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**Growth of Methanogens in the Presence of Perchlorate Salts: A Study for
Possible Life on Mars**

*An honors thesis submitted in partial fulfillment of the requirements of honors studies in
Biological Sciences.*

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Abstract

The Phoenix Lander collected soil samples from Mars, and it detected perchlorate salts in its analysis [1]. As oxidizing agents, perchlorates pose a threat to the hypothesis that there may be microbial life on Mars. Since Mars is very dry, perchlorate may continue to accumulate in the soil. If anaerobic methanogens inhabit Mars, then they must be able to grow in the presence of perchlorate salts.

There were four species of methanogens tested in this project. The methanogens were first exposed to small concentrations of calcium perchlorate, and then they were gradually tested at higher concentrations. Growth was measured by the production of methane, which is a biomarker for metabolic activity. Each species responded in a unique way to the increasing concentrations of calcium perchlorate salts.

The results showed that microorganisms that grow in minimal media had the lowest tolerance; while microorganisms that grow under increased levels of salt in their media could tolerate higher concentrations of calcium perchlorate.

Introduction

Perchlorate ions can exist dissolved in solution or in a solid salt form. These ions are damaging to living organisms. In humans, perchlorate functions to inhibit iodine uptake in the thyroid. In microorganisms, perchlorate can interrupt certain metabolic processes. Additionally, perchlorates are strong oxidizing agents known to cause damage to the cell membrane [2]. In 2008 the Phoenix Lander, a NASA spacecraft, landed on the Vastitas Borealis on Mars. The Vastitas Borealis has smooth topography, and it is speculated that an ocean once existed there. This is supported by studies focusing on the topography of Mars, which postulated that the land in the low northern hemisphere could have once been a large basin [3].

The Phoenix Lander had a wet chemistry lab, which contained ion-specific electrodes. The Hofmeister series electrode was present on the Phoenix Lander, and this was calibrated to identify and measure perchlorate anions. Soil sample analysis from the wet chemistry lab led to the discovery that martian surface soil contains 0.4% - 0.6% perchlorate (ClO_4^-) anions. The companion cations largely consisted of Mg^{2+} and Ca^{2+} with small amounts of K^+ and Na^+ [1]. NASA's robotic rover on Mars more recently detected the presence of magnesium perchlorate [$\text{Mg}(\text{ClO}_4)_2$] and calcium perchlorate [$\text{Ca}(\text{ClO}_4)_2$] with its sample analysis instrument [4]. These soil sample measurements taken by the Phoenix Lander suggest that perchlorate salts are present at approximately 1% concentrations, however they could be present at higher concentrations in certain areas.

Even though the levels of perchlorate on Mars's surface seem relatively small, it has been determined that perchlorate is responsible for much of the soluble chlorine in the martian soil [1]. Therefore, anaerobic microorganisms that could potentially inhabit Mars will inevitably be exposed to perchlorate. Some anaerobic microorganisms have been shown to use perchlorate as

a chlorine source in the Atacama Desert [5]. Methanogens are anaerobic, methane-producing Archaea that have been hypothesized to exist on Mars because a small amount of methane gas has been detected in the martian atmosphere [6]. Methanogens characteristically metabolize carbon dioxide (CO₂) and hydrogen (H₂) to produce methane (CH₄) and water (H₂O). Therefore, a balanced chemical equation to show this process is:



If methanogens are living on Mars they are most likely beneath the surface because environmental conditions on the surface of Mars are extremely harsh [7]. Since the Phoenix Lander collected soil samples from the surface of Mars, it is unclear exactly what the concentrations of perchlorate salts may be in the underground environment. Studies of the Atacama Desert in Northern Chile and Southern Peru may give us insight into the mechanism for the arrival of perchlorate salts on Mars. Quantities of perchlorate salts are deposited in the Atacama from gas phase oxidation of chlorine, likely interacting with ozone. A similar photochemical oxidation process is a possible mechanism for the origin of perchlorate salts on Mars. One of the reasons that Mars is described as a harsh environment is because it is extremely dry. Comparison control studies with the atmosphere of the Atacama Desert have shown that an arid environment supports dry deposition of perchlorate [5]. If the deposition rate exceeds the rate of dissolution, perchlorate will accumulate. Once perchlorate is formed, it tends to be unreactive and persistent in a dry environment. The unreactive nature of perchlorate is due to a stable tetrahedral conformation containing oxygen atoms around the central chlorine [5].

If perchlorate salts and methanogens are both present on Mars, then methanogens must be able to exist in the presence of these oxidizing agents. Due to the arid environment on Mars, perchlorate may accumulate to reach higher concentrations by the mechanism of dry deposition

into the soil. The purpose of this project was to explore the possibility for methanogens to grow in the presence of various concentrations of perchlorate. I have observed the effects of $\text{Ca}(\text{ClO}_4)_2$ on the ability of methanogens to grow. This was a continuation of a previous project, which tested methanogen viability in the presence of various perchlorate salts along with the ability for methanogens to adapt to increasing concentrations [8]. This project attempts to focus specifically on the effects of calcium perchlorate on methanogen growth.

The hypothesis of this project postulates that the methanogens will be able to tolerate the presence of calcium perchlorate, and that they may display variance in threshold tolerance. The outcomes of this study will help us understand the ability of methanogens to tolerate certain levels of calcium perchlorate, which is directly relevant and important to the possibility for methanogen life on Mars.

