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ORIGINAL PAPER

Lipoic Acid Does Not Affect The Growth of *Mycoplasma hominis* Cells *In Vitro*

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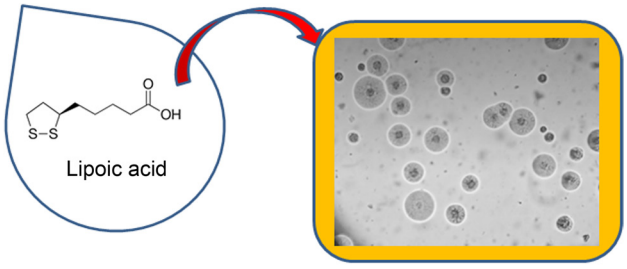
Abstract

Mycoplasma hominis is associated with various infections, for which the treatment can be complex. Lipoic acid (LA) plays a role as a cofactor in eukaryotes, most Bacteria, and some Archea. Research of recent years has increasingly pointed to the therapeutic properties of exogenously supplemented LA. The present study was conducted on 40 strains of *M. hominis* cultured with the following LA concentrations: 1,200 µg/ml, 120 µg/ml, and 12 µg/ml. The bacterial colonies of each strain were counted and expressed as the number of colony-forming units/ml (CFU). The number of CFU in *M. hominis* strains obtained in the presence of LA was compared with the number of CFU in the strains grown in the media without LA. The obtained results indicated that the presence of LA in the medium did not affect the growth of *M. hominis*. The investigation of the influence of LA on the growth and survival of microbial cells not only allows for obtaining an answer to the question of whether LA has antimicrobial activity and, therefore, can be used as a drug supporting the treatment of patients infected with a given pathogenic microorganism. Such studies are also crucial for a better understanding of LA metabolism in the microbial cells, which is also important for the search for new antimicrobial drugs. This research is, therefore, an introduction to such further studies.



effect of action LA	La concetration		
	1200 µl/ml	120 µl/ml	12 µl/ml
Incerease CFU/ml	21 (52.5%)	21 (52.5%)	21 (52.5%)
Decreace CFU/ml	18 (45%)	18 (45%)	17 (42,5%)
CFU/ml unchanged	1 (2.5%)	1 (2.5%)	2 (5%)

K e y w o r d s: *Mycoplasma hominis*, lipoic acid, lipoic acid metabolism in microbial cells, lipoyl carrier protein

Introduction

The species *Mycoplasma hominis* belongs to the genus *Mycoplasma* of the family *Mycoplasmataceae*. These bacteria are atypical bacteria because they do not have a cell wall, so they cannot be detected by the Gram-staining method, and their cells are uniquely small in size (Bébéar 2002; Krijnen et al. 2006). *M. hominis* is associated with various infections, mainly of the genitourinary system (such as cervicitis, pelvic inflammatory disease), and is responsible for infertility, obstetrical pathologies (premature delivery, premature rupture of membranes, chorioamnionitis), and neonatal infections (Koch et al. 1997; Waites et al. 2009; Bergin et al. 2017). Literature data indicate that *M. hominis* can cause also wound infections (Krijnen et al. 2006), meningitis (Zhou et al. 2016), postoperative infections (Whitson et al. 2014; Le Guern et al. 2015; Bergin et al. 2017; Qiu et al. 2017), and other disseminated infections in immunocompromised patients (Meyer et al. 1993; Miranda et al. 2005; Fernandez et al. 2017).

Lipoic acid (1,2-dithiolane-3-pentanoic acid; LA) and its reduced form, i.e., dihydrolipoic acid (DHLA;

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6,8-dimercaptooctanoic) play a role as a cofactor in eukaryotes, most bacteria, and some archaea. LA exists mainly as lipoamide in the living cells, and it is attached with an amide linkage to the ε-amino group of a particular lysine residue on lipoyl carrier protein (LCP). As a cofactor of several enzymatic complexes, of which pyruvate dehydrogenase is best known, LA is an essential component of the energy metabolism of living organisms.

There are two mechanisms by which LCP becomes lipoylated in the living cells, namely, via *de novo* LA biosynthesis, which is an endogenous pathway, and via LA scavenging (or salvage) – an exogenous pathway. Eukaryotic organisms (including humans and other mammals) have essentially only one mechanism of this type, namely the *de novo* LA synthesis pathway (Spalding and Prigge 2010). So, the non-protein bound LA is gained by human and other animal's cells from exogenous sources (drugs, dietary supplements, etc.). Due to its properties, LA is useful in treating numerous chronic and diet-related diseases (Salehi et al. 2019).

