Research article:

Bacillus subtilis response to mercury toxicity: A defense mediated by sulphur-rich molecules and oxidative prevention systems

Authors:

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Response to reviewers comments

We want to thank you for taking the time to review this manuscript. Please find the detailed responses below and the corresponding revisions/corrections highlighted/in track changes in the re-submitted files.

Point-by-point response to comments and suggestions for authors:

Reviewer 3

Comment 1. Please revise the abstract in subheads like background, objectives, methods, findings and important conclusions to make it more impactful and clear.

Response: We appreciate and agree with this comment. In order to attend this observation and to complain the numbers of words for abstract indicated in author instructions, the abstract was modify (highlighted in yellow in the revised manuscript file (*page 1, lines 17-34*) as follows:

"Upon reacting with cellular components, Hg(II) ions elicit the production of reactive oxygen species (ROS). While the ROS-promoted cytotoxic and genotoxic effects induced by Hg(II) have been widely described in eukaryotes, such effects have been less studied in bacteria. In this work, the prokaryotic environmental model *Bacillus subtilis* was employed to evaluate the cytotoxic and genotoxic impact of Hg(II) over strains proficient or deficient for the SOS, general stress and antioxidant responses; as well as the global transcriptional response elicited by this ion. The exposure to $HgCl_2$ significantly increased the mutation frequency to rifampicin resistence (Rif^R) in WT and mutant strains, suggesting a major contribution of these pathways in counteracting the genotoxic effects of Hg(II). Detection of A→T and C→G transversion mutations in the *rpo*B gene of Hg(II)-exposed cells suggested the generation of 8-oxo-guanines (8-OxoGs) and other oxidized DNA bases. The RNA-seq study revealed up-regulation of genes involved in efflux and/or reduction of metal ions, synthesis of sulphur-containing molecules, and down-regulation of genes implicated in iron metabolism and cell envelope

stress. Therefore, our results indicate that metal-extrusion and scavenging of Hg(II) by thiol-rich molecules, may constitute a line of defense of *B. subtilis* that counteracts the noxious effects of ROS resulting from an imbalance in iron metabolism elicited by this ion."

Instead of

"Introduction. Upon reacting with cellular components, the heavy metal mercury elicits the production of reactive oxygen species (ROS). While the ROS-promoted cytotoxic and genotoxic effects promoted by mercury have been widely described in eukaryotic cells such effects have been studied to a lesser extent in bacteria. **Methods.** Here, the impact of Hg (II) ions on cell toxicity and mutagenesis was determined in *Bacillus subtilis* cells proficient or deficient for the SOS (*recA*) and general stress (*sigB*) responses, antioxidant (*sodA* and *katA*/*katB*), or prevention/repair oxidized guanine system [GO; (*ytkD, mutM,* and *mutY*)]. Furthermore, employing an RNA-seq approach, we determined the global transcriptional response elicited by Hg (II) ions in vegetative *B. subtilis* cells. The mutation frequency to Rif^R significantly increased in *B. subtilis* cells treated with $HgCl_2$. Of note, mutagenesis was exacerbated by $HgCl_2$ treatment in *B. subtilis sodA*, *katA*/*katB*, *recA*, *sigB,* and GO-deficient strains, strongly suggesting a major contribution of these pathways in counteracting the genotoxic effects promoted by this heavy metal. The specific detection of A→T and C→G transversion mutations in the *rpo*B gene sequence of the Rif^R clones obtained from Hg(II)-exposed cells suggest induction of non-bulky DNA lesions as those caused by oxidative stress which are mainly repaired by the base excision repair mechanism. Results from RNAseq analysis revealed that genes involved in biosynthesis of metal efflux proteins, reductases and sulphur-containing molecules such as cysteine were up-regulated, whereas genes involved in metabolism and uptake of iron and cell envelope stress were down-regulated. **Discussion.** Metal-extrusion and the scavenging of Hg(II) ions by thiol-rich molecules could be the first line of defense against Hg(II) ions. The downregulation of genes involved in metabolism and uptake of iron, suggest that Hg(II) increases the intracellular pools of Fe(II); perhaps by the attack of mercury ions over [Fe-S]-proteins. We hypothesize that free iron can lead to i) transcriptional repression of genes involved in metabolism and iron sequestration and ii) acceleration of Fenton reaction, increased oxidative stress and activation of antioxidant responses to avoid oxygen radical damage to proteins, lipids and DNA."

Comment 2. Abstract and introduction sections lack clear objectives.

Response: We thank you for this observation. In order to attend this comment, the abstract and introduction sections was modified as follows to clarify that objective of the study is the evaluation of the impact of Hg(II) in toxicity and mutagenesis in bacteria using *Bacillus subtilis* as a model:

In abstract section

"In this work, the prokaryotic environmental model *Bacillus subtilis* was employed to evaluate the cytotoxic and genotoxic impact of Hg(II) over strains proficient or deficient for the SOS, general stress and antioxidant responses; as well as the global transcriptional response elicited by this ion."

Instead of
"Here, the impact of Hg (II) ions on cell toxicity and mutagenesis was determined in *Bacillus subtilis* cells..."

Please find this change highlighted in yellow in the revised manuscript file (*page 1, lines 20 - 23*).

In Introduction section
"Accordingly, the prokaryotic model *B. subtilis*, was employed in this work to evaluate the mutagenic potential of Hg (II) ions, the global transcriptional response to Hg (II) exposition, as well as the role of prevention/repair systems in counteracting the genetic damage inflicted by mercury ions over environmental bacteria."

Instead of
"Here, *B. subtilis* was employed to evaluate the mutagenic potential of Hg (II) ions, the global transcriptional response to Hg (II) exposition, and the role of prevention/repair systems in counteracting the genetic damage inflicted by mercury ions in this microorganism."

Please find this change highlighted in yellow in the revised manuscript file (*page 2, lines 83-86*).

