-7, Merck), $ZnNa_2EDTA.4H_2O$ (CAS: 14025-21-9, Merck), Zn-EDTA (CAS: 15954-98-0), $Na_2EDTA.2H_2O$ (CAS: 6381-92-6, Merck), Zn-glycinate.H_2O (CAS: 14281-83-5, Merck) and Zn-lysinate (CAS: 23333-98-4). $Zn(NH_3)_2$-glycinate and $Zn(NH_3)_2$-lysinate were prepared at the laboratory by adding two molar equivalent of NH_4OH to the solution of Zn-glycinate.H_2O and Zn-lysinate.

2.3 Determining growth kinetics in microplates

Growth kinetic measurements were performed in 48-well suspension culture plates (Greiner bio-one, Germany). Wells were filled with 500 µl liquid culture medium (Nutrient Medium, Merck for *B. subtilis*; MRS Medium, VWR Chemicals for *L. lactis* and Malt Extract Medium, Merck for *S. cerevisiae*). Test compounds were added into the liquid medium in 5-500 mg/L concentrations in duplicates. Each trial was performed in triplicates. Triplicate control wells with no treatment and triplicate blank well were included in each test, respectively. For each trial, mean values of the triplicates corrected against respective sterile media on the sample plate are reported. Wells were inoculated with 10 µl cell suspensions of each microorganism, containing 6 x 10^8 CFU/ml cells (McFarland Standard No. 2), resulting in a starting CFU number of 10^6/ml for all microorganisms.

Microplates were incubated with linear shaking at 37°C inside the chamber of the microplate reader (BioTek, Synergy H1), optical densities were read at every 15 minutes at 600 nm and were plotted against time. The time needed to reach the exponential growth phase determined the lag phase. The growth rate is calculated from the exponential phase of the bacterial growth curve, by least squared fitting of linear equation to the OD600 values, which is logarithmically proportional to the number of bacteria. The growth rates are assessed relative to the control well. The OD600 values reached at the stationary phase determined the maximal growth, which is also analyzed relative to the control wells based on the graphic presentation of the results.

2.4 Determining minimal inhibition concentrations (MIC)

MIC determination was performed in 48-well suspension culture plates (Greiner bio-one, Germany) using the European Committee's standard method on Antimicrobial Susceptibility Testing (EUCAST, 2020). Wells were filled with 500 µl liquid culture medium (Nutrient Medium for *B. subtilis*; MRS Medium for *L. lactis* and Malt Extract Medium for *S. cerevisiae*. Compounds were tested in a two-fold dilution in the range of 16,000 – 63 mg/L concentration. Wells were inoculated with 10 µl cell suspensions of each microorganism, containing 6 x 10^8 CFU/ml cells (McFarland Standard No. 2), resulting in a starting CFU number of 10^6/ml for all microorganisms, and the plates were incubated at

37°C for 24h with linear shaking. All compounds were tested in duplicate. Trials were performed in triplicates. Results are presented as mean values. The obtained optical densities were determined with a microplate reader (BioTek, Synergy H1), and MIC values were established as the lowest concentration where microbial growth was inhibited.

2.5 Statistical analysis

Lag phase, relative growth rates, and relative obtained maximal growth for each test compound concentration in each microorganism subjected to one-way analysis of variance as a completely randomized design using the Tibco Statistica software. Significant differences ($P \leq 0.05$) among the means were determined by Duncan's multiple range.

3. Results and discusion

Altogether the effect of eight test compounds in five different concentrations was monitored on the growth of three beneficial microbiome microorganisms, namely *Bacillus subtilis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae.* During the kinetic studies, the length of the lag phase, the growth rate, and the observed maximal growth were determined, as it is demonstrated in Figures 1 and 2. The results of the growth parameters of *L. lactis* in the presence of zinc compounds are summarized in Table 1. The addition of 5-10 mg/L ZnEDTA, 5 mg/L of $Zn(NH_3)_2$-glycinate and 5 mg/L of $Zn(NH_3)_2$-lysinate increased the growth rate, while the addition of 5-50 mg/L ZnEDTA, 5-100 mg/L of $Zn(NH_3)_2$-glycinate and 5-100 mg/L of $Zn(NH_3)_2$-lysinate increased ($P < 0.05$) the obtained maximal growth significantly when compared with control non-treated sample. However, microbial growth was significantly reduced by the addition of 10 (or above) mg/L $ZnSO_4$, 50 (or above) mg/L $ZnNa_2EDTA$, 50 (or above) mg/L Na_2EDTA, 50 (or above) mg/L Zn-glycinate, 100 (or above) mg/L $Zn(NH_3)_2$-glycinate, 100 (or above) mg/L Zn-lysinate and 100 (or above) mg/L $Zn(NH_3)_2$-lysinate. ZnEDTA reduced the microbial growth at only the 500 mg/L concentration. The relative maximal growth rates were significantly reduced by supplementing 50 (or above) mg/L $ZnSO_4$, 100 (or above) mg/L $ZnNa_2EDTA$, 500 mg/L ZnEDTA, 50 (or above) mg/L Na_2EDTA or 500 mg/L Zn-glycinate. Lag phases were elongated in the highest tested concentrations in all cases except for Na_2EDTA, where complete inhibition was observed at the 100 (or above) mg/L concentration; hence, no lag time was established (Table 1). MIC values indicated that Na_2EDTA inhibits the growth of *L. lactis* at 125 mg/L, while $ZnSO_4$ inhibits the growth at 500 mg/L. In the case of $ZnNa_2EDTA$, growth reduction was observed at the 100 mg/L concentration (52% relative growth rate and 62% obtained relative maximal growth). However, the MIC was established at 1000 mg/L. As shown in Table 4, the lowest MIC values were obtained for Na_2EDTA (125 mg/L), while the highest MIC values were obtained for ZnEDTA, $Zn(NH_3)_2$-glycinate, Zn-lysinate, $Zn(NH_3)_2$-lysinate (4000 mg/L) for the *L. lactis* commensal bacterium (Table 4).

