Article

Effects of Different Nutritional Zinc Forms on the Proliferation of Beneficial Commensal Microorganisms

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Abstract: This study compared the minimal inhibition concentrations (MICs) and their effects on the growth kinetics of seven different types of zinc (Zn) compounds and Na_2EDTA in the case of three typical commensal beneficial microorganisms (*Bacillus subtilis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae*). The seven Zn compounds included $ZnSO_4$, four Zn–amino acid chelates, and two Zn–EDTA complexes. Both MICs and growth kinetic parameters indicated that different microorganisms show different sensitivities; for example, *B. subtilis*, *L. lactis*, and *S. cerevisiae* were most sensitive to $ZnSO_4$, Na_2EDTA, and $Zn(NH_3)_2(Gly)_2$, respectively. Both ZnEDTA and $Zn(NH_3)_2(Lys)_2$ improved the growth rate of all beneficial commensal intestinal microorganisms at low concentrations (5–10 mg/L) and showed low toxicity towards all tested strains. At higher concentrations (100–500 mg/L), all compounds decreased the growth rate and increased the lag phase. In conclusion, both growth kinetic parameters and MICs tested effectively measured the inhibitory effects of the test materials; however, growth kinetics provides a more detailed picture of the concentration-dependent effects and those on the mechanisms of microbial growth inhibition.

Keywords: commensal beneficial microorganisms; growth kinetics; minimal inhibition concentration; zinc

1. Introduction

The gastrointestinal tract (GIT) is one of the most complex ecosystems known in nature, containing a diverse microbiota [1]. Within the microbiota, many microorganisms play essential roles in metabolic processes and in the modulation of the immune system [2,3]. Estimates suggest that the gut microbiome contains 500–1000 different species of microorganisms and that it outnumbers the host's total number of genes and cells by an estimated 10 fold [4,5]. The microecosystem of the GIT, which is a direct consequence of the mutualism between the host and its microbiota, is fundamental to maintaining a healthy individual [6]. The intestinal tract of humans and animals hosts a high and diverse number of different microorganisms, including bacteria, fungi, protozoa, and viruses [7]. The relationship between the gut microbiota and host can be commensal, pathogenic, a harmless co-existence, and mutualistic [8]. Intestinal commensal microorganisms can supply essential nutrients, synthesize vitamins, aid in the digestion, and promote angiogenesis and enteric nerve function [9–11]. In exchange, the host provides the microorganisms with nutrients and a stable environment [6]. Both host and indigenous microorganisms adapted to each other in a particular microevolution case to maintain the benefits this mutualism confers [12]. It has been reported that *Bacillus subtilis*, *Lactococcus lactis*, and *Saccharomyces cerevisiae* improved the microbial balance in the GIT by immune stimulation and by competitive

exclusion, and thus can be considered as beneficial microorganisms [13–15]. *B. subtilis, L. lactis,* and *S. cerevisiae* are present in the GIT of animals as well and are considered generally-recognized-as-safe (GRAS) organisms by the Food and Drug Administration (FDA) [16,17]. Both *B. subtilis* and *S. cerevisiae* have broad anti-competitive activity against animal enteric pathogens (e.g., *Clostridium* spp.), and were found to improve the immune status, modulate the intestinal microflora, and promote growth performance in animals [18,19]. Kobierecka et al. [20] determined that *Lactococcus lactis* significantly reduced enteric pathogen (e.g., *Campylobacter jejune*) colonization in animals.

Zinc (Zn) is an important trace element for humans and animals and a required dietary supplement for livestock (e.g., poultry and pigs) as a premix form. Zn is essential for microorganisms as well due to its role in their metabolism as part of many enzymes, such as alcohol dehydrogenase, Zn-dependent proteinases, DNA and RNA polymerases, phospholipase C, endopeptidases, and aminopeptidases [21–23]. Zinc deficiency in microorganisms manifests itself in metabolic disturbances and growth depression [24]. Conversely, the antimicrobial effect of excess Zn is well-known; high dietary ZnO levels (i.e., 2000–3000 mg Zn/kg) have been shown to reduce diarrhea and improve growth performance in weaning pigs [25,26]. The susceptibility to Zn, especially to ZnO and $ZnSO_4$, among microorganisms and even within strains of individual species can be highly variable [27–29]. Consequently, all microorganisms must precisely regulate intracellular Zn levels.

Currently, standard antibiotic efficacy testing relies on off-line, endpoint measurements, such as minimal inhibitory concentration (MIC) determination or the agar diffusion method [30]. However, these test methods do not allow for tracking changes in microbial growth profiles. Growth kinetics is a useful tool for designing and controlling biotechnological processes [31]. Four phases are distinguished in microbial growth: lag, exponential, stationary, and decay. Microbial growth is influenced by many factors, such as temperature, oxygen availability, light, and medium composition including micro- and macroelement concentrations such as Zn [32,33]. Sigmoidal mathematical models can describe the first three phases of the microbial growth curve and identify growth parameters [34]. Microbial growth may be followed by online optical density (OD) measurement, which gives real-time information about the number of cells [35]. While MIC measurements give precise information about the effect of a given molecule in a singular media, variations in growth kinetics may produce differential outcomes in a competitive microbial community, such as the intestine of humans, monogastric animals, or ruminants.

Previous studies investigated the growth kinetics of *B. subtilis, L. lactis,* and *S. cerevisiae* [31,36,37]. However, few in vitro studies are available on the impacts of Zn compounds on beneficial intestinal microorganisms. Therefore, in the present study, we investigated the effects of various formulations and concentrations of Zn (ZnSO4, $ZnNa_2EDTA$, ZnEDTA, $Zn(Gly)_2$, $Zn(NH_3)_2(Gly)_2$, $Zn(Lys)_2$, and $Zn(NH_3)_2(Lys)_2$) on the growth of three commensal beneficial microorganisms (*B. subtilis, L. lactis,* and *S. cerevisiae*). As $ZnNa_2EDTA$ and ZnEDTA are non-regular formulations of nutritional Zn, we sought to dissect the effects of metal and ligands on microbial growth by including Na_2EDTA in the study. In order to contrast the two techniques and completely understand the impact of the different forms of nutritional Zn on the commensal beneficial microorganisms, changes in growth kinetics were compared to the MIC values of the same compounds.