The situation is different in prokaryotic cells (but not all) where both of the mechanisms of LCP lipoylation mentioned above are present, that is *de novo* LA biosynthesis and LA scavenging (or salvage) – an exogenous pathway. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (the KEGG database is daily updated and freely available; http://www.genome.ad.jp/kegg/), it is known that, e.g., *Escherichia coli*, *Bacillus subtilis,* and *Staphylococcus aureus* have both pathways of acquiring LA: an endogenous pathway and LA salvage – an exogenous pathway. At this point, however, it should be noted that the results of some authors indicate that preparations containing LA also have antimicrobial activity (Zhao et al. 2018).

So the question arises whether LA is "a friend" to bacterial cells or their "enemy"? We know even less about the bacteria belonging to the family *Mycoplasmataceae*. The LA metabolic pathway in these cells is not well understood. It is unclear whether these cells have two pathways to metabolize LA or only one classical – *de novo* LA biosynthesis. There are no studies to solve this problem. The studies on the LA effect on bacteria multiplication and its bactericidal activity against these cells have not yet been published. The only paper that showed the antimicrobial activity of LA on bacterial cells belonging to the family *Mycoplasmataceae* (these were strains of *Ureaplasma urealyticum* and *Ureaplasma parvum*) was published by Biernat-Sudolska et al. (2020).

Considering the above, the aim of the present study was to determine for the first time whether LA influences the growth and multiplication of *M. hominis* strains. These studies are of a pilot nature.

Experimental

Materials and Methods

General. The study was conducted on 40 strains of *M. hominis*, isolated from the genital tract of women reporting for vaginal microbiota testing to the Microbiological Diagnostics Laboratory of the Department of Microbiology of the Collegium Medicum of the Jagiellonian University in Krakow in the years 2012–2015.

Detection of *M. hominis*. The collected genital secretions were transferred to the R2 BioMerieux transport medium, then. according to the manufacturer's instructions, to the Mycoplasma IST 2 BioMerieux diagnostic strip, and the permanent PPLO media prepared followed the Difco 11th Edition. Mycoplasma IST 2 test results for *M. hominis* were read after 48 hours. In addition, after 48-hour incubation at 37°C, the growth of mycoplasmas on a solid medium was examined microscopically for the appearance of characteristic bacterial colonies. The solid medium consisted of PPLO agar (Difco) with 25% yeast extract, 5% thallium acetate (final concentration of 0.05%), penicillin 500 U/ml, and 10% horse serum. *M. hominis* colonies with agar were excised with a scalpel and stored at –70°C on shaped agar blocks until used in this experiment. Additionally, after 48-hour incubation, R2 medium was used as a DNA isolation material to confirm the presence of *M. hominis* by PCR. To achieve this goal, the R2 medium was centrifuged at 14,000 × *g* for 20 minutes, and the pellet was suspended in 100 µl of distilled water and denatured at 95°C for 10 minutes. The PCR reaction was performed according to the previously described procedure (Biernat-Sudolska et al. 2006). H1 and H2 for 16S rRNA primers were used to detect *M. hominis* described by Luki et al. (1998). DNA from the *M. hominis* sample, strain PG21 (ATCC Cat #23114) was used as a positive control and to check for possible PCR inhibitors in the test sample.

The effect of lipoic acid on *M. hominis*. In the present study, we used a formulation Thiogamma Turbo-Set (Wörwag Pharma, Germany). It is an injection solution containing a racemic mixture of LA as a pharmacologically active substance of 600 mg/50 ml. This formulation is used in Germany in inpatient care as an adjunctive drug in treating diabetes and diabetic complications. The formulation Thiogamma Turbo-Set was diluted with *M. hominis* liquid medium to obtain final LA concentrations of 1,200 µg/ml, 120 µg/ml, and 12 µg/ml. The influence of each obtained LA concentrations on the growth and survival of *M. hominis* was studied. The highest concentration of 1,200 µg/ml used in the studies was also the highest non-toxic concentration of LA for cell culture of the RK13 line, which was previously determined *in vitro*.

Frozen agar blocks with *M. hominis* were thawed by placing in 1 ml of PPLO broth (Difco) containing 25% yeast extract, 25% L-arginine (final concentration 0.05%), 10% horse serum, 5% thallium acetate (up to 0.05% concentration), penicillin 500 U/ml and 0.5% phenol red (up to a concentration of 0.002%). The broth was incubated at 37°C for 48 hours.