Materials and Methods

This experiment studied four types of methanogenic Archaea growing in their ideal media:

1. MM medium for *Methanothermobacter wolfeii*
2. MS medium for *Methanosarcina barkeri*
3. MSF medium for *Methanobacterium formicicum*
4. MSH medium for *Methanococcus maripaludis*

Using anaerobic methods, we exposed methanogens to calcium perchlorate and measured growth by gathering methane gas measurements over time. Methane levels are expected to increase as methanogens persist in growth because they metabolize CO_2 and H_2 in a closed, anaerobic setting.

The first step in the experiment involved making 50 mL of each medium, which was prepared in beakers according to the protocol called for in Appendix A. Ten milliliters of each medium were measured into individual test tubes, yielding five replicates for each trial. A control trial was conducted without any calcium perchlorate along with experimental trials contained calcium perchlorate in concentrations of 1%, 2%, 5%, 7%, and 10%. Calcium perchlorate was weighed and added to each individual test tube instead of being added to the media beakers prior to pouring. Media were prepared in a bicarbonate buffer. The buffer solution was prepared by dissolving 4g of NaOH in 1 liter of deionized water, followed by saturation with CO₂ gas. The acidity created from the CO₂ is offset by the basic NaOH, which maintains a stable pH. With this in mind we considered the density of the media solution to be approximately 1.0 g/mL. Therefore with 10mL of media in each test tube, 0.1g of calcium perchlorate were placed in each tube for the 1% experimental trial, 0.2g for the 2% trial, 0.5g for the 5% trial, 0.7g for the 7% trial, and 1.0g for the 10% trial.

The creation of the media began in the aerobic environment where each component from Appendix A was measured and placed in the beaker. Next the media, test tubes, and rubber caps were placed in the anaerobic chamber, which is where the bicarbonate buffer was added to the media solutions. The media were allowed to deoxygenate in the chamber for at least 24 hours. After the deoxygenating process was complete, 10 mL of media were measured and poured into the individual test tubes within the anaerobic chamber. After the media was poured, rubber stoppers were used to seal the test tubes. When the test tubes were completely anaerobic, they were ready to be removed from the anaerobic chamber. Once the test tubes containing media were removed from the chamber, aluminum crimps were placed on all of the test tubes to ensure the rubber stoppers were not removed until the trials were complete. The media was then

autoclaved to prepare for inoculation. After autoclaving the media, 0.125 mL of a sterile 2.5% Na₂S solution were added to each test tube to remove residual oxygen.

Any exchange of material, including inoculation of media and gas collection, involved using a syringe to pierce through the rubber stoppers. For the inoculation procedure, 0.5 mL of exponential-phase methanogens were added to their respective medium as indicated above. All newly inoculated tubes were then pressurized with 200kPa of H₂ and then incubated at specific growth temperatures for each methanogen. *M. wolfeii* grew at 55°C, *M. maripaludis* grew at room temperature (25°C), and *M. barkeri* and *M. formicicum* grew at 37°C. On a periodic basis over the course of twelve weeks, the trial replicates were tested for methane production. The data were collected by injecting a 1 mL sample of gas from each replicate into a Varian CP-4900 Micro Gas Chromatograph. The result of this test identified the concentration of methane gas as a percentage of the total sample injection. Monitoring the methane levels over time provide empirical data on the growth of the microbes. Methane is a biomarker for methanogens, generally accepted as an indicator of growth, when methane increases over time. The gas chromatography results are also important because they can identify oxygen contamination from the aerobic environment, which is a source of error when working with strict anaerobes. Another indicator of oxygen contamination is a red dye called Resazurin, which is in Solution C of the media preparation (Appendix A).

Results

Several experimental trials were carried out in this project including a 1% $\text{Ca}(\text{ClO}_4)_2$ trial, two 2% $\text{Ca}(\text{ClO}_4)_2$ trials, a 5% $\text{Ca}(\text{ClO}_4)_2$ trial, a 7% $\text{Ca}(\text{ClO}_4)_2$ trial, and a 10% $\text{Ca}(\text{ClO}_4)_2$ trial. The results are provided by graphical representation in order of increasing concentrations. Each data point in the graph contains vertical error bars, which represent the standard deviation of the replicate data set. Replicates of *M. wolfeii* were discontinued after the 5% $\text{Ca}(\text{ClO}_4)_2$ because the threshold was determined after conducting two trials at 2% $\text{Ca}(\text{ClO}_4)_2$. Due to oxygen contamination in all replicates, the 7% trials for *M. maripaludis* have been excluded from the data presentation.

M. wolfeii was able to grow at 1% $\text{Ca}(\text{ClO}_4)_2$ concentration, however, it was not able to sustain growth in a 2% concentration of $\text{Ca}(\text{ClO}_4)_2$. Therefore, it is reasonable to conclude that *M. wolfeii* has a threshold between 1% and 2%. These results indicate that *M. wolfeii* is the least tolerant of the four methanogens tested in this project. *M. wolfeii* did not grow at any concentration except 1%.

M. barkeri grew at a 1% and 2% concentrations of $\text{Ca}(\text{ClO}_4)_2$, although the growth was notably slower than the control. Inconsistent results were observed at 5% concentrations; however, this is most likely due to oxygen contamination in at least two of the five replicates. *M. barkeri* appears to be reluctant to grow well in environments containing perchlorate, but its threshold is higher than that of *M. wolfeii*. The threshold concentration for *M. barkeri* measured in this project is between 5% and 7% $\text{Ca}(\text{ClO}_4)_2$. This species did not grow at 7% or 10% $\text{Ca}(\text{ClO}_4)_2$.