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 (Merck, Darmstadt, Germany), MRS Agar (VWR Chemical, Radnor, PA, USA), and Malt Extract Agar (Merck, Darmstadt, Germany), respectively. Microorganisms were cultivated under aerobic conditions at 37 °C.

2.2. Test Compounds

The test compounds $ZnSO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$ were purchased from Merck (Darmstadt, Germany). The test compound $Zn(Gly)_2$ was purchased from BASF, Animal Nutrition (Mannheim, Germany). The starting materials ZnO, Urea, NH_4OH, Lysine, Glycine, and EDTA were purchased from Merck, (Darmstadt, Germany), and the test compounds $Zn(NH_3)_2(Gly)_2$, $Zn(Lys)_2$, $Zn(NH_3)_2(Lys)_2$, and ZnEDTA were produced according to the procedures described by EP2866799B1. The test compound $ZnNa_2EDTA$ was synthetized by mixing ZnO and Na_2EDTA in equimolar amounts in an aqueous solution.

2.3. MIC

The determination of the MIC was performed in 48-well suspension culture plates (Greiner bio-one, Frickenhausen, Germany) using the European Committee's standard method for antimicrobial susceptibility testing [38]. The wells were filled with 500 μL of liquid culture medium. The compounds were tested in a two-fold dilution at a concentration in the range of 63–16,000 mg/L. The wells were inoculated with 10 μL of cell suspensions of each microorganism, containing 5×10^7 colony-forming units (CFU)/mL, resulting in a starting CFU number of 10^6/mL for all microorganisms. The plates were incubated at 37 °C for 24 h with linear shaking. All compounds were tested in duplicate. After 24 h, the OD (λ = 600 nm) of each well was determined with a microplate reader (Synergy H1, BioTek, Winooski, VT, USA). The highest concentration where microbial growth was absent ($OD_{600} = OD_{600}$ negative control) determined the MIC of the given test compound. Trials were performed in triplicate; however, there was no difference between the replicates.

2.4. Correction between CFU and OD

For each microorganism, the OD_{600} adsorption values were correlated with CFU numbers by the culturing method. A cell suspension was created for each microorganism from the agar plates by suspending two inoculum loops in 1 mL of liquid culture medium. Serial dilutions were created in the growth media in the same 48-well format plate as used for the growth kinetics measurements. The OD values were read at 600 nm using a microplate reader (Synergy H1, BioTek, Winooski, VT, USA). The serial dilutions were further diluted for culturing purposes using the corresponding medium detailed in Section 2.1. Cells were counted after 24 h of culturing at 37 °C. The OD values were correlated with the cell counts. The fitted parameters and the graphical representations of the results are shown in Figure 1. A linear correlation was found between the OD values and the viable cell numbers, as shown by the R^2 values above 0.99. These results are in agreement with previous reports for all three microorganisms: *S. cerevisiae* [39], *L. lactis* [40], and *B. subtilis* [41].

(a)   (b)   (c)
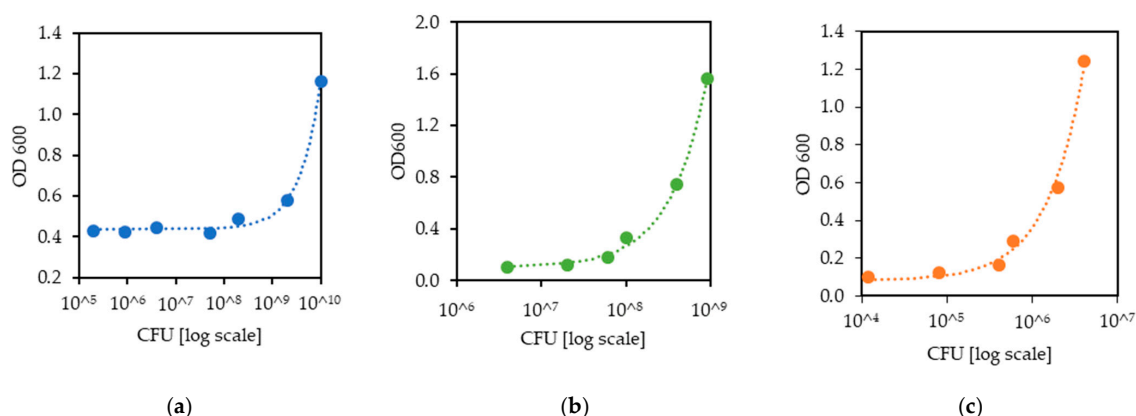
Figure 1. Correlation between OD_{600} and cell number for (**a**) *L. lactis*, for which the regression equation is $OD_{600} = 7.257 \times 10^{11}$ CFU + 0.436, where the R^2 is 0.995; (**b**) *B. subtilis*, for which the regression equation is $OD_{600} = 1.622 \times 10^9$ CFU + 0.104, where the R^2 is 0.997; (**c**) and *S. cerevisiae*, for which the regression equation is $OD_{600} = 5.629 \times 10^8$ CFU + 0.082, where the R^2 is 0.991.

2.5. Growth Kinetics

Growth kinetic measurements were performed in 48-well suspension culture plates. Wells were filled with 500 μL of liquid culture medium containing the test compounds in 5–500 mg/L concentrations in duplicates. Each trial was repeated three times. Three positive control wells without test compounds and three blank wells were included in each test. Wells were inoculated with 10 μL of cell suspensions of each microorganism, containing 5×10^7 CFU/mL cells, 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, and OD was read every 15 min at $\lambda = 600$ nm.

The OD was converted to cell numbers using the relationships determined prior. The growth parameters were determined by fitting the Gompertz equation [34] as follows:

$$\left(\ln \frac{N}{N_0} = A\exp[\frac{\mu_m * e}{A}(\lambda - t) + 1] \right) \tag{1}$$

where N is the cell number, N_0 is the starting cell number, and t is for time. The advantage of using the Gompertz equation for the description of microbial growth kinetics is that biological meaning is attributed to the parameters of the equation. The parameters A, μ_m, and λ represent the maximal growth, the maximal growth rate, and the lag phase length. A is proportionate to the number of cells at saturation: the maximal cell density reached given the space and nutrient limitation [34]. The μ_m parameter of the Gompertz equation is the slope of the growth curve that corresponds to the maximum specific growth rate of the microorganism, which is reciprocal to the doubling time of the organism [34]. The λ parameter of the equation shows the lag time of the growth curve, the time needed for the microorganism to become adapted to the conditions and for the exponential phase to begin [34]. All primary data analysis and equation fitting was carried out using R (R version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria). In some cases, growth was absent at the highest tested concentration of 500 mg/L during the 24 h of the test; therefore, equation fitting was hindered, and the parameters could not be determined. In these cases, A and μ_m were assumed to be 0 and λ 24 h, the duration of the experiment.