After incubation, the liquid culture of each strain was centrifuged (10 minutes/11,000 rpm) to remove any agar residue. The supernatant was discarded, and the pellet was resuspended in 0.6 ml of *M. hominis* liquid medium. An aliquot (0.1 ml) of each *M. hominis* strain was sieved into 0.9 ml of the liquid medium with the addition of three tested concentrations of LA and the liquid medium without the addition of LA as a control. All cultures were incubated at 37°C for 48 hours. After 48 hours, the control and cultures incubated with three concentrations of LA were centrifuged (10 minutes/11,000 rpm), the pellet was resuspended in 1 ml of saline and centrifuged again to remove LA. After the second centrifugation, each pellet was suspended in 100 µl of *M. hominis* liquid medium without adding LA. From this suspension, logarithmic dilutions of –1 to –10 were made in 96-well plates. 10 µl of each dilution were transferred to *M. hominis* solid medium and incubated at 37°C for 48 hours. Then, the bacterial colonies of each strain grown on the medium were counted and expressed as the number of colony-forming units/ml (CFU), assuming that the number of colonies is equal to the number of microorganisms in the sample. In every case, untreated cells were utilized as controls for treated cells. So, the CFU of the cultures grown with LA were compared with those of the control cultures.

Statistical analysis. Statistical calculations were carried out with the IBM SPSS Statistics v.26.0. Statistical software. The differences between groups (comparing CFU in the control samples and CFU achieved after 48 hours incubation with the specific LA concentration) were analyzed using the nonparametric Kruskal-Wallis test.

Results and Discussion

The mean value of log CFU for control bacterial cultures was 6.90 (median 6.77, IQR (5.87; 7.74)), while the mean values of log CFU for bacteria cultured in the presence of LA at a concentration of 1,200 µg/ml, 120 µg/ml, and 12 µg/ml were 6.94 (6.87, (5.87; 7.78)), 6.86 (6.95, (5.81; 7.78)) and 7.17 (7.00, (6.38; 7.93)), respectively. These differences are not statistically significant ($p = 0.807$) (Fig. 1). Thus, the obtained results indicated that the presence of LA did not affect the growth of *M. hominis*.

Based on principal biochemical and microbiological knowledge, it should be hypothesized that LA should rather be "a friend" to bacterial cells than "an enemy" of them. It should be recalled that LA was isolated from bovine liver in 1950. The authors of this discovery – Reed and colleagues, describing their research, pointed out that the crystalline compound obtained turned out to be highly active against the growth of *Streptococcus lactis,* and also an important activator of the pyruvate

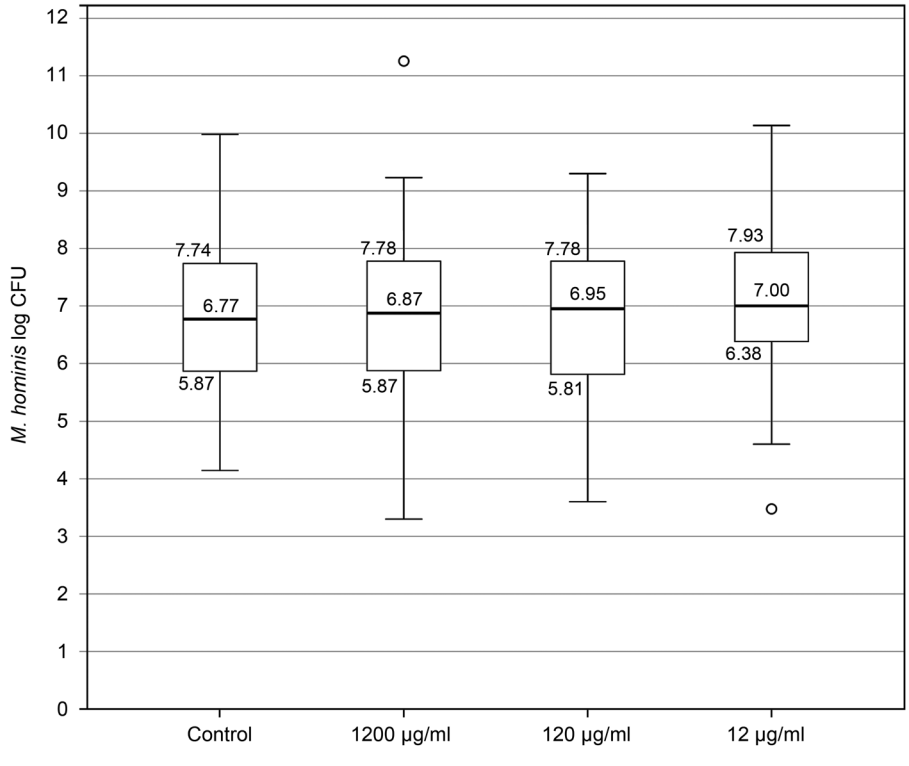

Fig. 1. The graph shows the dependence of the log CFU *Mycoplasma hominis* on LA dose. Mean value, median value, and IQR value are given for each concentration of LA. There were no significant differences ($p = 0.807$) according to the Kruskal-Wallis test.