M. formicicum showed the strongest results for concentrations of 1%, 2%, and 5%

Ca(ClO₄)₂. In fact, *M. formicicum* impressively outperformed the control at all data points in the second trial of the 2%. However, the growth slowed down tremendously at concentrations of 7% and 10% Ca(ClO₄)₂. Nonetheless, *M. formicicum* revealed its ability to grow at all concentrations tested in this project. Consequently, a threshold for *M. formicicum* cannot be concluded from these results. This species experiences rapid growth at concentrations less than 7%.

M. maripaludis may be the strongest performer in the presence of Ca(ClO₄)₂. Steady growth is observed at all concentrations except for the 7% Ca(ClO₄)₂ concentrations. However, it was later determined that oxygen contamination was present in at least four of the five replicates involved in the 7% trial. We believe this happened due to permeable rubber stoppers, which were being reused. In an effort to reach this conclusion we pressurized the replicates with H₂, and gas escaping could be heard immediately after the tubes were pressurized. This revealed that gas exchange was occurring with the aerobic environment due to permeable rubber stoppers. Therefore, in light of the results for *M. maripaludis* at a 10% Ca(ClO₄)₂ concentration, we have concluded that this species is the strongest performer in the presence of Ca(ClO₄)₂. A threshold concentration could not be determined for *M. maripaludis*, but it is most likely above 10% Ca(ClO₄)₂.

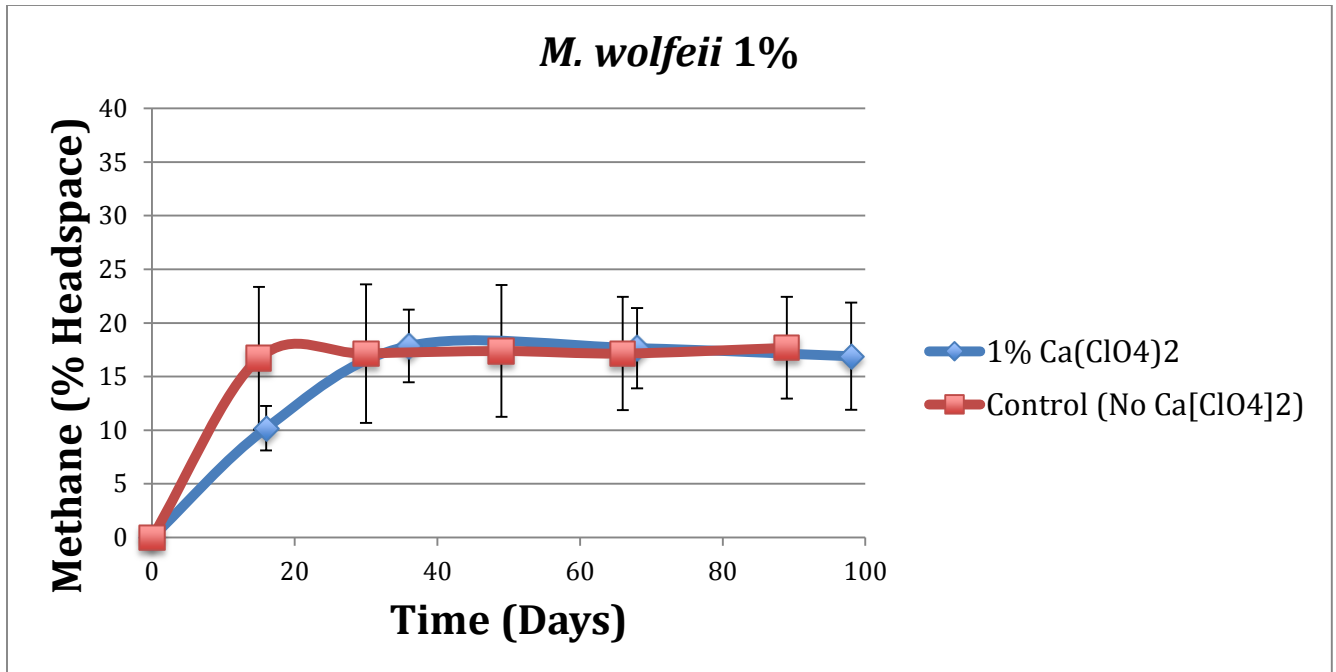


Figure 1: Methane production by *Methanothermobacter wolfeii* in the presence of 1% calcium perchlorate under anaerobic conditions.