2.6. Statistical Analysis

The effects of different formulations of nutritional Zn and Na_2EDTA on the growth parameters were compared to the untreated control to obtain a complete picture of the effect of the treatments on the individual growth parameters; therefore, relative values compared to the respective untreated controls were calculated for each parameter. The relative maximal growth (Rel. *A*), relative growth rate (Rel. μ_m), and relative lag phase (Rel. λ) for each test compound (dependent factor) in each concentration (categorical predictor) in each microorganism (categorical predictor) were subjected to one-way analysis of variance as a completely randomized design using the Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Dunnett's multiple range test determined significant differences ($p < 0.05$) among the means. Data were statistically analyzed using the General Linear Models (GLM) procedure (PC-SAS® ver. 9.2, SAS Institute Inc. Cary, NC, USA) following two factorial treatments at the same concentration. Treatment groups consisted of 3 microorganisms (*B. subtilis*, *L. lactis*, and *S. cerevisiae*) × 8 compounds.

3. Results

3.1. MIC

All tested beneficial commensal intestinal microorganisms were less susceptible to ZnEDTA than other Zn compounds or Na_2EDTA (Figure 2). In addition, all tested microorganisms were more sensitive to $ZnNa_2EDTA$ than ZnEDTA except *S. cerevisiae*. The MICs of all tested compounds for the three microorganisms were 500 mg/L or higher, except for Na_2EDTA for *L. lactis* (125 mg/L) and $ZnSO_4$ for *B. subtilis* (250 mg/L). *B. subtilis*, *L. lactis*, and *S. cerevisiae* were most sensitive to $ZnSO_4$, Na_2EDTA, and $Zn(NH_3)_2(Gly)_2$,

respectively. The MICs of $Zn(Gly)_2$ and $Zn(Lys)_2$ were both lower compared to the MICs for $Zn(NH_3)_2(Gly)_2$ and $Zn(NH_3)_2(Lys)_2$ in *B. subtilis*. In contrast, the MICs of $Zn(Gly)_2$ and $Zn(Lys)_2$ were both higher than the MICs of $Zn(NH_3)_2(Gly)_2$ and $Zn(NH_3)_2(Lys)_2$ in *S. cerevisiae*. *B. subtilis* proved to be the most susceptible to all tested compounds (except for Na_2EDTA and $Zn(NH_3)_2(Gly)_2$) with MIC values between 250 and 2000 mg/L. *S. cerevisiae* tolerated the highest concentration of all the tested compounds except for ZnEDTA and $Zn(NH_3)_2(Lys)_2$ with MIC values of 1000–4000 mg/L.
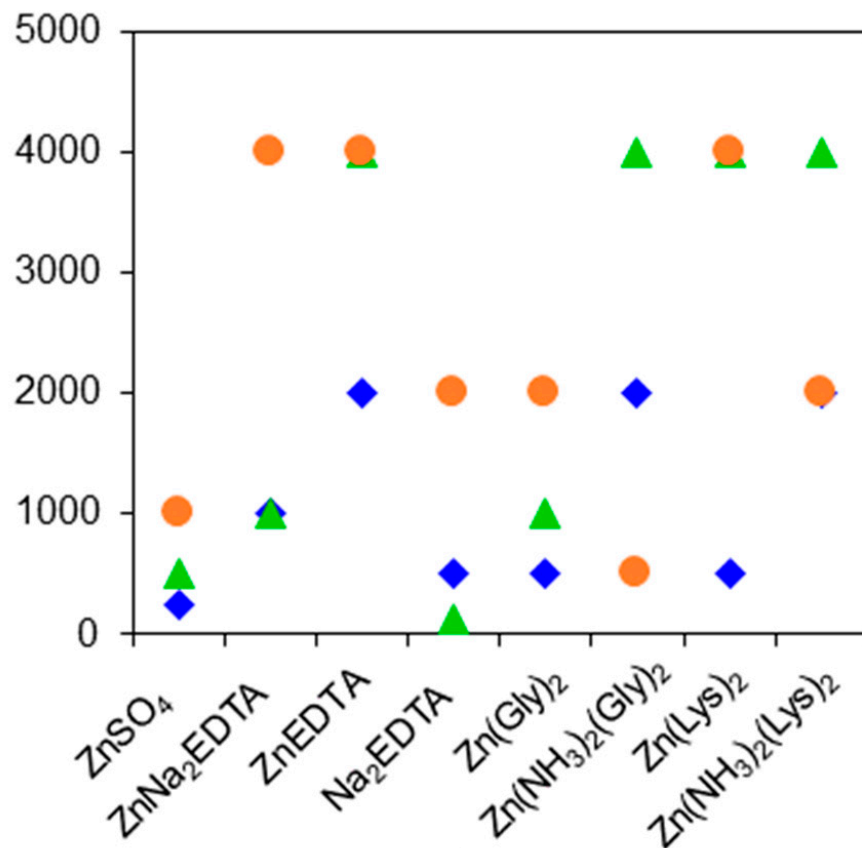
Figure 2. Minimal inhibition concentrations (MICs) of the eight investigated compounds on the three beneficial microorganism strains. MIC values are shown by green triangles for *L. lactis* (▲), blue rhombi for *B. subtilis* (♦), and orange dots for *S. cerevisiae* (●).