Comment 3. What was the basis for selection of *subtilis* for this designed study? Please mention in the introduction section.
Response: We thank you this observation. In order to indicate the basis for selection of *B. subtilis* for this study, we add this information to the introduction section as follows and highlighted in yellow in the revised manuscript file (*page 2, lines 77-86*):

"The evolution has equipped *B. subtilis* with an arsenal of adaptive responses to monitor and rapidly respond to adverse growing conditions, including, nutrient limitation, sudden temperature changes, and exposure to distinct biologically toxic compounds. In contrast to *E. coli*, *B. subtilis* can proliferate in a wide diversity of habitats making this bacterium an ideal model of study to unravel the cellular strategies deployed by bacteria to survive challenging environmental conditions [20–24]. Accordingly, the prokaryotic model *B. subtilis*, was employed in this work to evaluate the mutagenic potential of Hg(II) ions, the global transcriptional response to Hg(II) exposition, as well as the role of prevention/repair systems in counteracting the genetic damage inflicted by mercury ions over environmental bacteria."

Instead of:
"The Gram-positive bacterium *B. subtilis* has been successfully employed as an excellent model of cell differentiation as well as to unravel the mechanisms underlying the cell responses elicited by distinct environmental stressors [20–24]."

Comment 4. Manuscript has high plagiarism content which should not be more than 10%.

Response: We thank this observation, to this respect, we can ensure that this manuscript was originally wrote based on original research. In order to revise and detect similarity we analyze the content of different manuscript sections (Abstract, Introduction, Materials and Methods, Discussion, and Conclusion) using the Quetext software, a report of this analysis is present at the end of this archive. The results indicate that in all the sections submit to the analysis (sections of approximately 500 words) no more than 10% of plagiarism was detected.

Comment 5. In Statistical analysis section, T student test should be Student's t-test and P value should be p value ($p < 0.05$).

Response: We thank you the observation. The change was done in the revised manuscript file and highlighted in yellow (*page 4, lines 171, 172 and 175*).

Comment 6. It will be more impactful if authors will add short information related to mutant like mutated gene/genes and its product roles in cellular defense system in Materials and Methods section.

Response: We appreciate this contribution. In order to attend this comment, information related to mutated genes and its roles in cellular defenses was included in Table 1. (*page 3, line 98*).

Comment 7. Please verify the data of Wild type 168 and ΔsodA mentioned in Table 2. SOD plays very important role in cellular defense system and both wild and mutant have almost similar LD50 value.

Response: We thank you for this important observation, we revise the data of Wild type 168 and Δ*sodA* presented in Table 2 (in the revised manuscript the data of Table 2 are presented in Figure 1 as recommended by Reviewer 1) and a misinterpretation occurred with respect to lethal doses of *sodA* mutant. Indeed, in accordance to the statistical analysis, the lethal doses 90/50's values of *sodA* mutant did not shown statistical differences with respect to the wild type strain. In order to amend this mistake, the text was changed as follow and highlighted in yellow in the revised manuscript file:

In Results section (*page 5, lines 190-192*)

"In contrast, the SodA-deficient strain exhibited similar levels of susceptibility to mercury treatment as those observed in the wild type strain (Figure 1)."

Instead of

"In contrast, the strain deficient for SodA exhibited higher lethal doses 90/50´s to mercury treatment that the wild-type strain (Table 2)."

In Discussion section (*page 12, lines 304-312*)

"Notably, the lack of SodA, an antioxidant enzyme that converts superoxide radicals into hydrogen peroxide [35] did not show differences in the resistance to Hg(II) ions respect to resistance observed in the wild-type strain. In agreement with this observation, it has been reported that the SodA-deficiency neither increased the susceptibility of *B. subtilis* cells to Cr(VI) [16]. Therefore, it is feasible

that in the absence of SodA, additional systems confer protection to this bacterium from oxidative stress promoted by heavy metals. Indeed, in low-(G+C)-content gram-positive bacteria like *Bacillus*, bacillithiol (BSH), a LMW thiol present [36], could efficiently protect the cells against superoxide and metal stress in absence of SodA [37]. "

Instead of

"Notably, the lack of SodA, an antioxidant enzyme that converts superoxide radicals into hydrogen peroxide [35] increased *B. subtilis* resistance to mercury ions, above that of the wild-type strain. In agreement with this result, indeed a decreased susceptibility to chromate of a *B. subtilis* strain deficient for SodA [16] was reported. This suggests that in the absence of SodA, additional systems could be involved in protection against superoxide stress. In *Bacillus*, bacillithiol (BSH), a LMW thiol present in low-(G+C)-content gram-positive bacteria [36], could efficiently protect the cells against superoxide and metal stress in absence of SodA [37]."

Comment 8. Please check the data presented in the Table 3 and Figure 1. Both data are unmatched.
Response: We thank you for this observation. The data of Figure 1 (changed to Figure 2 in the revised manuscript) and Table 3 (changed to Table 2 in the revised manuscript) are unmatched due to a mistake of the data plotted in the graph of Figure 1, which correspond to values obtained in one of the 3 independent experiments, thus, Figure 1 (Figure 2 in the revised manuscript) was modified to show the correct graph obtained by plotting the data of mean +/- SD, corresponding to data showed in Table 3 (Table 2 in the revised manuscript).

Please find this change in Figure 2 of the revised manuscript (*page 7, line 210*).

Comment 9. Figure 1, $p < 0.05$ are for * or **. Please clearly mention the level of significance $p < 0.05$; $p < 0.01$; $p < 0.001$ in the figure legend.
Response: Thank you for this comment. In the legend of Figure 1 (Changed to Figure 2 in the revised manuscript) p-value was specified. (*page 7, lines 215-218)*.

Comment 10. The name of gene should be uniform throughout manuscript.
Response: The name of genes were revised and uniformed throughout the manuscript, please find the changes highlighted in fuchsia *(page 7, line 212; and page 11, line 271 Table 5)* in the revised manuscript.