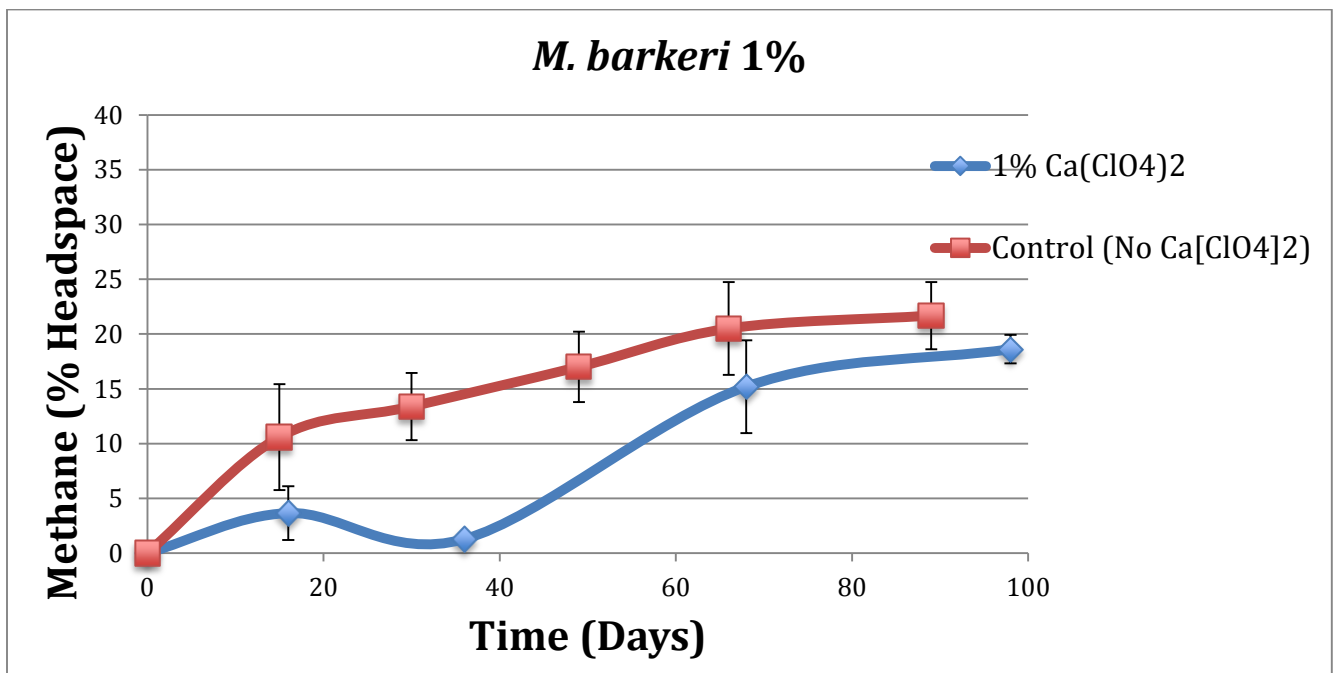


Figure 2: Methane production by *Methanosarcina barkeri* in the presence of 1% calcium perchlorate under anaerobic conditions.

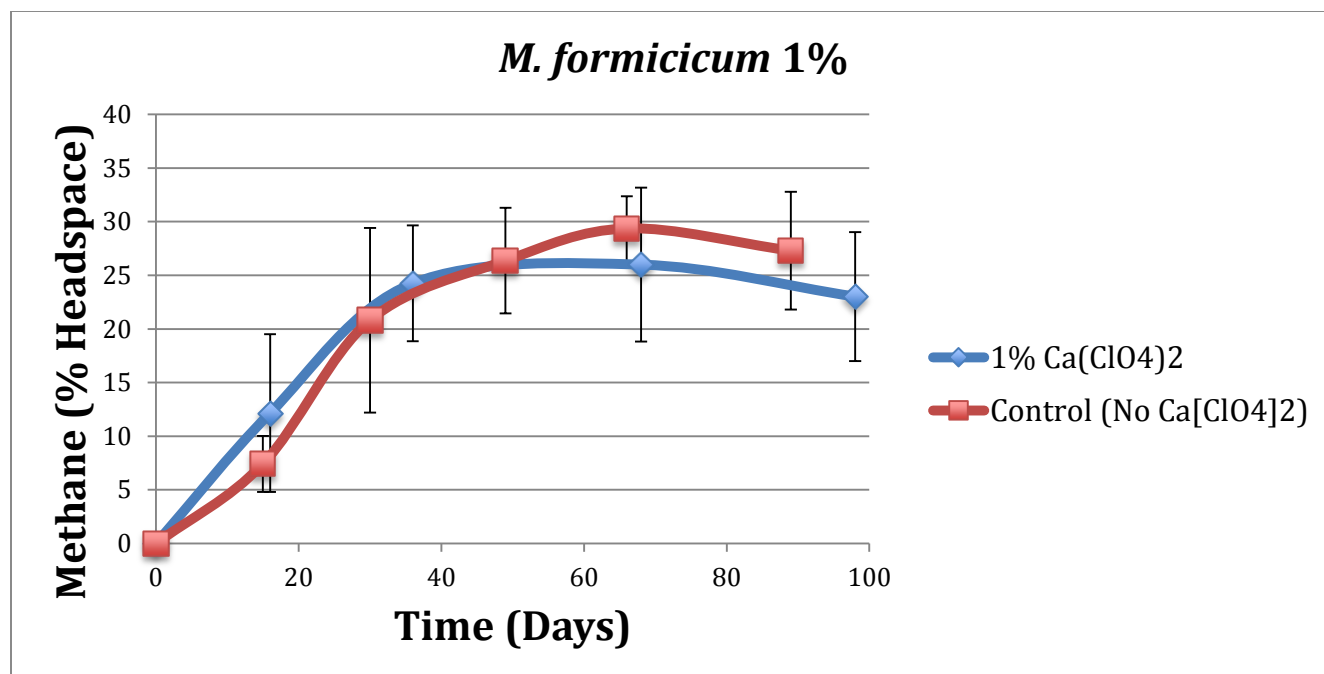


Figure 3: Methane production by *Methanobacterium formicicum* in the presence of 1% calcium perchlorate under anaerobic conditions.