3.2. Growth Kinetic Parameters

The effects of increasing concentrations of different Zn compounds and Na_2EDTA on the growth parameters of *B. subtilis*, *L. lactis*, and *S. cerevisiae* are shown in Figures 3–5, respectively. In general, the Rel. *A* and Rel. μ_m decreased with increasing concentrations of most tested compounds in *B. subtilis*, *L. lactis*, and *S. cerevisiae*. In contrast, the Rel. λ increased as the concentrations of most test compounds increased in three tested beneficial microbes. In the case of *B. subtilis* (Figure 3), the Rel. *A*, the Rel. μ_m, and the Rel. λ were not affected by the increasing concentration of $Zn(NH_3)_2(Lys)_2$. Furthermore, the Rel. λ was not affected by the increasing concentrations of $ZnNa_2EDTA$, ZnEDTA, or $Zn(NH_3)_2(Gly)_2$. *B. subtilis* was most sensitive to the increasing concentrations of $ZnSO_4$. In the case of *L. lactis* (Figure 4), the Rel. *A* was not affected by any concentrations of ZnEDTA, $Zn(Gly)_2$, or $ZnLys_2Zn(NH_3)_2Lys_2$. The Rel. λ of *L. lactis* was not affected by the increasing concentrations of $ZnNa_2EDTA$, ZnEDTA, or $Zn(NH_3)_2Gly_2$. *L. lactis* was most sensitive to Na_2EDTA. In the case of *S. cerevisiae* (Figure 5), the Rel. *A* and Rel. μ_m were not affected by any concentration of $Zn(Lys)_2$ or $Zn(NH_3)_2Lys_2$. Furthermore, the Rel. *A* and Rel. λ of *S. cerevisiae* were not affected by the concentration changes in the ZnEDTA

treatment. *S. cerevisiae* was most sensitive to $Zn(NH_3)_2(Gly)_2$. It is noteworthy that the small concentrations of almost all test materials had growth-enhancing effects compared to the untreated control for all three microorganisms, as shown by the Rel A and Rel μ_m values above 100%.

Figure 3. Effect of increasing doses of test compounds on the growth parameters of *B. subtilis* (**a**) relative maximal growth (Rel. A), (**b**) relative growth rate (Rel. μ_m), and (**c**) relative lag phase (Rel. λ). The growth parameters are shown relative to the test-compound-free *B. subtilis* growth parameters. When no growth was observed, A and μ_m were considered to be 0, while λ, the lag phase, was considered to be the duration of the experiment, 24 h.

The effects of seven Zn compounds and Na_2EDTA on the growth parameters of three commensal intestinal microorganisms are shown in Table 1. Compared to Figures 3–5, the results given in Table 1 compare whether there was an interaction between different microorganisms and different compounds at the same concentration. The Rel. A, Rel. μ_m, and Rel. λ were all significantly affected by the species at all tested concentrations ($p < 0.05$), except for 10 mg/L. The type of compound had a significant effect on the Rel. A, Rel. μ_m, and Rel. λ with all tested concentrations ($p < 0.05$). There was a significant interaction between the tested species and compound types ($p < 0.0001$) on the Rel. A, Rel. μ_m, and Rel. λ. From the lowest concentration (5 mg/L) to the highest concentration (500 mg/L), the highest Rel. A was observed in *L. lactis* with the $Zn(NH_3)_2(Gly)_2$ treatment. In contrast, *B. subtilis* treated with $ZnSO_4$ had the lowest Rel. A in all tested concentrations from all tested compounds. In addition, the Rel. A and Rel. μ_m of *B. subtilis* were significantly decreased by $ZnSO_4$ in all tested concentrations ($p < 0.05$), corresponding to an inhibition of growth. The low and moderate concentrations (5–50 mg/L) of ZnEDTA had