Comment 11. On Page no. 12; Please verify the statement: Notably, the lack of SodA, an antioxidant enzyme that converts superoxide radicals into hydrogen peroxide [35] increased *B. subtilis* resistance to mercury ions, above that of the wild-type strain However in Table 2 LD50 are 1.19 ± 0.04 for wild type and 1.18 ± 0.09 for Δ*sodA*.
Response: Thanks for this observation, the statement was revised and accordingly to your comment 7, a misinterpretation occurred with respect to lethal doses of *sodA* mutant. Indeed, based on the statistical analysis, the lethal doses 90/50's values of *sodA* mutant did not shown statistical differences

with respect to the wild type strain. In order to amend this mistake the wrong statement was replaced in the revised manuscript (*page 13, lines 304-312*) by the statement:

"Notably, the lack of SodA, an antioxidant enzyme that converts superoxide radicals into hydrogen peroxide [35] did not show differences in the resistance to Hg (II) ions respect to resistance observed in the wild-type strain. In agreement with this observation, it has been reported that the SodA-deficiency neither increased the susceptibility of *B. subtilis* cells to Cr(VI) [16]. Therefore, it is feasible that in the absence of SodA, additional systems confer protection to this bacterium from oxidative stress promoted by heavy metals. Indeed, in low-(G+C)-content gram-positive bacteria like *Bacillus*, bacillithiol (BSH), a LMW thiol present [36], could efficiently protect the cells against superoxide and metal stress in absence of SodA [37]. "

Instead of

"Notably, the lack of SodA, an antioxidant enzyme that converts superoxide radicals into hydrogen peroxide [35] increased *B. subtilis* resistance to mercury ions, above that of the wild-type strain. In agreement with this result, indeed a decreased susceptibility to chromate of a *B. subtilis* strain deficient for SodA [16] was reported. This suggests that in the absence of SodA, additional systems could be involved in protection against superoxide stress. In *Bacillus*, bacillithiol (BSH), a LMW thiol present in low-(G+C)-content gram-positive bacteria [36], could efficiently protect the cells against superoxide and metal stress in absence of SodA [37]."

Comment 12. Conclusion section is missing. Please add this section.
Response: To attend this comment, the final paragraph of discussion section was modified as follow to be presented as the conclusion in the revised manuscript (*pages 16-17, lines 471-480)*:

"In this work, we employed *Bacillus subtilis* as a Gram-positive model to investigate the cytotoxicity and genotoxicity, as well as the global transcriptional response elicited by the heavy metal pollutant mercury, over environmental bacteria. In summary, our results indicate that upon interacting with rich-cysteine molecules, Hg(II) promote iron and cysteine metabolism stresses. Such processes, i) increase the transcription of genes encoding rich-cystein molecules to counteract mercury toxicity, ii) high intracellular iron concentrations generate ROS-promoted DNA damage; in support of these notion, iii) antioxidant and BER proteins were found to prevent the cytotoxicity and genotoxicity of Hg(II). However, the mechanistic aspects by which DNA mutations are induced by mercury in *B. subtilis*, represent an important issue for future research."

Instead of last paragraph of the discussion section.

"In this work the mutagenesis and bacterial response to mercury toxicity in prokaryotes was presented. The close relation between mercury toxicity and iron metabolism observed, as well as the mechanism by which DNA mutations are induced, could be important issues to investigate in future."

Additional Changes

Change 1. With respect to the general comment indicated by e-mail:

2. After checking the manuscript, we found that reference [11](Transcriptional Responses of Escherichia coli during Recovery from Inorganic or Organic Mercury Exposure) has published correction(https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-4413-z#cit). If it is possible, we suggest you to replace or remove the reference during the revision process; If the reference is indispensable, please cite the corrected reference correctly.

Response: Thanks for the observation, we have revised the correction of this article and consider that correction in the manuscript do not affect the information provided in our manuscript, moreover, we consider that this reference is indispensable for this work and we decided to mantain this reference. Its important to mention that the data of this reference as well as doi of this article remains without changes (*page 17, lines 516-517*) as you can revised in https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-4413-z#change-history where the correction and update if the manuscript is adressed as follow:

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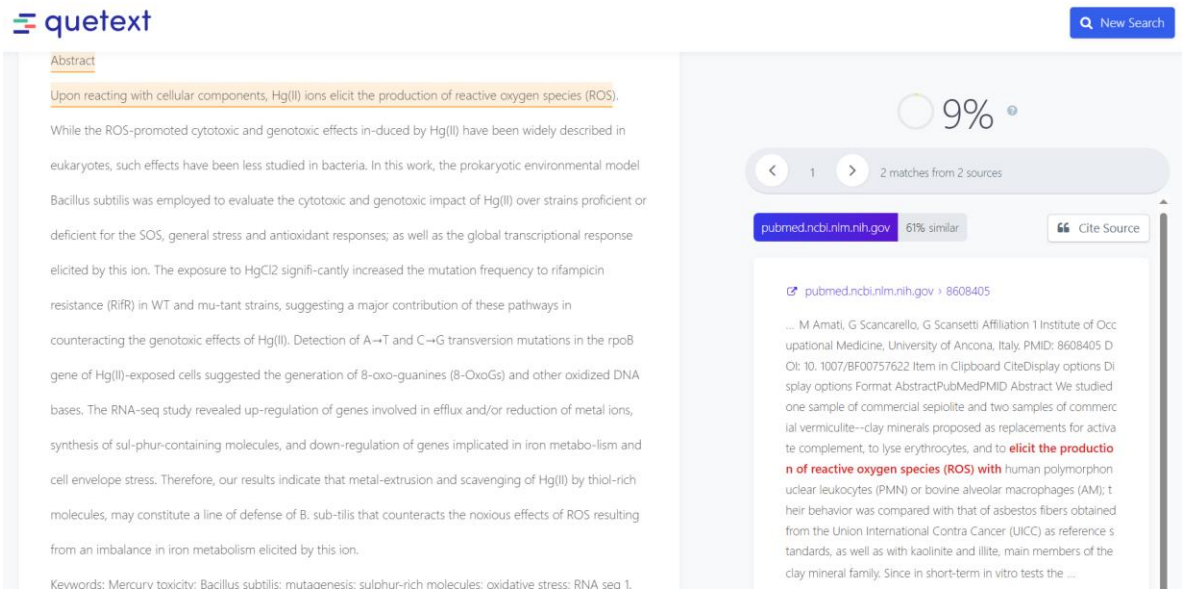
18 April 2018

After publication of the original article [1] the authors noted that the key displayed in Figure 1a was incorrect, as the PMA and HgCl2 conditions had been switched.

Change 2. Additional changes to clarify the impact of this work and those derived from modifications in figure numbers, insertion of references, etc. were highlighted in green in the revised manuscript.