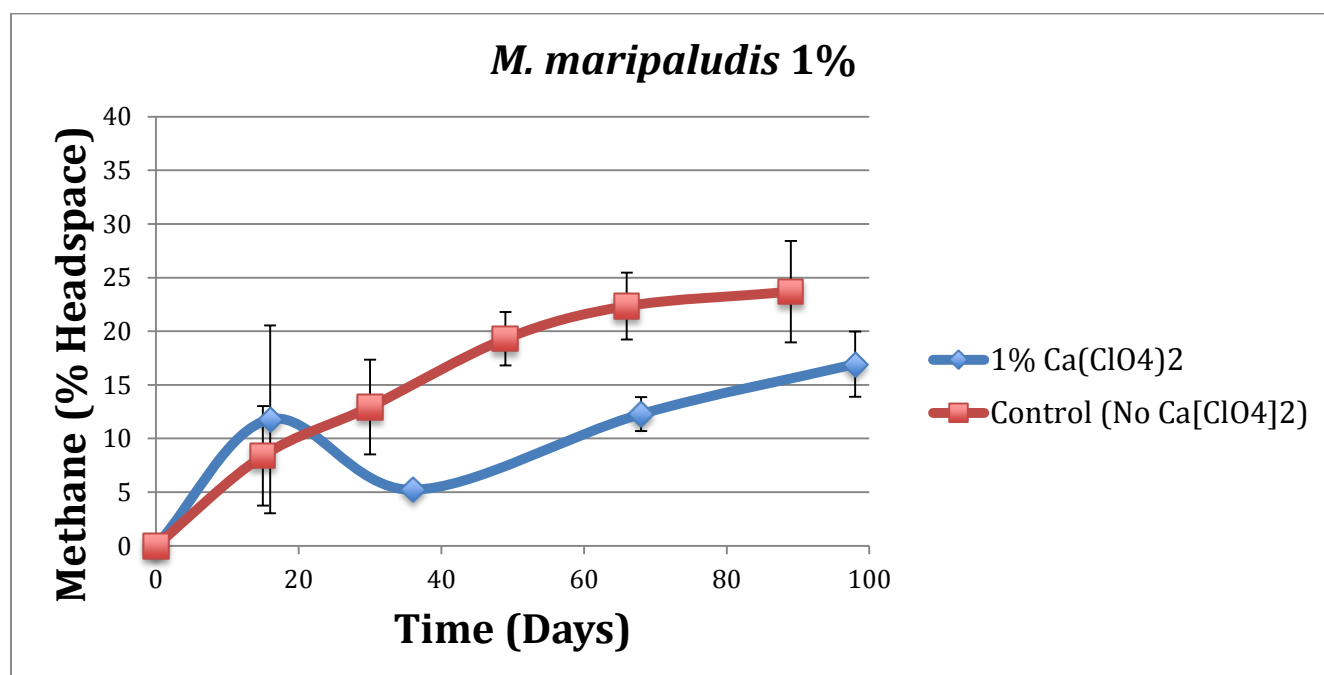


Figure 4: Methane production by *Methanococcus maripaludis* in the presence of 1% calcium perchlorate under anaerobic conditions.

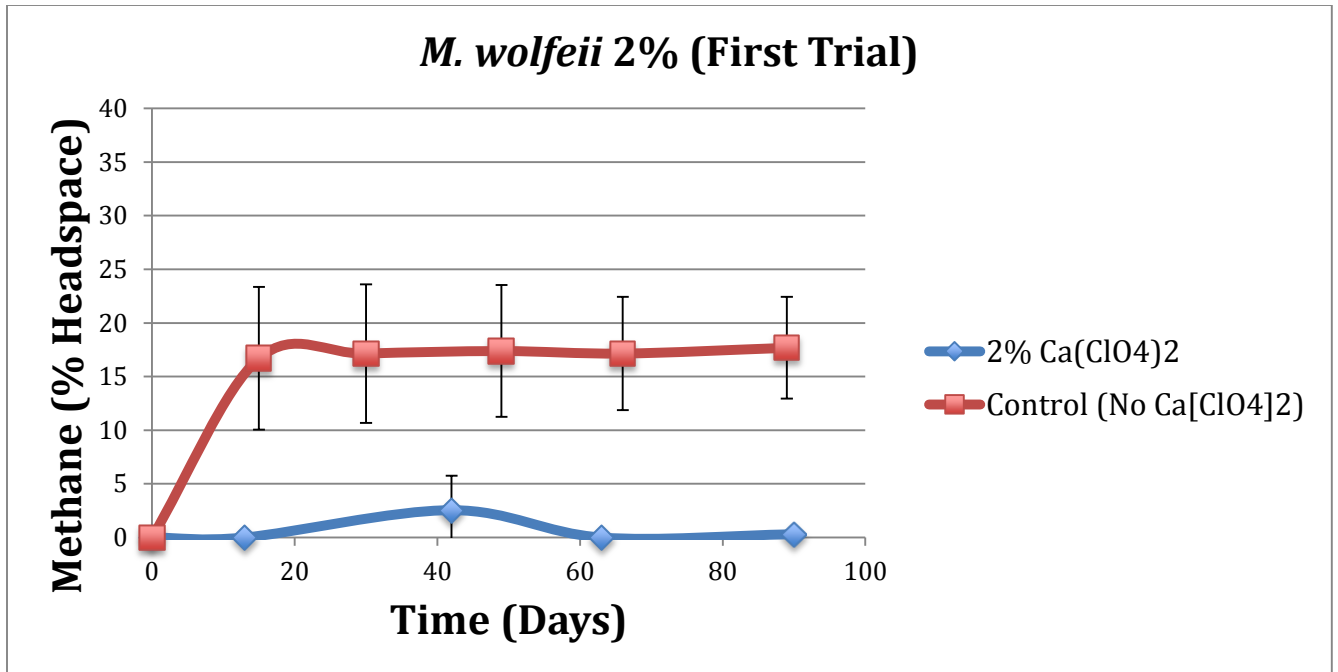


Figure 5: Methane production by *Methanothermobacter wolfeii* in the presence of 2% calcium perchlorate under anaerobic conditions – first trial.