the most positive effects on the Rel. *A* of all tested microorganisms ($p < 0.05$). Furthermore, the complete inhibition of microbial growth was observed for all three microorganisms (Rel. *A* and Rel. μ_m indicated zero) for some tested compounds in the highest concentrations of 500 mg/L, such as *B. subtilis* treated with $ZnSO4$, Na_2EDTA, $Zn(Gly)_2$, and $Zn(Lys)_2$, *L. lactis* treated with Na_2EDTA, and *S. cerevisiae* treated with $Zn(NH_3)_2(Gly)_2$. In the case of Na_2EDTA, growth was also inhibited at the 100 mg/L concentration for *L. lactis.* The Rel. *A* of all tested microorganisms significantly decreased ($p < 0.05$) when any of the tested compounds was present at the highest concentration in the culture medium, except for *B. subtilis* treated with $Zn(NH_3)_2(Lys)_2$; *L. lactis* treated with $Zn(NH_3)_2(Gly)_2$ and $Zn(NH_3)_2(Lys)_2$; and *S. cerevisiae* treated with ZnEDTA. *L. lactis* had the highest Rel. *A* and Rel. μ_m at 5–100 mg/L of $Zn(NH_3)_2(Gly)_2$ and $Zn(NH_3)_2Lys_2$, which indicates a beneficial effect on its growth. Regarding the Rel. λ, no significant difference was observed among the three microorganisms treated with the same concentrations of ZnEDTA ($p > 0.05$). The highest Rel. λ of *B. subtilis, L. lactis,* and *S. cerevisiae* was presented in the growth medium containing $ZnSO_4$, Na_2EDTA, and $Zn(NH_3)_2(Lys)_2$ at 100 and 500 mg/L, respectively, which is indicative of longer lag phase.
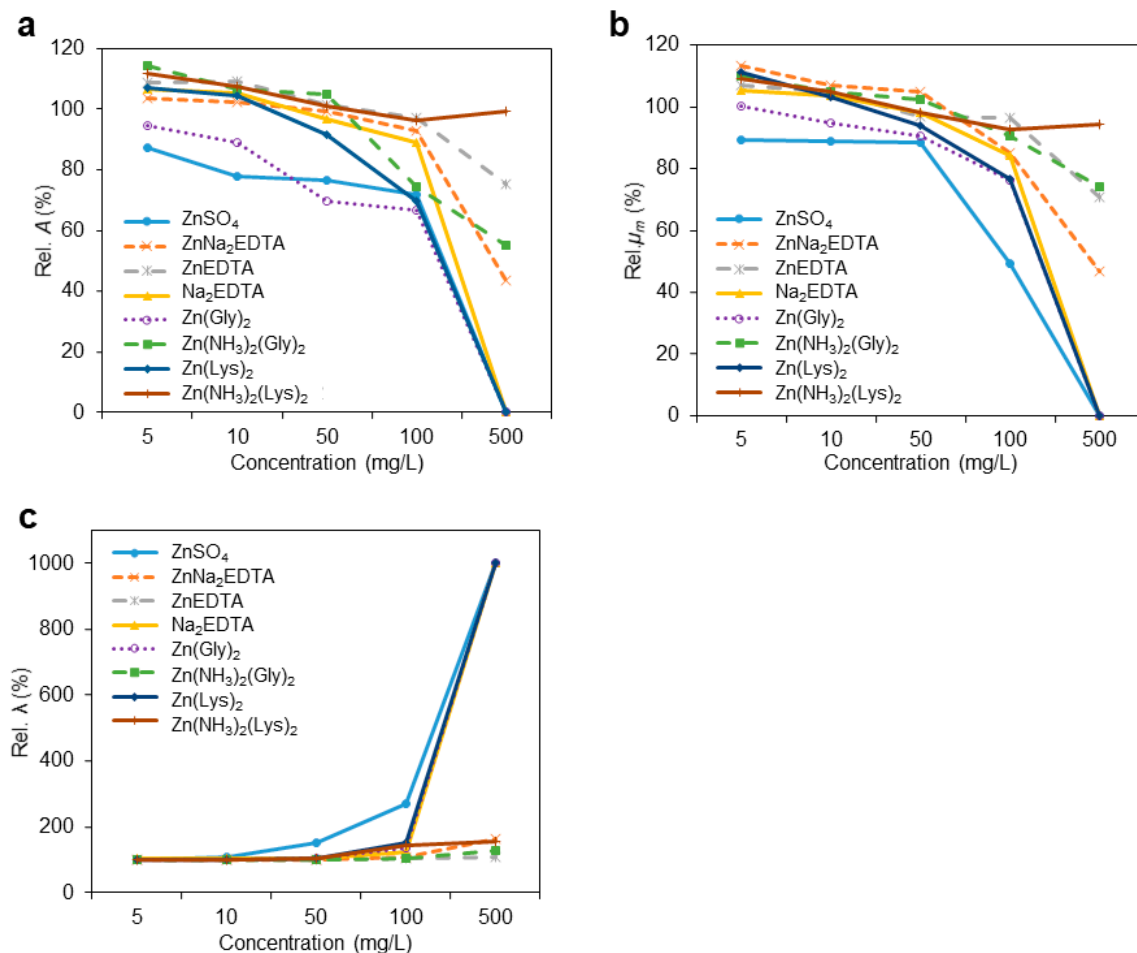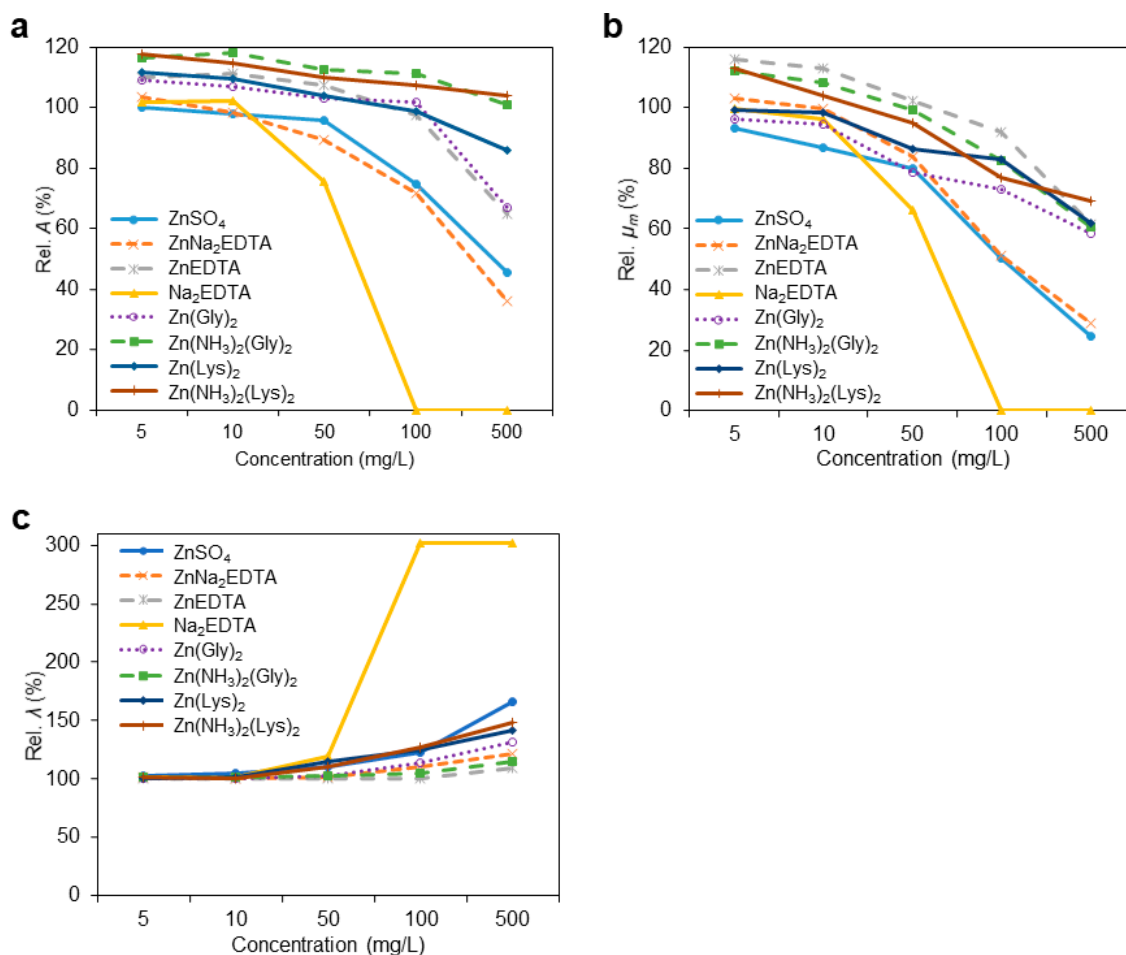

Figure 4. Effect of increasing doses of test compounds on the growth parameters of *L. lactis*, (**a**) relative maximal growth (Rel. *A*), (**b**) relative growth rate (Rel. μ_m), and (**c**) relative lag phase (Rel. λ). The growth parameters are shown relative to the test-compound-free *L. lactis* growth parameters. When no growth was observed, *A* and μ_m were considered to be 0, while λ, the lag phase, was considered to be the duration of the experiment, 24 h.

Figure 5. Effect of increasing doses of test compounds on the growth parameters of *S. cerevisiae*, (**a**) relative maximal growth (Rel. *A*), (**b**) relative growth rate (Rel. μ_m), and (**c**) relative lag phase (Rel. λ). The growth parameters are shown relative to the test-compound-free *S. cerevisiae* growth parameters. When no growth was observed, *A* and μ_m were considered to be 0, while λ, the lag phase, was considered to be the duration of the experiment, 24 h.

