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Possible methods for evaluation of hydrogen sulfide toxicity against lactic acid bacteria

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ABSTRACT

An integral part of the intestinal microbiota is undoubtedly formed by lactic acid bacteria (LAB). Their presence in the digestive tract is essential for its proper functioning. During inflammatory bowel disease, such as ulcerative colitis (UC), LAB occurrence is reduced while sulfate-reducing bacteria (SRB) occur widely in the intestine resulting in an increase of their metabolite, hydrogen sulfide. Inhibitory concentration and mechanism of action of hydrogen sulfide on LAB are not fully known yet. The aim of this paper is to find the proper testing methods for evaluation of the interaction between hydrogen sulfide and lactic acid bacteria, including minimal inhibitory concentration (MIC) determination. Spectrophotometric, colorimetric, agar plate methods and combination of these methods were tested. Thousands of people from all over the world are affected by inflammatory bowel disease every year. The gained results could help to understand and improve the stability of intestinal microbiome, improve the treatment of bowel inflammation diseases, or prevent bowel disease altogether.

Keywords: *hydrogen sulfide, toxicity, lactic acid bacteria, sulfate-reducing bacteria.*

1. INTRODUCTION

Lactic acid bacteria, including especially Lactobacillus, Streptococcus, and Bifidobacterium genera, are known to play an important role in the intestinal environment. They are part of the wide group of non-sporing, non-motile, anaerobic or aerotolerant Gram-positive cocci and rods with fermentative metabolism and lactic acid formation as the main end-product [1]. They represent a heterogeneous group of bacteria which could be found in various environments from the human and animal body to plants. Certain specific probiotic strains show positive effects on the intestine which might be useful for the prevention of bacterial translocation and gut infections caused by bacteria [2-4].

Sulfate-reducing bacteria are presented in the intestine consuming the sulfate or sulfite ions as a terminal electron acceptor while sulfide is produced [5-9]. This group of microorganisms is widespread in nature in particular in the intestines of humans and animals as well as in bioreactors [10-12]. According to acidic intestinal environment, sulfide is hydrolyzed to hydrogen sulfide. There is an overproduction of hydrogen sulfide in patients suffering from ulcerative colitis. Many research papers show evidence that higher occurrence of SRB and their hydrogen sulfide overproduction is one of the factors in ulcerative colitis [13-15]. The prevailing intestinal SRB belong mainly to five genera, namely Desulfovibrio, Desulfobacter, Desulfomonas, Desulfobulbus, and Desulfotomaculum [16-19]. Ulcerative colitis is an inflammatory disease of the large intestine. Although the exact cause remains unclear, the prerequisite appears to be related to a combination of environmental and genetic factors [20]. There are several pathological findings related to ulcerative colitis including oxidative stress, increase in specific inflammatory mediators, abnormal content of glycosaminoglycan in the mucosa, reduced oxidation of short chain fatty acids or increased intestinal permeability [19]. Moreover, overgrowth of sulphate-reducing bacteria resulting in high production of hydrogen sulfide is also considered as one of the causes of UC formation. According to the scientific papers, it has been shown that high concentration of H2S has a negative effect on the colonic epithelial cells [21-25].

The number of lactic acid bacteria during colonic inflammation is decreased, while the number of SRB and their metabolites (hydrogen sulfide and acetate) is increased. The effect of these metabolites on lactic acid bacteria has never been explored. Lactic acid bacteria play a fundamental role in the intestinal microbiome, and therefore it is important to investigate this issue more deeply.

This study is focused on the various methods that could be possibly used for evaluation of the hydrogen sulfide effect on the lactic acid bacteria. Samples of lactic acid bacteria were isolated from colon affected by ulcerative colitis, probiotic supplements and various dairy products. The obtained results proved that lactic bacteria show high sensitivity to hydrogen sulfide.

2. MATERIALS AND METHODS

Pure cultures of LAB were obtained from different sources such as intestinal environment, dairy products and probiotic supplements. All LAB was cultivated at 37 °C. The purity of the cultures has been verified by microscopy and sequencing. MRS broth was used for the pure LAB cultures isolation. Used MRS broth was the following composition: yeast extract (5 g/l), beef extract (10 g/l), peptone (10 g/l), glucose (20 g/l), tween 80 (5 ml/l), K_2HPO_4 (2 g/l), sodium acetate (5 g/l), di-ammonium citrate (2 g/l), $MgSO_4 \times 7H_2O$ (0.2 g/l), $MnSO_4 \times 4H_2O$ (0.05g/l). Final pH was adjusted to 6.2–6.6. Sodium sulfide (Na2S) was used as a source of H2S. In this research, several methods were tested where the most of them were considered as unusable.