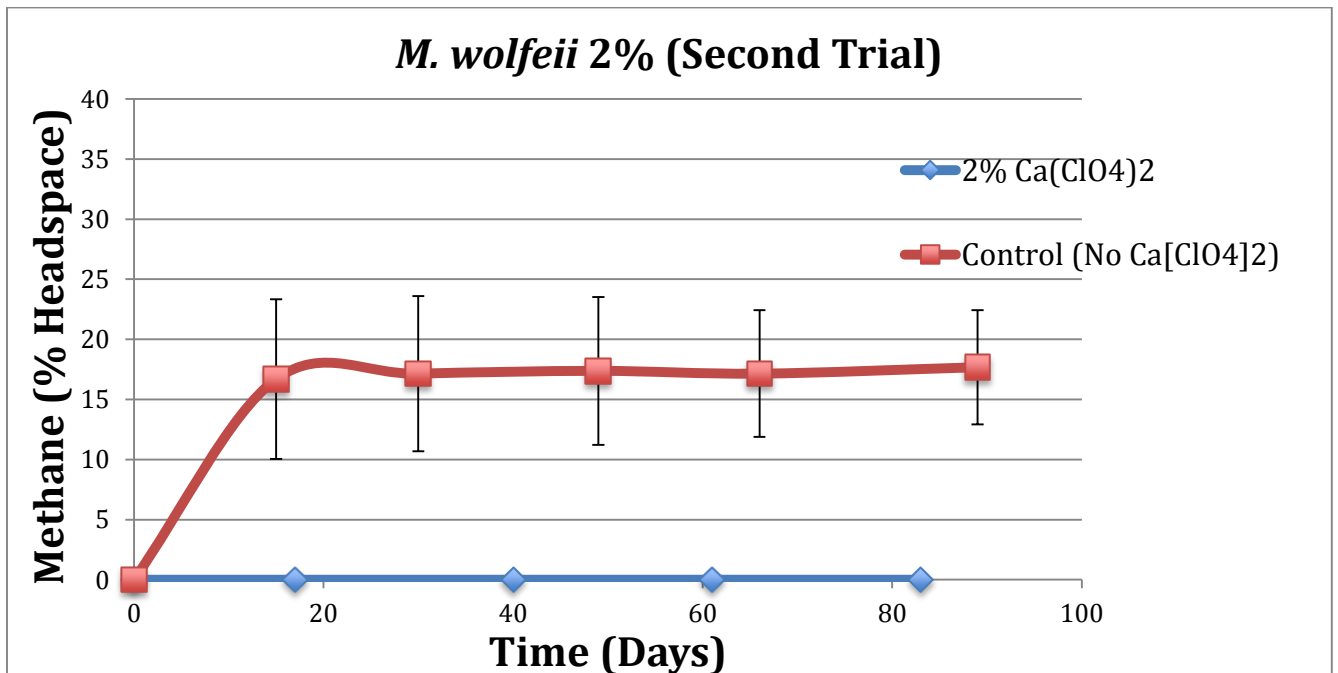


Figure 6: Methane production by *Methanothermobacter wolfeii* in the presence of 2% calcium perchlorate under anaerobic conditions – second trial.