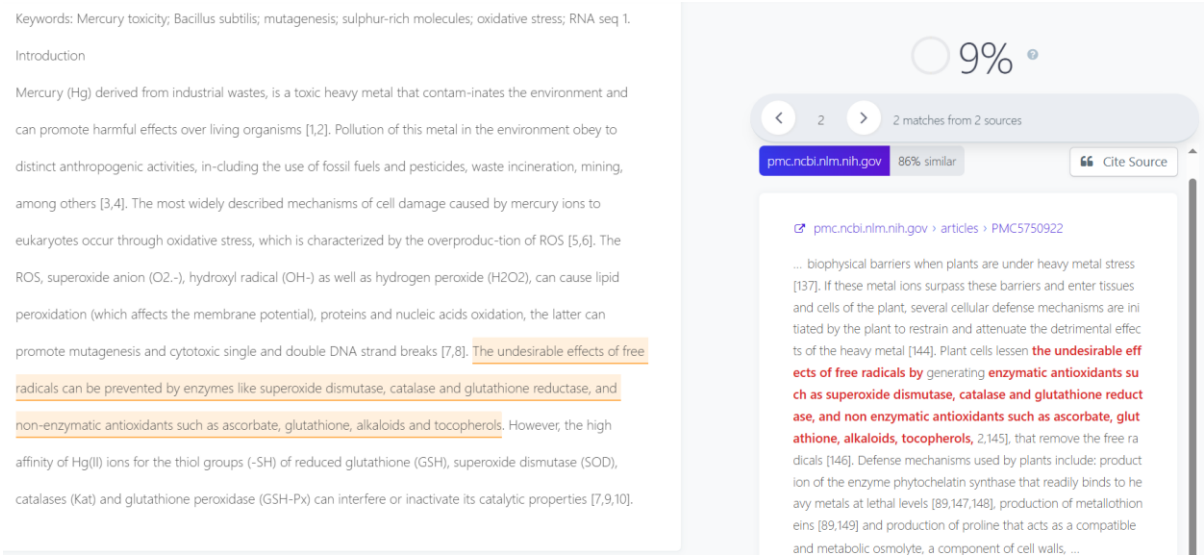
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Report of Abstract section

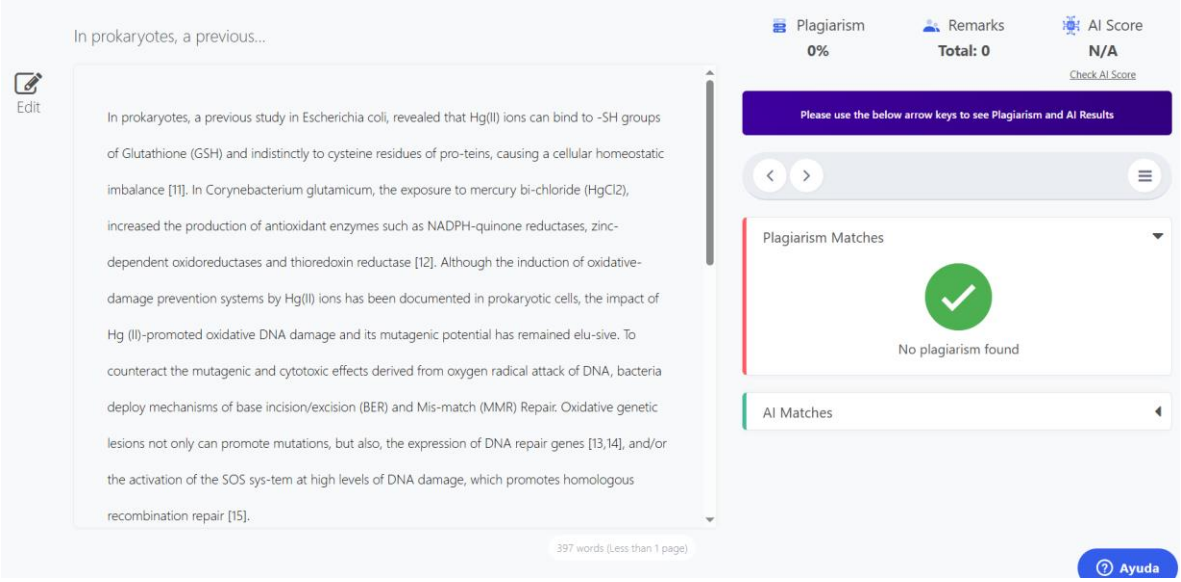


Report of First part of the Introduction section

A 9% was detected, however, identified plagiarism correspond to information that is appropriately cited (in the revised manuscript corresponds to reference 7).