Table 2 shows the results of the growth parameters of *Bacillus subtilis* in the presence of zinc compounds. The addition of 5-10 mg/L $ZnNa_2EDTA$, 5 mg/L $Zn(NH_3)_2$-glycinate or 5 mg/L Zn-lysinate increased the growth rate, while the addition of 5-10 mg/L ZnEDTA, 5-10 mg/L $Zn(NH_3)_2$-glycinate, 5 mg/L Zn-lysinate or 5-10 mg/L $Zn(NH_3)_2$-lysinate increased the relative maximal growth significantly. Addition of 100 (or above) mg/L $ZnSO_4$, 500 mg/L $ZnNa_2EDTA$, 100 (or above) mg/L Na_2EDTA, 100 (or above) mg/L Zn-glycinate and 100 (or above) mg/L Zn-lysinate significantly reduced the growth rates. ZnEDTA, $Zn(NH_3)_2$-glycinate, and $Zn(NH_3)_2$-lysinate reduced the growth rate in only 500 mg/L concentrations. The relative maximal growth rates were significantly reduced by supplementing 10 (or above) mg/L $ZnSO_4$, 500 mg/L $ZnNa_2EDTA$, 500 mg/L ZnEDTA, 500 mg/L Na_2EDTA, 50 (or above) mg/L Zn-glycinate, 100 (or above) mg/L $Zn(NH_3)_2$-glycinate, 100 (or above)

mg/L Zn-lysinate and 500 mg/L $Zn(NH_3)_2$-lysinate. In all cases where a significant reduction in either growth rates or relative maximal growths were obtained, the lag phase elongated significantly, indicating the compounds' inhibitory effect on bacterial growth. MIC results (Table 4) obtained for *B. subtilis* indicated that in the case of $ZnNa_2EDTA$, ZnEDTA, $Zn(NH_3)_2$-glycinate and $Zn(NH_3)_2$-lysinate, MIC values are higher (1-2000 mg/L), compared to kinetic studies, where growth limitations were observed in lower, 500 mg/L, concentrations. $ZnNa_2EDTA$ showed a four times lower MIC value than ZnEDTA (1,000 mg/L and 4,000 mg/L, respectively) due to the EDTA compound's sodium salt structural form. Based on the results detailed in Table 4, the lowest MIC values were established for ZnSO4 (250 mg/L), followed by Na_2EDTA, Zn-glycinate, and Zn-lysinate (500 mg/L). In contrast, the highest MIC values were obtained for ZnEDTA, $Zn(NH_3)_2$-glycinate and $Zn(NH_3)_2$-lysinate for the *Bacillus subtilis* probiotic (Table 4).

Table 3 summarizes the results of the growth parameters of *S. cerevisiae* in the presence of zinc compounds. The addition of 5-50 mg/L $ZnSO_4$, 5-10 mg/L $ZnNa_2EDTA$, 5-50 mg/L ZnEDTA or 5-50 mg/L Na_2EDTA increased the relative maximal growth significantly. However, the addition of 500 mg/L $ZnSO_4$, 500 mg/L $ZnNa_2EDTA$, 500 mg/L Na_2EDTA, 500 mg Zn-glycinate or 100 (or above) mg/L $Zn(NH_3)_2$-glycinate reduced the growth rate of the yeast significantly and the addition of 500 mg/L $ZnSO_4$, 500 mg/L $ZnNa_2EDTA$, 500 mg/L Na_2EDTA or 500 mg/L $Zn(NH_3)_2$-glycinate reduced the relative maximal growth of the yeast significantly. Elongation of the lag phase was also observed at higher concentrations of the tested compounds (Table 3). MIC tests (Table 4) indicated inhibitory concentrations for $ZnSO_4$ and $Zn(NH_3)_2$-glycinate at 1000 mg/L, while the rest of the test compounds had higher MIC values (2000-4000 mg/L).