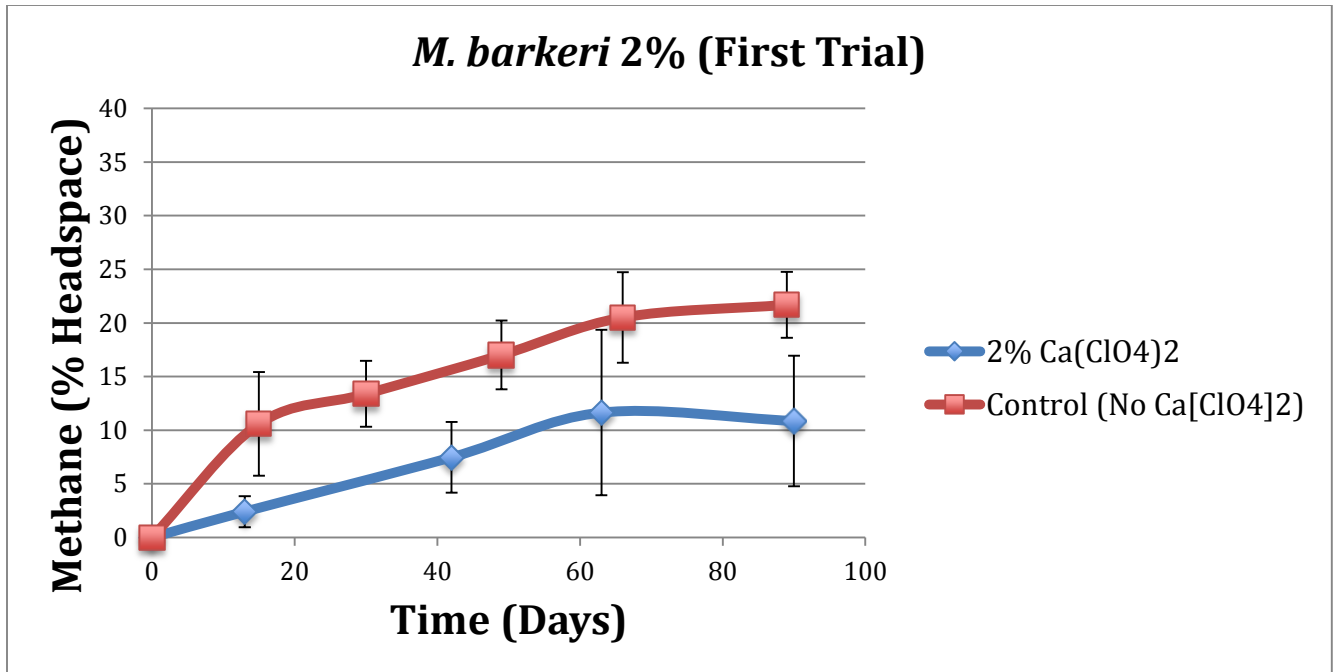


Figure 7: Methane production by *Methanosarcina barkeri* in the presence of 2% calcium perchlorate under anaerobic conditions – first trial.

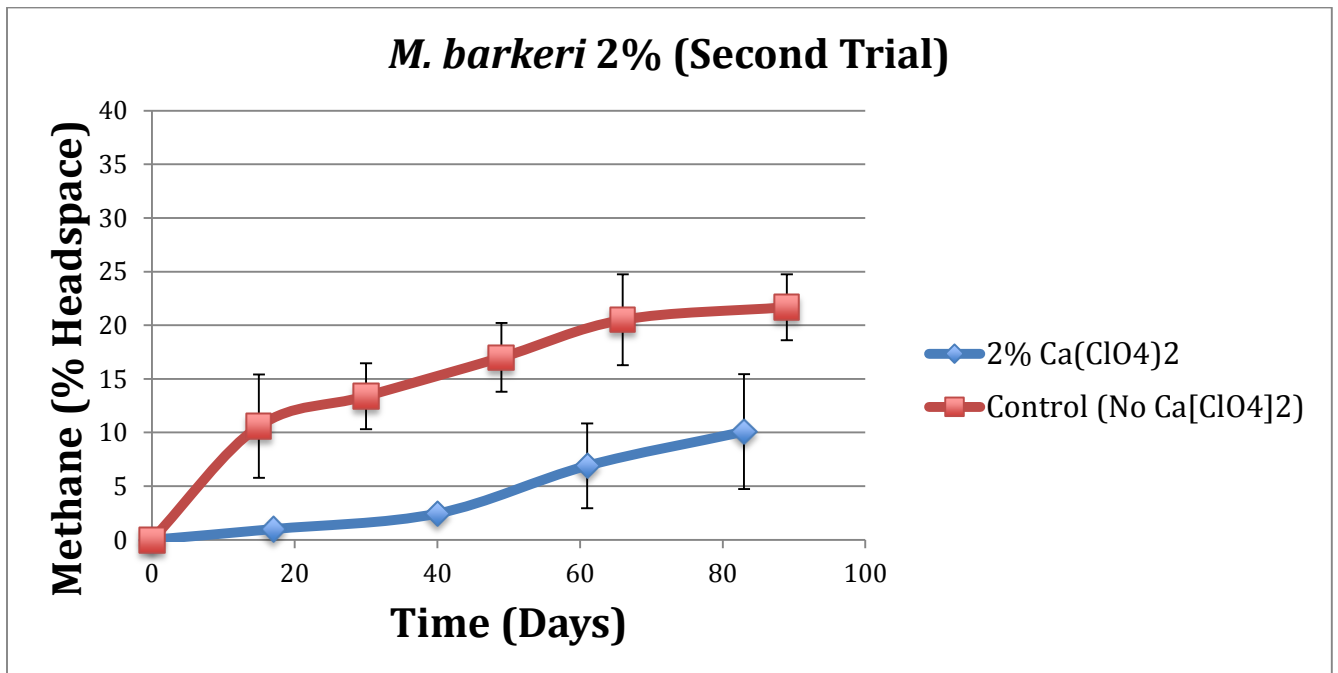


Figure 8: Methane production by *Methanosarcina barkeri* in the presence of 2% calcium perchlorate under anaerobic conditions – second trial.