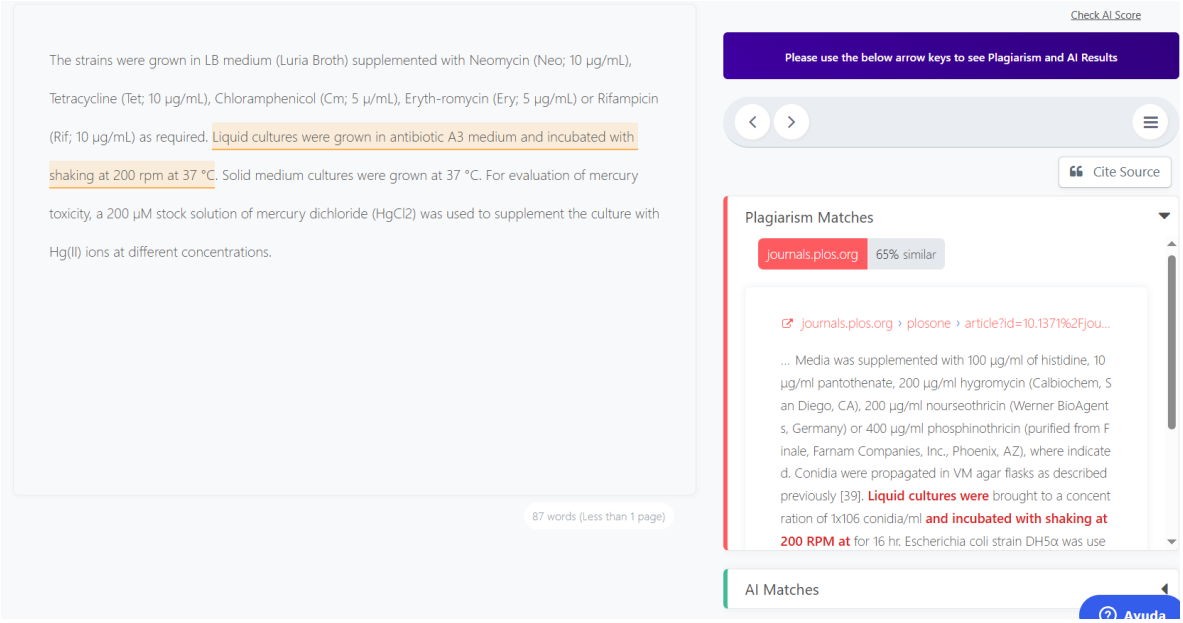


Report of Last part of the Introduction section

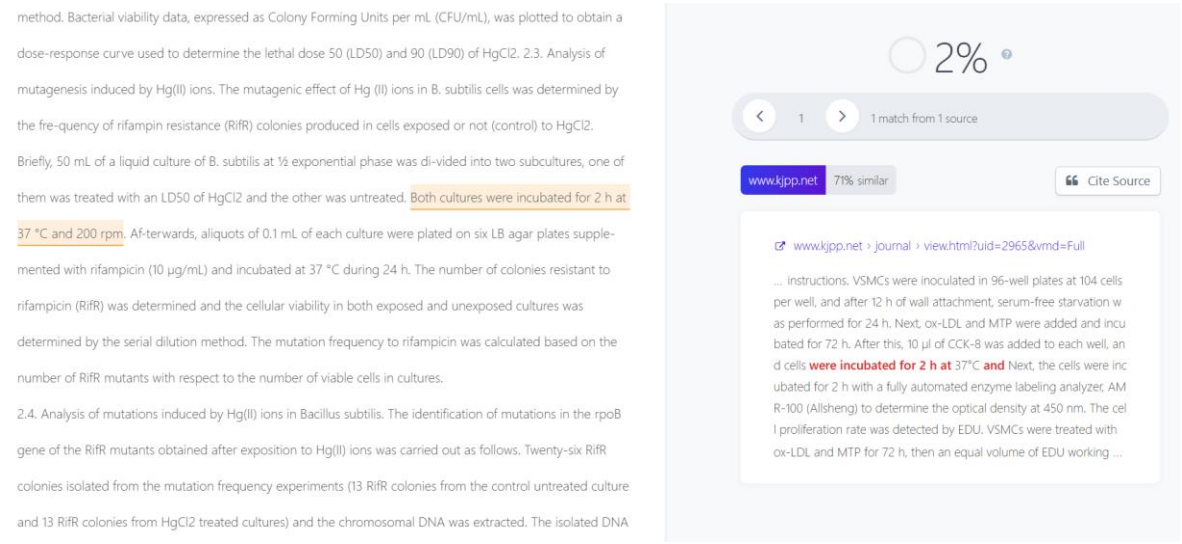


Report of <u>Materials and Methods 2.1 and 2.2 sections</u>

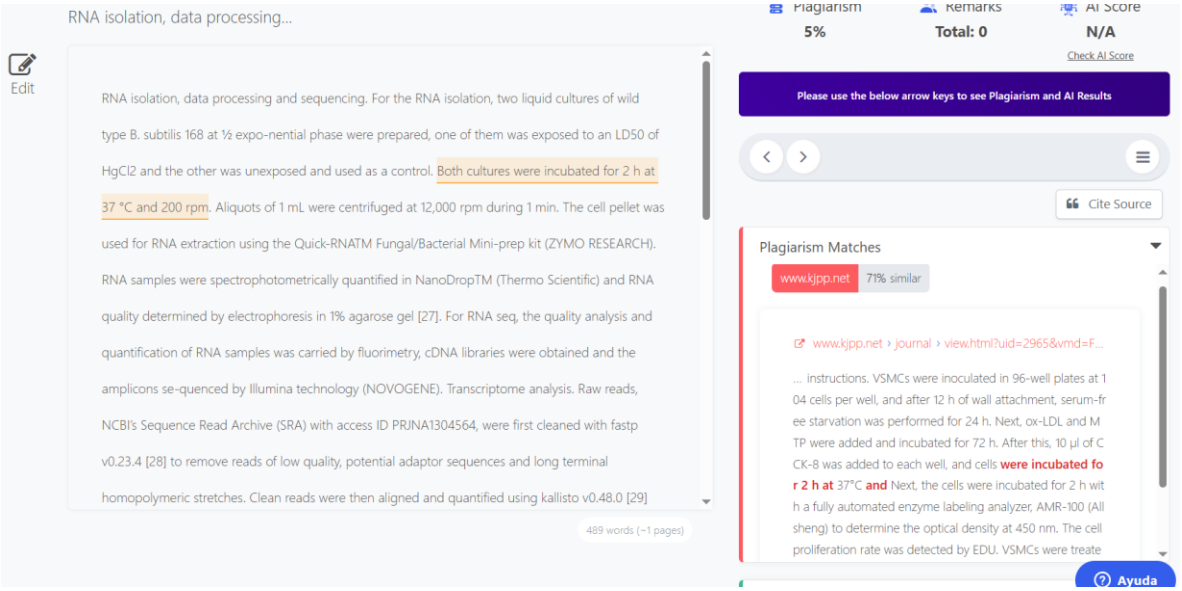
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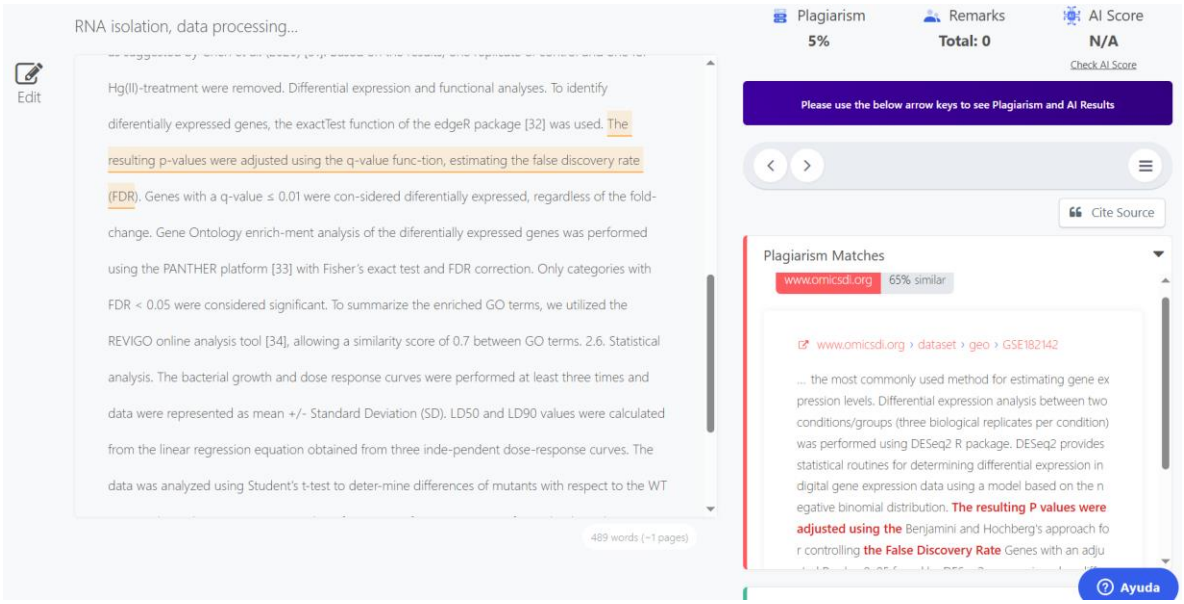
Report of <u>Materials and Methods 2.3 and 2.4 sections</u>