Growth kinetic studies provide a complex set of data and results regarding different compounds' effects on microbial growth. As it is detailed in Table 1-3, larger quantities of the tested zinc-compounds can elongate the lag phase of commensal microorganism, similarly to the effect of antibiotics (Theophel et al., 2014), resulting in differences in the growth kinetic profiles of probiotics, which may also result in reduced efficiency of the applied probiotic supplementation. Based on the type of the supplemented zinc compound as a feed additive, it may have a growth-stimulating or growth-inhibiting impact on the applied probiotic supplementation.

The type of the applied zinc compound has a significant influence on both the MIC and the beneficial microbe's growth kinetics. For example, the MIC and complete kinetic growth inhibition occurred at 500 mg/L of Zn-glycinate for *B. subitilis*. Conversely, $Zn(NH_3)_2$-glycinate only reduced the growth rate to 74% and the relative maximal growth in 24h to 63%, compared to the control with a MIC value of 2000 mg/L. $ZnNa_2EDTA$ at 500 mg/L, for example, reduces the growth rate and obtained maximal growth of *L. lactis* to 31 and 36%, respectively. In contrast, ZnEDTA reduces the growth rate and obtained maximal growth of *L. lactis* to 62 and 66%, respectively. MIC values for $ZnNa_2EDTA$ and ZnEDTA were established at 1000 and 4000 mg/L, respectively, for *L. lactis*. These results indicate that in terms of growth kinetics, $ZnNa_2EDTA$ shows a more substantial inhibitory effect with a four-times lower MIC value. However, the molecular structure of $ZnNa_2EDTA$ chelate differs in only a few atoms from ZnEDTA chelate. These results indicate that even slight changes in the form of the applied zinc compound may result in different MIC values and the tested beneficial microorganisms' growth kinetic behaviors. Therefore, selecting the optimal zinc supplementation is necessary to achieve optimal growth of either the beneficial microbiome or the applied probiotic.

In some cases, lower concentrations of zinc compounds may stimulate probiotics or beneficial bacteria in the gastrointestinal tract, while in larger concentrations, reduction in growth rates or even growth inhibition may occur. Furthermore, this study's results also confirmed that growth kinetic studies

provide more sophisticated data in terms of microbial behaviors and reactions toward different compounds than MIC evaluations; whereby, only endpoints are measured, and other effects on growth are not studied. MIC values provide less information on the potential stimulatory or inhibitory effects of a given compound than dynamic growth kinetic analysis.

Conflict of Interest

Dr. Bata Ltd. employs V. Molnar-Nagy and S. Bata; Vetanco S.A. employs B. Vecchi, and BV Science employs J. Hall and S. Layton. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

V. Molnar-Nagy and S. Bata: Conceptualization, Methodology, and Software. K-H. Tso and B. Vecchi: Visualization, Investigation, and Data curation. V. Molnar-Nagy, S. Bata, J. Hall and S. Layton: Supervision, Writing - Original draft preparation. J. Hall, S. Layton, X. Hernandez-Velasco: Reviewing and Editing. All the authors reviewed, edited, and approved the manuscript.

Funding

This research was supported by the National Research, Development and Innovation Office, Hungary, under project of Funding to SMEs and large companies for RDI activities (2018-1.1.2-KFI).

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317

318 **Table 1.** Growth parameters of *Lactococcus lactis* ATCC 19435 in the presence of zinc compounds.

Test compound	Conc. [mg/l]	LAG [h]	Rel. growth rate [%]	Rel. maximal growth [%]
-	-	6.00	100	100
$ZnSO_4$	5	6.00	97	99
	10	6.00	85*	94
	50	6.00	66*	85*
	100	10.00	13*	77*
	500	11.50	17*	24*
$ZnNa_2EDTA$	5	6.00	102	99
	10	6.00	101	91
	50	6.00	85*	88
	100	7.00	52*	62*
	500	11.00	31*	36*
ZnEDTA	5	6.00	116*	117*
	10	6.00	113*	111*
	50	6.00	102	109*
	100	6.00	91	94
	500	6.25	62*	66*
Na_2EDTA	5	6.25	101	100
	10	6.75	95	87
	50	7.50	62*	68*
	100	-	-*	1*
	500	-	-*	1*
Zn-glycinate	5	6.50	96	111*
	10	6.25	95	105
	50	6.25	79*	104
	100	7.75	75*	100
	500	10.00	63*	67*
$Zn(NH_3)_2$-glycinate	5	6.25	114*	119*
	10	6.25	108	118*
	50	6.50	101	116*
	100	6.50	83*	113*
	500	7.50	66*	103
Zn-lysinate	5	6.25	99	115*
	10	6.25	98	110*
	50	6.75	88	102
	100	8.00	84*	98
	500	11.50	62*	86
$Zn(NH_3)_2$-lysinate	5	6.00	112*	119*
	10	6.00	106	117*
	50	7.00	95	114*
	100	8.25	78*	113*
	500	11.25	72*	106

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320 **Indicates significant difference from the control values $P < 0.05$.*

321

322 **Table 2.** Growth parameters of *Bacillus subtilis* ATCC 6633 in the presence of zinc compounds.