dehydrogenase of *Streptococcus faecalis*. The researchers called the compound α-lipoic acid (Reed et al. 1951). The results of research published by Kafkewitz and colleagues in 1996 conducted on the selected strains of *Pseudomonas* were also interesting. The authors showed that the addition of a solution containing B vitamins (biotin, folic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, niacin, pantothenic acid, and cyanocobalamin), p-aminobenzoic acid, and LA to the culture medium resulted in a 7–16% increase in the concentration of target compounds (2-chlorophenol, 4-chlorophenol, and 4-chlorobiphenyl) degraded over the incubation period that was required for the concentration of the compound in the cultures to drop to approximately zero (Kafkewitz et al. 1996).

EA.hy 926 is a human cell line that exhibits highly differentiated functions characteristic of the human vascular endothelium. Inoculation of these cells with *Rickettsia rickettsi* resulted in a productive infection. Eremeeva and Silverman (1998) indicated that supplementing the culture medium with 100, 200, and 500 μM LA led to an increase in the viability of the infected EA.hy 926 cells after 96 hours to 45%, 51%, and 70%, respectively, compared with 26% for the samples untreated with LA.

Zorzoli et al. (2016) also obtained exciting results in the murine sepsis model, who in the murine sepsis model due to *S. aureus* infection found that *de novo* biosynthesis or salvage of LA promoted *S. aureus* survival. When both LA biosynthesis and salvage pathways were blocked, then *S. aureus* was rendered avirulent.

Several authors showed the second bacterial-unfriendly "face" of LA. The research of Jariwalla et al. (2008) indicated that supplementation with LA acid might positively impact patients with HIV and acquired immune deficiency syndrome. In addition, it is worth mentioning the preprint by Zhong et al. (2019) on the clinical efficacy and safety of LA for critically ill patients with coronavirus disease 2019 (COVID-19).

Nevertheless, the antiviral activity of LA seems less surprising than its antibacterial activity because LA is an integral part of the energy metabolism of living organisms, including bacteria, as mentioned before. Noteworthy, there are over a dozen studies whose results have revealed the antibacterial properties of LA. We present a few of them here. Among the new methods of antimicrobial therapy, particular consideration should be given to antimicrobial peptides (AMP), including the Bac8c (RIWVIWRR-NH2), the natural AMP exhibiting a high antibacterial activity against Gram-negative and Gram-positive bacteria. Zhou et al. (2020) used LA as a fatty acid hydrophobic ligand to modify Bac8c (LA-Bac8c). The authors reported that minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays showed that LA-Bac8c exhibited lower MIC and lowered MBC values against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) than Bac8c. The authors also demonstrated that LA-Bac8c showed better activity against *S. aureus* and MRSA biofilms, which have been formed or are being formed, than Bac8c (Zhou et al. 2020). It was also shown that LA exerted moderate inhibitory effects against *Cronobacter sakazakii* strains (Shi et al. 2016).

In our previous research on *Ureaplasma urealyticum* and *Ureaplasma parvum*, we observed that LA only at a concentration of 1,200 μg/ml had a statistically significant ($p < 0.001$) inhibitory effect on cell division of *Ureaplasma* strains compared to the control (Biernat-Sudolska et al. 2020).