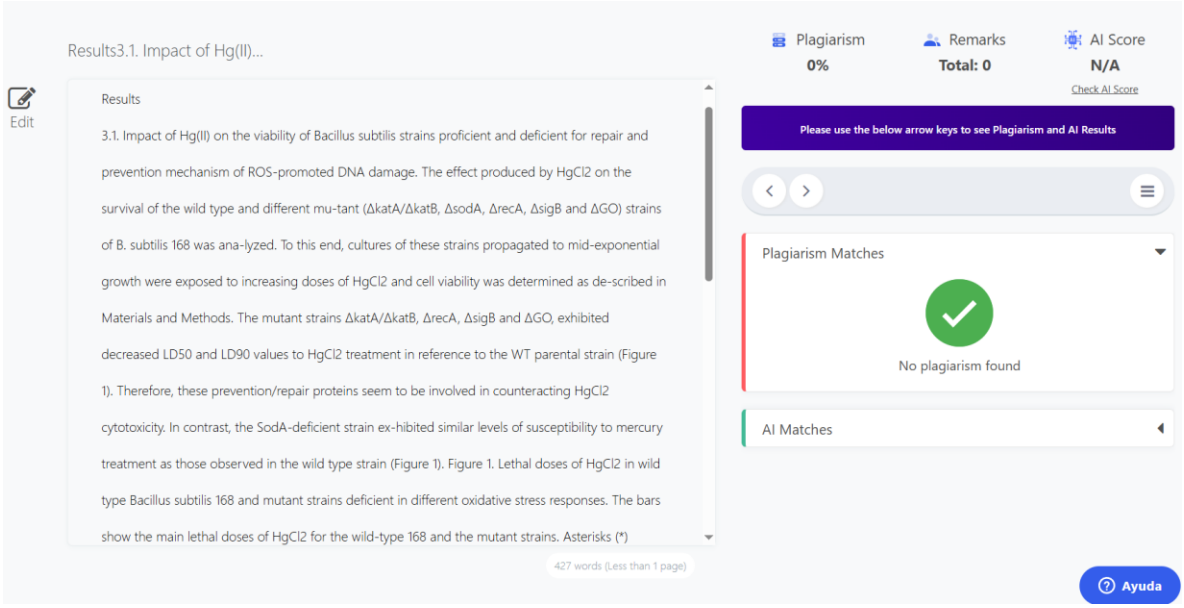
Report of <u>Materials and Methods 2.5 section</u>



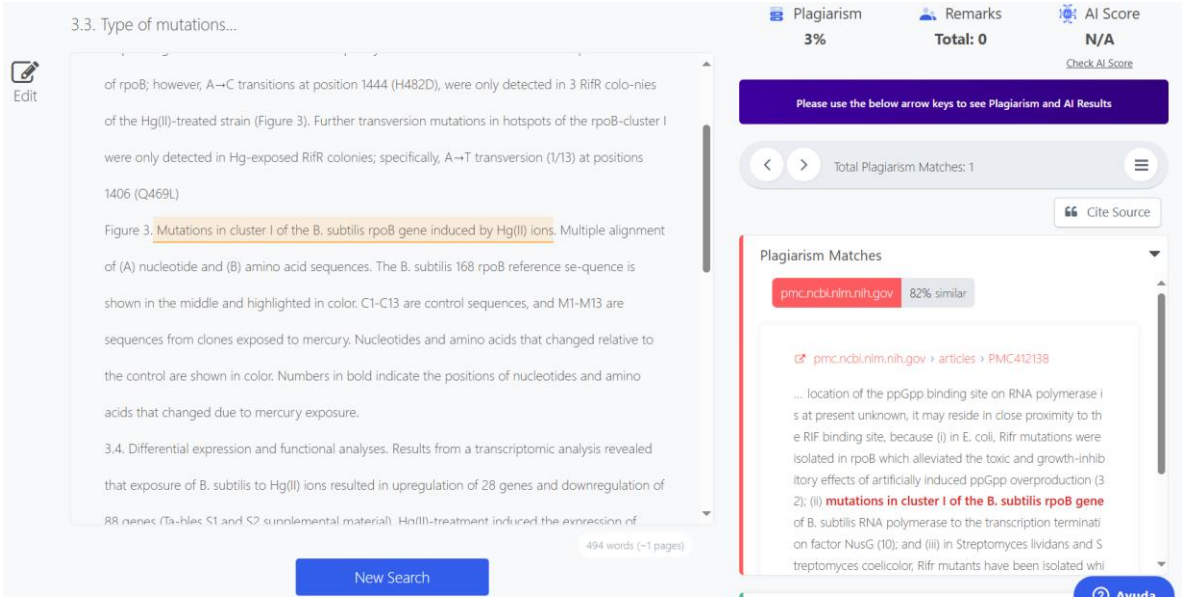
Report of <u>Materials and Methods 2.5.3. section</u>