Table 1. Effects of different Zn-compounds and Na$_2$EDTA on growth parameters of *B. subitis*, *L. lactis*, and *S.cerevisiae*.

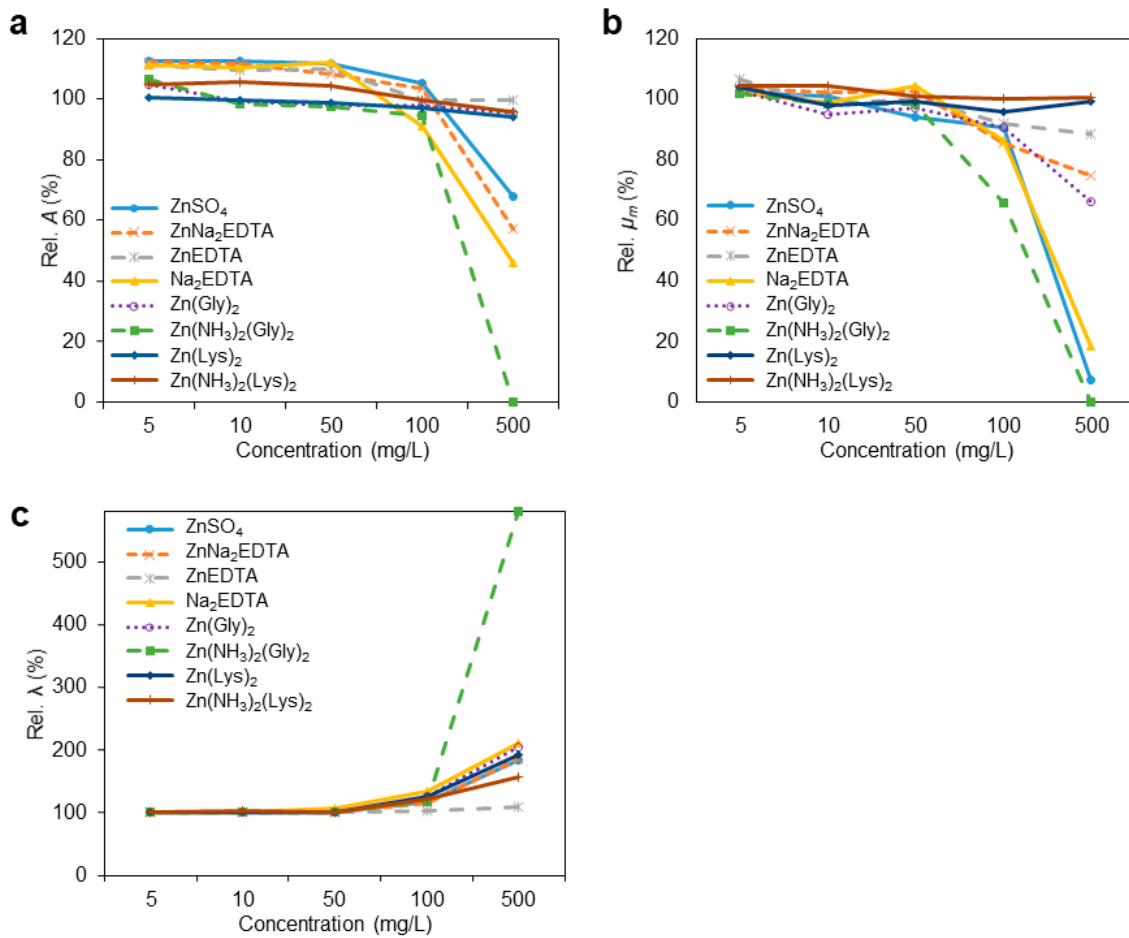| Microorganism (M)	B. subtilis								L. lactis								S. cerevisiae								SEM	p Value		
Compounds (C)	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8		M	C	M*C
5 mg/L																												
[1] Rel. *A*, %	87.5 *i	103.7 *ef	108.7 *d	106.8 *de	94.8 *h	114.2 *b	107.2 *de	111.6 *c	100.1 g	103.4 f	110.8 *cd	101.6 fg	109.0 *d	116.1 *ab	111.3 *cd	117.6 *a	113.6 *bc	113.3 *bc	112.2 *cd	112.6 *cd	105.7 *ef	107.5 *e	101.4 g	105.7 *ef	0.8692	<0.0001	<0.0001	<0.0001
Rel. μ_m, %	89.3 *f	113.1 *b	107.0 *bcd	105.1 cd	100.2 cde	109.9 *bc	111.1 *b	109.0 *bc	93.6 *f	103.0 e	116.1 *a	99.9 e	96.2 ef	112.4 *a	99.3 de	113.0 *a	102.1 ab	103.4 cde	106.7 cde	102.2 cde	102.8 cde	101.9 cde	103.9 cde	104.3 cd	1.6138	0.0264	<0.0001	<0.0001
Rel. λ, %	99.9 b	99.0 b	100.3 b	102.2 a	101.2 ab	100.0 b	100.9 ab	99.7 b	102.4 a	101.0 ab	99.9 b	101.3 ab	100.5 b	101.2 ab	100.8 ab	101.1 ab	100.8 ab	99.8 b	100.6 ab	101.6 ab	101.3 ab	101.7 ab	101.5 ab	101.0 ab	0.5620	0.0392	0.0105	0.0809
10 mg/L																												
Rel. *A*, %	77.8 *i	102.4 ef	109.3 *cd	105.2 *de	88.9 *h	106.7 *de	104.6 *e	107.5 *d	97.8 g	98.0 g	111.1 *c	102.1 f	106.5 *de	117.8 *a	109.5 *cd	114.6 *b	113.7 *bc	112.8 *c	110.5 *c	111.4 *c	100.0 g	99.5 g	100.4 g	106.5 *de	0.8229	<0.0001	<0.0001	<0.0001
Rel. μ_m, %	89.0 *e	107.0 *bc	104.7 bc	103.5 c	94.9 d	104.8 bc	103.2 c	104.8 bc	87.1 *e	99.9 cd	113.3 *a	96.5 d	94.7 d	108.3 *b	98.3 d	104.0 bc	101.1 cd	102.3 cd	98.4 d	99.0 cd	95.1 d	98.2 d	98.0 d	104.6 bc	1.6139	0.0636	<0.0001	<0.0001
Rel. λ, %	108.1 *a	99.8 c	101.1 bc	103.4 b	100.6 c	100.4 c	100.2 c	100.0 c	104.3 *b	100.7 c	100.8 c	101.4 bc	100.4 c	101.0 c	101.5 bc	100.8 c	100.4 c	99.9 c	100.8 c	101.2 bc	101.4 bc	101.2 bc	101.3 bc	102.1 bc	0.5523	0.0520	<0.0001	<0.0001
50 mg/L																												
Rel. *A*, %	76.6 *j	99.2 e	101.6 g	96.6 *k	69.6 *l	104.7 *d	91.7 *h	101.0 ef	95.7 ef	89.0 f	107.0 c	75.5 *k	102.9 de	112.5 *a	103.7 de	109.6 *ab	112.6 *ab	109.2 *c	111.1 *c	113.2 *a	99.2 fg	98.5 fg	99.9 fg	105.4 *d	0.6911	<0.0001	<0.0001	<0.0001
Rel. μ_m, %	88.3 *d	104.9 a	96.7 bc	98.2 bc	90.4 *d	102.4 ab	94.0 cd	98.1 bc	80.3 *ef	83.9 *e	102.4 ab	66.4 *g	78.8 *f	99.6 b	86.5 *de	95.1 c	94.0 *cd	102.5 ab	100.1 ab	104.2 a	97.0 bc	98.2 bc	99.1 bc	101.1 ab	1.5130	<0.0001	<0.0001	<0.0001
Rel. λ, %	150.5 *a	100.4 f	101.6 ef	104.6 *e	103.6 ef	100.9 f	104.3 *ef	104.1 *ef	110.6 *d	101.7 f	100.5 f	119.6 *b	102.5 ef	102.1 ef	114.7 *c	110.0 *d	99.8 f	100.1 f	101.4 ef	107.6 *de	101.5 ef	101.7 ef	101.2 ef	101.4 ef	1.1722	<0.0001	<0.0001	<0.0001
100 mg/L																												
Rel. *A*, %	71.8 *j	92.8 *fg	97.3 e	89.2 *h	66.9 *k	74.3 *i	69.8 *j	96.2 *fg	74.3 *i	71.7 *j	97.1 e	0.0 *l	101.7 d	111.0 *a	98.6 e	107.2 *b	106.1 *c	104.6 *cd	100.9 de	91.9 *gh	99.0 e	95.3 f	98.2 e	100.8 de	0.7979	<0.0001	<0.0001	<0.0001
Rel. μ_m, %	49.4 *f	84.9 *c	96.5 ab	84.1 *c	76.1 *d	90.4 *bc	76.8 *d	92.7 *b	50.5 *f	51.2 *f	92.0 *b	0.0 *g	73.3 *d	82.4 *c	83.2 *c	77.0 *d	90.9 *b	85.4 *c	92.1 *b	86.6 *c	90.8 *b	65.6 *e	95.9 ab	100.1 a	1.5349	<0.0001	<0.0001	<0.0001
Rel. λ, %	269.5 *b	107.3 *k	104.3 *kl	123.3 *g	133.6 *e	103.4 l	150.8 *c	144.5 *d	122.5 *g	110.6 *h	100.7 l	301.5 *a	114.1 *i	104.6 kl	125.0 *f	126.8 *f	117.0 *hi	116.0 *hi	103.6 l	134.2 *e	126.4 *f	117.5 *h	124.6 *fg	120.8 *g	1.0996	<0.0001	<0.0001	<0.0001
500 mg/L																												
Rel. *A*, %	0.0 *k	43.5 *i	75.1 *e	0.0 *k	0.0 *k	55.3 *h	0.0 *k	99.2 b	45.2 *i	36.0 *j	64.5 *g	0.0 *k	66.8 *fg	100.9 ab	85.7 *d	103.5 *a	68.5 *f	57.7 *h	100.7 b	46.2 *i	96.5 *c	0.0 *k	94.9 *c	96.7 *c	0.9675	<0.0001	<0.0001	<0.0001
Rel. μ_m, %	0.0 *l	46.7 *g	70.9 *de	0.0 *l	0.0 *l	74.2 *d	0.0 *l	94.3 b	24.6 *j	28.9 *i	61.6 *f	0.0 *l	58.5 *f	60.7 *f	62.1 *f	69.2 *e	7.4 *k	74.8 *d	88.5 *c	18.3 *j	66.2 *e	0.0 *l	99.1 a	100.6 a	1.1913	<0.0001	<0.0001	<0.0001
Rel. λ, %	1003.2 *a	161.9 *l	107.3 *t	1003.2 *a	1003.2 *a	128.4 q	1003.2 *a	156.4 *m	165.6 *k	121.1 *r	109.0 *t	301.5 *c	131.6 *p	114.8 s	141.7 *o	147.8 *n	182.7 *j	186.7 *i	108.3 *t	211.5 *d	205.1 *e	579.3 *b	192.0 *f	156.9 *m	0.9927	<0.0001	<0.0001	<0.0001