Test compound	Conc. [mg/l]	LAG [h]	Rel. growth rate [%]	Rel. maximal growth [%]
-	-	1.50	100	100
$ZnSO_4$	5	1.50	94	86
	10	1.75	91	75*
	50	2.50	89	75*
	100	6.25	53*	68*
	500	-	-*	2*
$ZnNa_2EDTA$	5	1.50	111*	102
	10	1.50	108*	100
	50	1.50	104	97
	100	1.75	89	91
	500	3.25	43*	38*
ZnEDTA	5	1.50	107	111*
	10	1.50	104	112*
	50	1.50	97	104
	100	1.75	96	100
	500	1.75	75*	71*
Na_2EDTA	5	1.75	106	107
	10	1.75	103	105
	50	1.75	97	98
	100	2.00	83*	91
	500	4.25	-*	3*
Zn-glycinate	5	1.50	101	96
	10	1.50	98	89
	50	1.75	90	69*
	100	2.25	70*	65*
	500	-	-*	-*
$Zn(NH_3)_2$-glycinate	5	1.50	110*	116*
	10	1.50	105	109*
	50	1.75	104	104
	100	1.75	90	74*
	500	2.25	74*	63*
Zn-lysinate	5	1.50	110*	109*
	10	1.50	103	104
	50	1.75	95	93
	100	2.75	72*	76*
	500	-	-*	-*
$Zn(NH_3)_2$-lysinate	5	1.50	107	114*
	10	1.50	104	109*
	50	1.75	98	101
	100	2.75	93	99
	500	3.25	63*	78*

323 **Indicates significant difference from the control values $P < 0.05$*
324

Table 3. Growth parameters of *Saccharomyces cerevisiae* ATCC 2341 in the presence of zinc compounds.

Test compound	Conc. [mg/l]	LAG [h]	Rel. growth rate [%]	Rel. maximal growth [%]
-	-	4,50	100	100
$ZnSO_4$	5	4,50	101	114*
	10	4,50	99	115*
	50	4,50	94	113*
	100	5,25	91	106
	500	8,50	9*	68*
$ZnNa_2EDTA$	5	4,50	104	114*
	10	4,50	103	114*
	50	4,50	102	108
	100	4,75	86	105
	500	7,25	79*	59*
ZnEDTA	5	4,50	107	113*
	10	4,50	102	112*
	50	4,50	99	113*
	100	4,50	92	101
	500	4,75	87	102
Na_2EDTA	5	4,50	102	113*
	10	4,50	100	111*
	50	4,75	105	114*
	100	5,50	87	93
	500	8,75	18*	46*
Zn-glycinate	5	4,50	95	109
	10	4,50	89	103
	50	4,50	95	101
	100	5,25	93	101
	500	7,75	65*	102
$Zn(NH_3)_2$-glycinate	5	4,50	103	106
	10	4,50	96	99
	50	4,50	98	99
	100	4,75	65*	100
	500	5,00	2*	5*
Zn-lysinate	5	4,50	104	100
	10	4,50	96	101
	50	4,50	100	101
	100	5,25	98	97
	500	8,00	98	93
$Zn(NH_3)_2$-lysinate	5	4,50	108	109
	10	4,50	105	107
	50	4,50	102	104
	100	5,00	101	103
	500	6,50	98	95

**Indicates significant difference from the control values $P < 0.05$.*

Table 4. MIC results (mg/L) of the zinc compounds.

Test compound	***Lactococcus lactis***	***Bacillus subtilis***	***Saccharomyces cerevisiae***
$ZnSO_4$	500	250	1000
$ZnNa_2EDTA$	1000	1000	4000
ZnEDTA	4000	2000	4000
Na_2EDTA	125	500	2000
Zn-glycinate	1000	500	2000
$Zn(NH_3)_2$-glycinate	4000	2000	1000
Zn-lysinate	4000	500	4000
$Zn(NH_3)_2$-lysinate	4000	2000	2000

Endpoint MIC studies were performed with the eight test compounds in the concentration range of 63-16,000 mg/L, with using a two-fold dilution method (EUCAST, 2020).