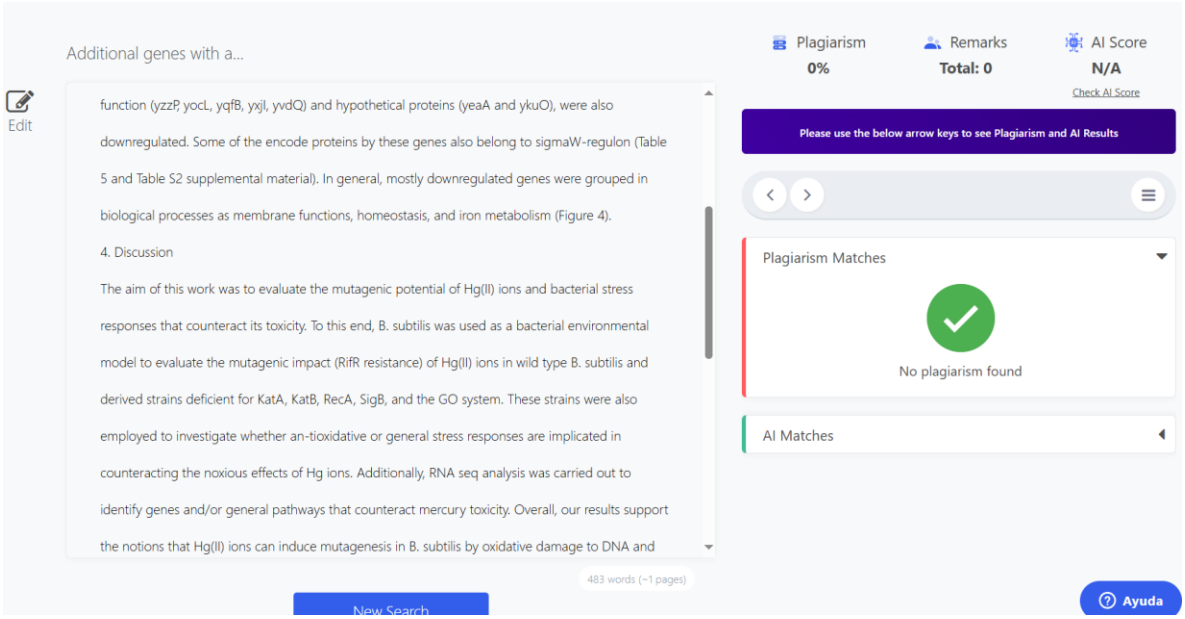
Report of First part of Results section



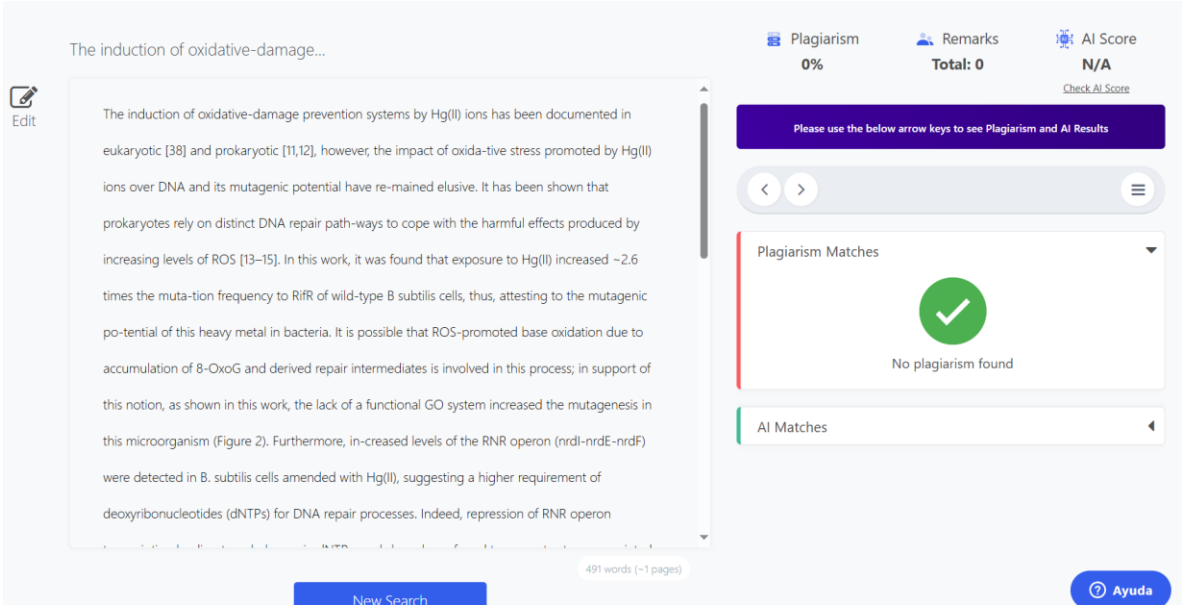
Report of Second part of Results section