a–t Means without the same superscripts in the same row differ (*p* < 0.05). * Means indicate from the control group (*p* < 0.05). [1] Fitted parameters of the Gompertz equation, A: maximal growth, μm: growth rate, and λ: lag time Representative of compounds from 1 to 8 were ZnSO$_4$, ZnNa$_2$EDTA, ZnEDTA, Na$_2$EDTA, Zn(Gly)$_2$, Zn(NH$_3$)$_2$(Gly)$_2$, Zn(Lys)$_2$, and Zn(NH$_3$)$_2$(Lys)$_2$, respectively.

4. Discussion

Zinc is an essential nutrient for the physiological processes of microorganisms, including DNA replication and protein synthesis [42]. Various studies investigated the bioavailability of Zn in both animal [43] and human nutrition [44]. A high dietary ZnO concentration used to be a common method for the prevention of diarrhea in weaning piglets [45,46]. One substantial effect of high concentrations of ZnO in the nutrition of weaning piglets is the modulation of the GIT microbiota composition [47]. It was shown that free Zn ions and protein-bound Zn correlated with various and partially different parameters of intestinal microbiota in the colon of pigs fed high dietary ZnO concentrations [48]. The major effect is likely exerted by bacteriostatic or toxic effects of Zn in the intestine [47,48]. However, little is known about the effects of different forms and quantities of Zn on the intestinal microbiota. In the present study, measurements regarding growth kinetics and MIC values of water-soluble organic and inorganic Zn sources were performed to better understand which Zn compounds or concentrations could stimulate or inhibit the intestinal microbial growth.