2.1. Bioscreen C spectrophotometer.

The BioScreen C Spectrophotometer (100 well plate) was used for comparing the growth curves of bacteria with and without the presence of the inhibitor (hydrogen sulfide). 330 µl of the MRS medium inoculated with 24 hours LAB culture was pipetted into a 100 well plate. 10 µl of H2S was added to achieve the desired concentration of the H2S in the reaction mixture in each well. The anaerobic environment was ensured by the addition of 10 µl of paraffin. 10 different concentrations in 5 repeats were tested in one run of the experiment. Many metabolites are produced by lactic acid bacteria. The optical density measured by the BioScreenC Spectrophotometer does not correspond to biomass concentration but to the turbidity caused by the sediment of the metabolites. The resulting curves had appeared to be irregular and therefore unusable for the evaluation. The method was considered as inappropriate for our interests.

2.2. Agar plate enriched with inhibitor.

Noticeably better results were achieved with agar plate medium enriched with hydrogen sulfide where CFU was counted after 24 hours of cultivation (Figure 1).

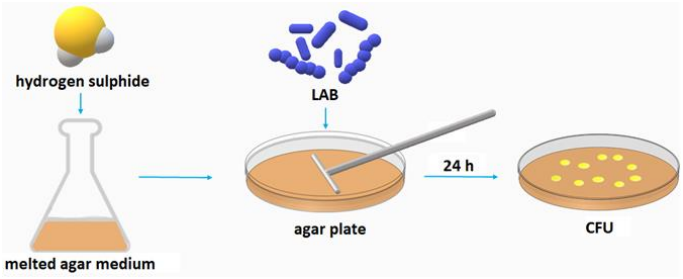

Figure 1. Scheme of agar plate medium enriched with hydrogen sulfide: colony forming units (CFU) were counted after 24 hours.

In total, 20 ml of MRS agar medium was melted in an Erlen flask and the H2S has been added. This agar mixture with H2S was poured into a Petri dish. 100 µl of diluted 24 hours LAB culture was placed. CFU was calculated after 24 hours. Inhibition effect was noted, however, due to the difficult handling and high temperature requirement as well as the impossibility to measure the real H2S concentration in the solid medium, the method was rejected.

2.3. Colorimetric methods.

Colorimetric methods as Resazurin and Tetrazolium salt assays are usually used as cell viability tests. Resazurin is known to be used as an oxidation-reduction indicator in cell viability assays where metabolically active living cells are able to reduce non-fluorescent resazurin to fluorescent resorufin due to NADH activity which has been suggested to be linked with this reaction [26]. The H_2S and 300 µl of the 0,02% resazurin solution was added to the tube with 2 ml of MRS media inoculated with 24 hours LAB culture. The mixture was cultivated for 24 hours at 37°C. However, resazurin assays cannot be used to measure lactic acid bacteria viability. Color change in the reaction mixture has not been caused by the metabolic activity of the cells but by low pH of the environment due to lactic acid production. According to Jung, et al. 2017 [28] it was showed that tetrazolium salt assay can be used for lactic acid viability evaluation. This colorimetric method is widely used for cell metabolic activity evaluation. Tetrazolium salt (colorless) is reduced by NAD(P)H-dependent oxidoreductase and dehydrogenase of the viable cells resulting in formazan (red color) production [27]. However, this assay is not possible to use when H_2S is used as a cell growth inhibitor. The H_2S and 300 µl of tetrazolium salt solution (0.1g/100ml) was added to the tube with 2 ml of MRS media inoculated with 24 hours LAB culture. After addition of the inhibitor to the reaction mixture, an immediate reaction (color change) with tetrazolium salt occurred.

2.4. Optical density and Colony forming units.

Combination of 24 h LAB culture cultivation in tubes in the presence of hydrogen sulfide for 24 hours and optical density (OD) measurement followed by culturing on the agar plate for 24 hours (Figure 2) was chosen as the most suitable method for assessing the toxicity of hydrogen sulfide to the lactic acid bacteria. The H_2S was added to the tube with 2 ml of MRS media inoculated with 24 hours LAB culture followed by 24 hours cultivation. Optical density of the samples was measured by spectrophotometer. 100 µl of the diluted sample was placed on MRS agar plate and CFU was counted after 24 hours.