Report of <u>Third part of Results and First part of Discussion</u> sections

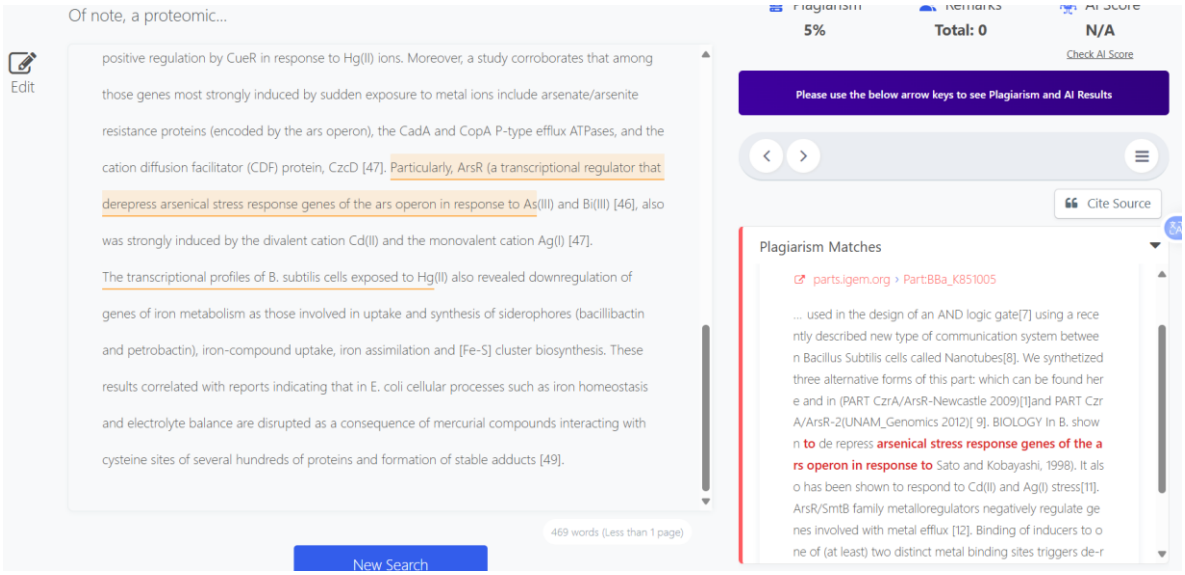


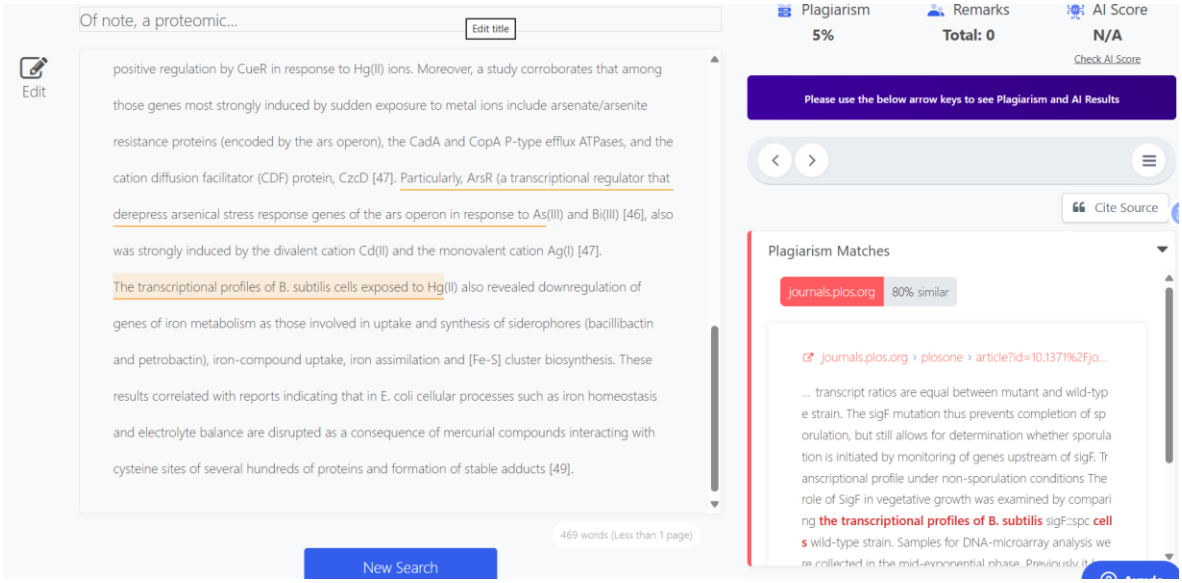
Report of the <u>Second part of Discussion</u> section



Report of the <u>Third part of Discussion</u> section

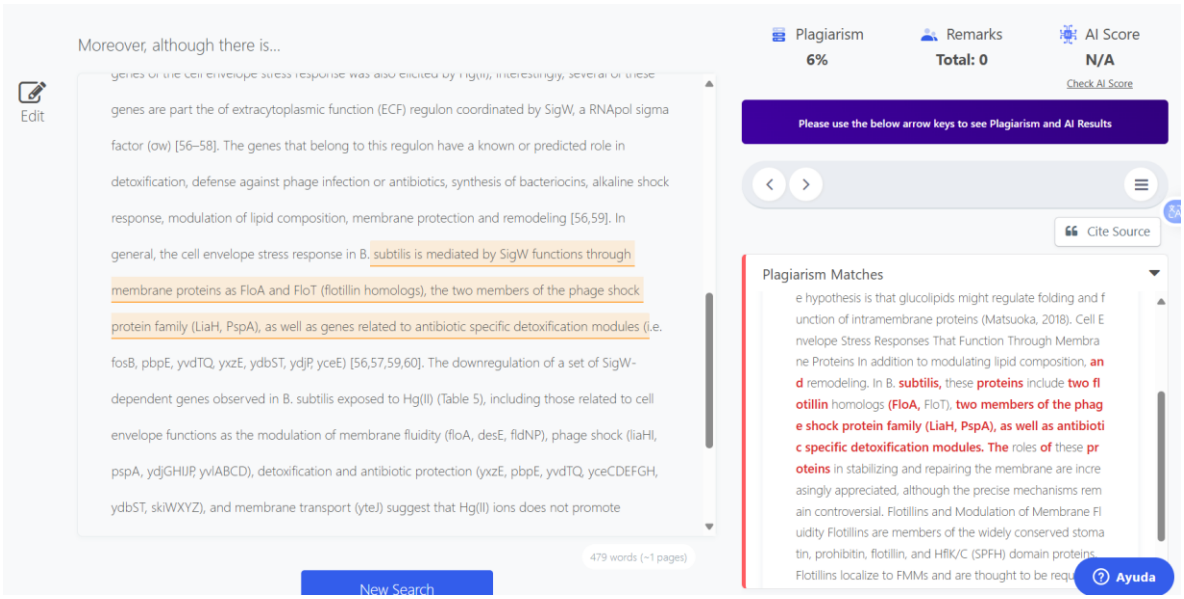
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Report of the <u>Fourth part of Discussion</u> section

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Report of the Fifth part of Discussion and Conclusion sections

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