The MIC is a parameter that is widely used to assess the susceptibility of microorganisms to antibiotics and other compounds. It is defined as the lowest antimicrobial drug concentration that prevents the visible growth of the microorganism after overnight incubation. An advantage of the MIC is that it is quantitative, and if standardized procedures are used [38], the values obtained by different laboratories can be compared [33]. Our results confirm that the sensitivities of the three microorganisms to Zn compounds, in the MIC test and in the growth kinetics tests, are in agreement. This result is similar to previous results for essential oils or antibiotics against pathogenic bacteria [49,50]. It is worth noting that the growth kinetic parameters, besides showing the inhibitory effects, can also detect potential beneficial effects at lower concentrations than the MIC; thus, the growth kinetics test can reveal more subtle changes and mechanisms in microbial growth. Researchers may observe subtle dose-dependent effects with changing lag phase, growth rate, and maximal growth density. These details would be missed by only using the endpoint readings of the MIC.

The growth kinetics revealed that ZnEDTA and $Zn(NH_3)_2(Lys)_2$ stimulated the growth of all tested microorganisms, which also tolerated these two compounds most in the MIC tests. It is worth noting that the maximal tested concentration (500 mg/L) of ZnEDTA still enhanced the growth rate of *S. cerevisiae*. Furthermore, the highest MIC value for each tested microorganism was obtained with ZnEDTA. It indicates that ZnEDTA is the least toxic formulation of Zn amongst those tested and has the potential to promote the growth of beneficial commensal intestinal microorganisms. In contrast, both the MIC test and growth kinetics indicated that *B. subtilis*, *L. lactis,* and *S. cerevisiae* were the most sensitive to $ZnSO_4$, Na_2EDTA, and $Zn(NH_3)_2(Gly)_2$ compounds, respectively. Results show that the tested microorganisms were most sensitive to $ZnSO_4$; thus, this compound has the highest potential to inhibit the growth of the beneficial commensal intestinal microorganisms. Results also correlate with previous studies, indicating that Zn from inorganic Zn sources is toxic to microorganisms [47,48]. The high concentrations of all tested Zn compounds also elongated the lag phase of commensal microorganisms, similar to the effect of antibiotics [24], resulting in differences in the growth kinetic profiles of these microorganisms. Our results also found the lag phase to be the least sensitive growth kinetic parameter, while the growth rate was the most sensitive indicator of the effect of the test compounds.

Spears et al. [51] reported that Zn bioavailability in Zn–amino chelates is higher than in $ZnSO_4$ in animals. Many in vitro studies also indicated that Zn–glycinate and Zn–lysinate are more stable than $ZnSO_4$ [52,53]; therefore, $Zn(Gly)_2$ and $Zn(Lys)_2$ chelates may be more available for absorption than $ZnSO_4$ in commensal intestinal microorganisms. In addition to bioavailability, Pieper et al. [54] showed that $Zn(Lys)_2$, as an organic Zn source, promoted the formation of a differentially composed microbiome that was enriched in genes capable of promoting glycan foraging and maintaining the formation of beneficial metabolites. The present study indicated that $Zn(Gly)_2$ and $Zn(Lys)_2$ had equal effects on

the growth kinetic parameters of *L. lactis* and *S. cerevisiae*. Thus, in terms of the overall microbiota, the bioavailability between $Zn(Gly)_2$ and $Zn(Lys)_2$ is not expected to be different. Lin et al. [55] detected no significant differences in growth performance and immune response between $Zn(Gly)_2$ and $Zn(Lys)_2$ in shrimp; this result is consistent with our observations. The growth of *B. subtilis* and *L. lactis* with diamine–zinc–amino acid chelates was increased compared to the use of simple Zn–amino chelates at the same concentrations. The diamine–zinc–amino acid chelates and Zn–amino acid chelates showed similar MIC results; *B. subtilis* tolerated higher concentrations of diamine–zinc–amino acid chelates than Zn chelates. Ammonia complex formation is known to improve the stability and solubility of Zn chelates (US4312815A); thus, it is possible that diamine–zinc–amino acid chelates enhanced Zn bioavailability for the commensal intestinal microorganisms and, as a consequence, stimulated their growth at low concentrations.

The tested microorganisms are beneficial commensal intestinal microorganisms that improve the absorption of nutrients and produce a healthier intestinal system [56]. The interaction of the host and its GIT microbiome is an important and direct factor in gastrointestinal functionality and health condition, including effective digestion, nutrition absorption, and immune status [57]. The growth of *B. subtilis* is easily impaired by the excessively high concentration of Zn in a diet [58]. Damaskos and Kolios [59] also proposed *B. subtilis* could enhance the lactic acid bacterial counts in the GIT of many animals and exert a positive effect on protection against intestinal pathophysiology. The intestinal microbiome limits the growth of pathogens such as *Salmonella*, a mechanism referred to as colonization resistance [60]. Based on the results of previous studies and the current research, it is clear that low concentrations of ZnEDTA and $Zn(NH_3)_2(Lys)_2$ simulate the growth of beneficial intestinal microorganisms, which can improve nutrient availability and promote intestinal health.

5. Conclusions

MICs have been widely used to assess the susceptibility of microorganisms to antimicrobial agents. To our best knowledge, this study is the first research to compare the effects on growth kinetics and MIC values of different members of the commensal beneficial microbiome for various Zn compounds. Our results indicate that growth kinetics provides more sophisticated data in terms of microbial behaviors and reactions toward different Zn-containing compounds and concentrations than MIC evaluations. We quantitatively evaluated the growth of microorganisms in the presence of different Zn compounds by growth kinetics in order to quantify the promotion or the inhibition of growth. Low concentrations of ZnEDTA and $Zn(NH_3)_2(Lys)_2$ promoted the growth of all tested microorganisms; thus, prebiotic effects in vivo may be expected of these two compounds. With increasing concentrations, all test compounds inhibited the growth of these beneficial microorganisms, which were most sensitive to $ZnSO_4$, Na_2EDTA, and $Zn(NH_3)_2(Gly)_2$ for *B. subtilis*, *L. lactis*, and *S. cerevisiae*, respectively. In the future, researchers are encouraged to use growth kinetics studies to investigate the modulatory effects on other microorganisms (such as pathogen and neutral microorganisms) of different Zn compounds.

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