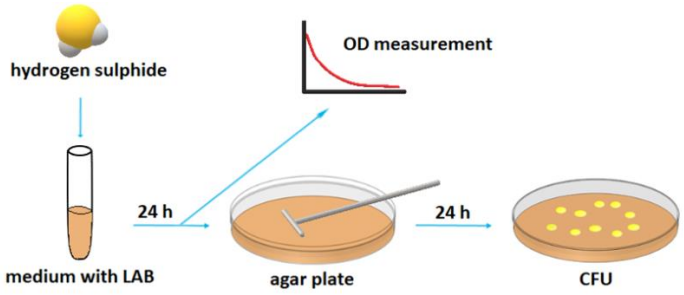

Figure 2. Suitable method for assessing the toxicity of hydrogen sulfide to the lactic acid bacteria: 24 h culture of lactic acid bacteria was cultivated with H2S. After 24 h of the cultivation, the optical density (OD) was measured and reacting mixture was spread on the agar plate. Colony forming units was calculated after further 24 h.

3. RESULTS

The method chosen for assessing the hydrogen sulfide toxicity on the LAB consists of a combination of LAB cultivation in the presence of H_2S, an OD measuring by spectrophotometer and the determination of the viability of LAB grown in the presence of H_2S determined by agar plate method. In this way, specific numbers of surviving cells can be demonstrated, since only uninhibited cells are capable to grow after cultivating of the reacting mixture on the agar plate.

Several LAB species were measured using this method. Showed results refer to the isolates HF1 – isolated from the intestinal environment and LB1 – isolated from a probiotic supplement. The remaining data are not published. Tested strains show sensitivity to hydrogen sulfide. The values gained for the HF1 strain (MIC > 36 µM) appeared to be much higher than values for the LB1 strain (MIC > 0.5mM). This is probably caused by the origin of the isolate where LB1 is isolated from the

probiotic supplement. Commercially available probiotic supplements are expected to withstand the hard conditions of the gastrointestinal tract and to act effectively at the target site – in the intestine. They could be genetically improved and therefore show increased resistance to the substances with an inhibitory effect.

As mentioned above, the LAB cultivation in the presence of H2S, an OD measuring by spectrophotometer and the determination of the viability of LAB grown in the presence of H2S determined by agar plate method was chosen as the most appropriate. Though the method is time consuming, however, it is very inexpensive. The obtained results seem to be satisfactory.

The percentage of the hydrogen sulfide toxicity against bacterial cells and their viability is presented in Table 1.

An integral part of the intestinal microbiota is undoubtedly formed by lactic acid bacteria. Their presence in the digestive tract is essential for its proper functioning. During IBD, such as UC, lactic acid bacteria occurrence is reduced while SRB occurs widely in the intestine resulting in an increase of their metabolite, hydrogen sulfide. Thousands of people from all over the world are affected by IBD every year. The gained results could help to understand and improve the stability of intestinal microbiome, improve the treatment of bowel inflammation diseases, or prevent bowel disease altogether [29].

It should be also noted that hydrogen sulfide can be toxic not only for intestinal cells, but also for its producers. The highest toxicity of H2S was measured in the presence of concentrations higher than 6 mM, where growth was stopped, though metabolic activities were not 100 % inhibited. The presence of 5 mM H_2S resulted in two times longer lag phase and generation time was eight times longer. The results are confirming H_2S concentrations toxicity toward Desulfovibrio [30]. Other research showed that not only hydrogen sulfide and decreased level of LAB in the intestine can be the cause of the disease, but also low pH [31].

Table 1. The hydrogen sulfide toxicity (MIC) and viability (IC_{50}) of bacterial cells.

Strains of *Lactobacillus*	Source of isolation	Toxicity (MIC)	Viability (IC_{50})
L. pentosus	Human feces	> 0.15 mM	> 0.076 mM
L. paracasei		> 1.1 mM	> 0.527 mM
L. plantarum	Probiotic supplement	> 0.45 mM	> 0.266 mM
L. fermentum	Yogurt	> 0.45 mM	> 0.373 mM
L. reuteri	Mice faces	> 1.1 mM	> 0.572 mM

Note: MIC is minimal inhibition concentration, IC_{50} is inhibition concentration where 50 % bacterial cells are viable

4. CONCLUSIONS

Lactic acid bacteria play a fundamental role in the human body. Their function in the large intestine is irreplaceable. For this reason, it is necessary to deal more with the physiology of LAB and the factors that influence their growth. It was necessary to optimize the method for investigating the toxicity of hydrogen sulfide to LAB. As shown by the results so far, the hydrogen sulfide has a significant inhibitory effect on the LAB. This is related to the fact that there is an increased production of H_2S by SRB and decrease of LAB during the UC. Further research is needed in this area.

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