So, what is the mechanism by which LA displays its "Janus face" in microorganisms? Our hypothesis assumes involvement of sulfane sulfur. Sulfane sulfur is a divalent, reactive, and labile sulfur atom covalently bonded to another sulfur. This form of sulfur, with six valence electrons, occurs in the oxidation state of 0 or −1, and quickly leaves the compound being transferred to various acceptors, including the thiol groups (-SH) of the cysteine residues of proteins. This is another method of covalent modification of proteins, changing their biological activity (Iciek et al. 2018). It is known that in bacteria, the concentration of sulfane sulfur can be higher than 100 μM (Ran et al. 2019). It has recently been demonstrated that sulfane sulfur increases the virulence in *Pseudomonas aeruginosa* PAO1 by targeting its quorum sensing (QS) systems (Xuan et al. 2021). However, we recall the long known antimicrobial activity of garlic, for which, as we know today, diallyl sulfides are responsible, which are the source of sulfane sulfur. For example, it is known that diallyl disulfide (DADS) from garlic reduces the pathogenicity and biofilm development of *P. aeruginosa* PAO1 by targeting its QS systems (Li et al. 2018) "Cognitive dissonance"? Not necessarily. The concentration of DADS used by the researchers cited above was 1.28 mg/ml (about 8.8 mM). Hou et al. (2019), in studies on *E. coli* cells, showed that at high concentrations, sulfane sulfur was toxic to bacteria. Why did we devote so much attention to the problem of sulfane sulfur? Since, in our opinion, the biological activity of LA is also associated with the generation of sulfane sulfur compounds (Bilska et al. 2008; Bilska-Wilkosz et al. 2017).

The second problem that arises when studying the antibacterial properties of LA is its metabolism in these cells, which was mentioned in the Introduction. Unfortunately, the LA metabolic pathway in *Mycoplasma* species cells is not well understood. It is unclear whether these cells have two pathways to metabolize LA. The available data suggest that, like eukaryotic organisms, *Mycoplasma* cells do not have the LA salvage pathway. It would mean that the presence of LA in the medium should not affect the growth of these bacterial cells. The first research on this problem dates back to the 1960s.

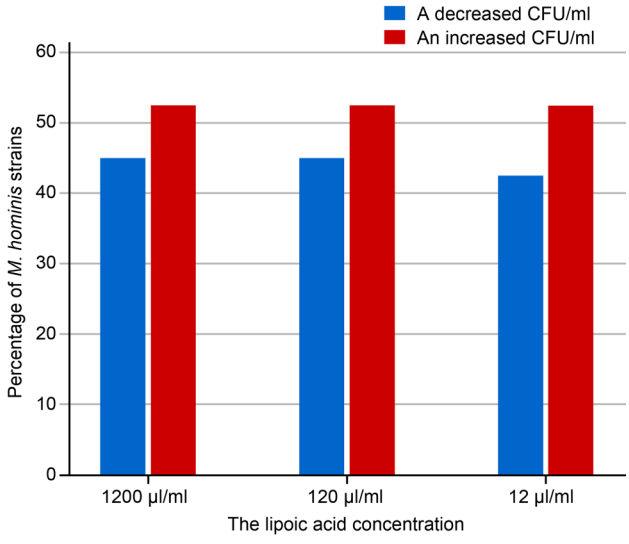

Fig. 2. The graph shows the dependence of the bacterial CFU value on the LA dose. Data are presented as a percentage relative to all strains tested (100%).

The results obtained at that time showed that although the presence of LA in the medium stimulated the growth of *Mycoplasma* strain Y in primary culture, on the other hand, LA had little or no effect on its growth in subculture in the same medium (Rodwell 1969).

Based on our results, none of the above hypotheses can be reliably confirmed or rejected because our data did not reach statistical significance. We did not obtain statistical significance because for 52.5% of *M. hominis* strains, the CFU increased for all tested concentrations of LA. It could indicate that *M. hominis* cells do not have the LA salvage pathway. In opposite, at concentrations of 1,200 µg/ml and 120 µg/ml, a decrease of the CFU was observed for 45% of the tested strains (Fig. 2, Table I). After averaging these values and subjecting the results to statistical analysis, it turned out that LA does not affect the growth of *M. hominis* cells in i*n vitro* studies.

The problem is interesting but requires further research. Perhaps this research should be repeated on the selected strains of *M. hominis*. It seems, however, that the research using the molecular system able to knock out a gene and/or genes relevant to *de novo* LA biosynthesis pathway is needed for a definite answer to this problem. Further studies would also need to show that LA generates sulfane sulfur in *M. hominis* cells and affects the mechanisms and/or structures necessary for cell survival and/or growth. Thus, the study presented here is a modest contribution and an invitation to research on the influence of LA on bacteria from the *Mycoplasmataceae* family. This type of research can provide several answers to questions important for a biochemist, microbiologist, and physician. Taken together, this research is an introduction to further investigations aimed at a better understanding of LA metabolism in the microbial cells, which is also essential for the search for new antimicrobial drugs.

Table I
The effect of lipoic acid on the value of CFU of *M. homins* strains.

The change in the CFU value	Concentration of lipoic acid (µg/ml)		
	1200	120	12
	Number of strains		
increased	21	21	21
decreased	18	18	17
unchanged	1	1	2

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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