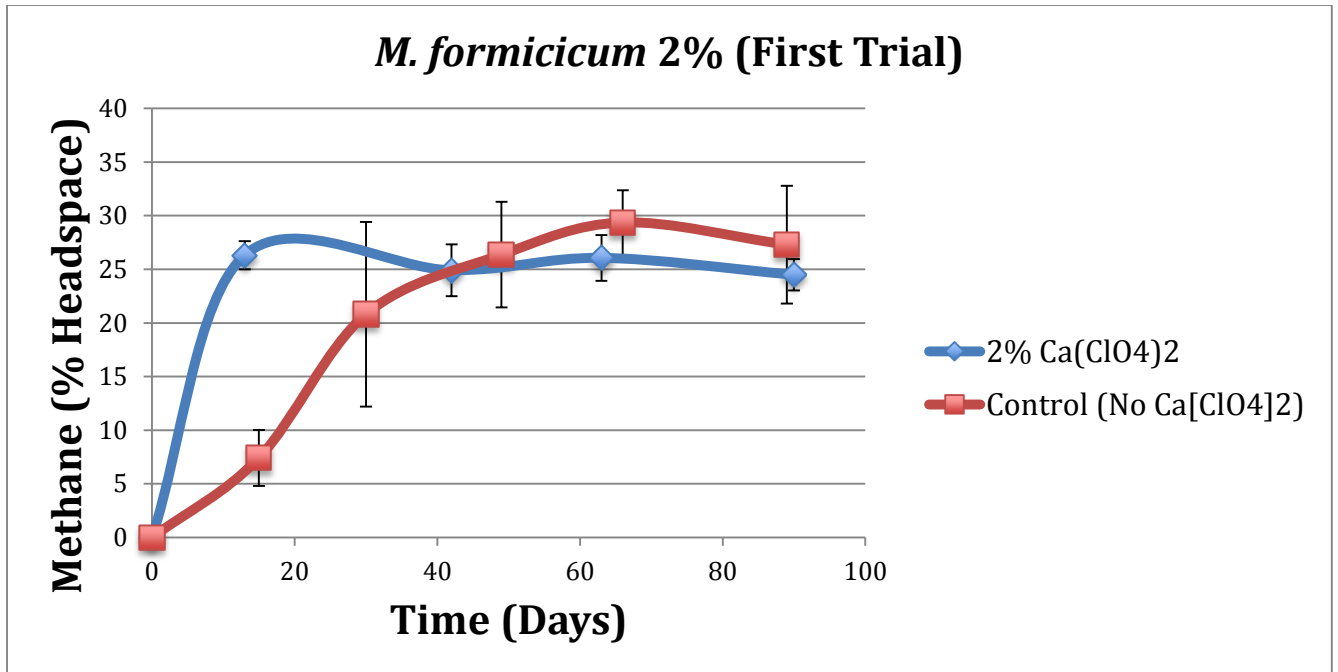


Figure 9: Methane production by *Methanobacterium formicicum* in the presence of 2% calcium perchlorate under anaerobic conditions – first trial.

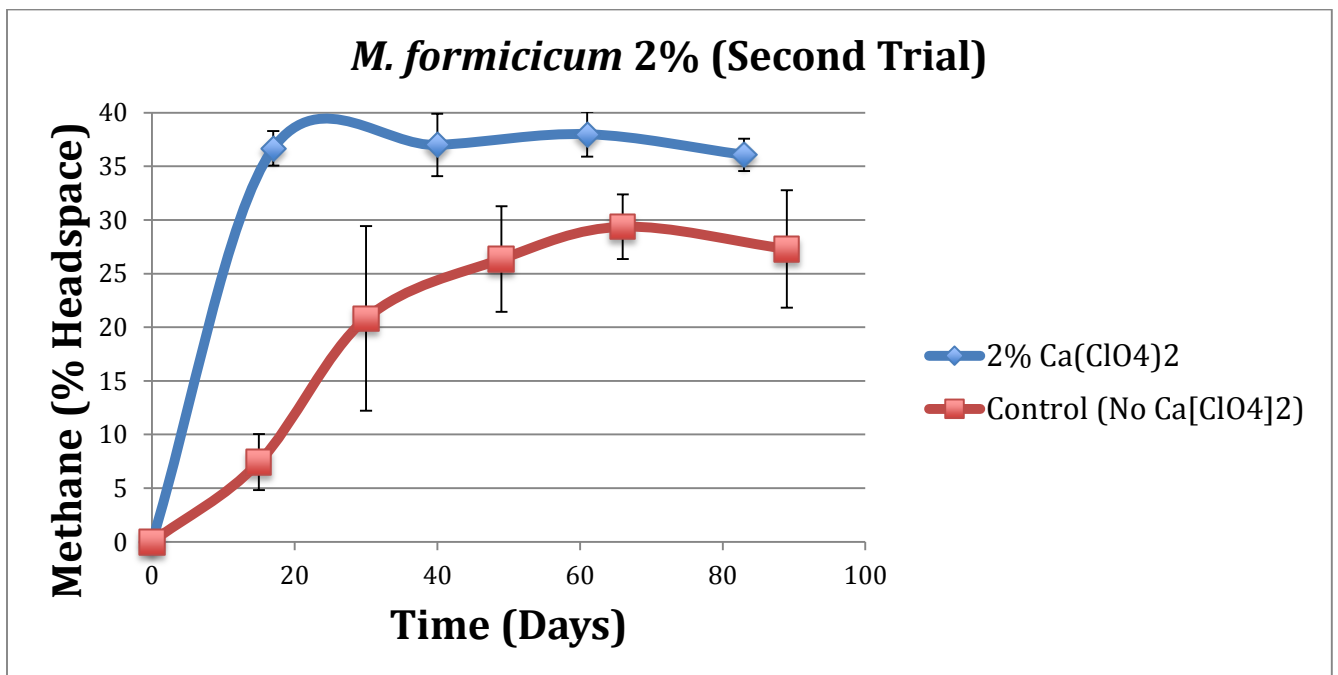


Figure 10: Methane production by *Methanobacterium formicicum* in the presence of 2% calcium perchlorate under anaerobic conditions – second trial.

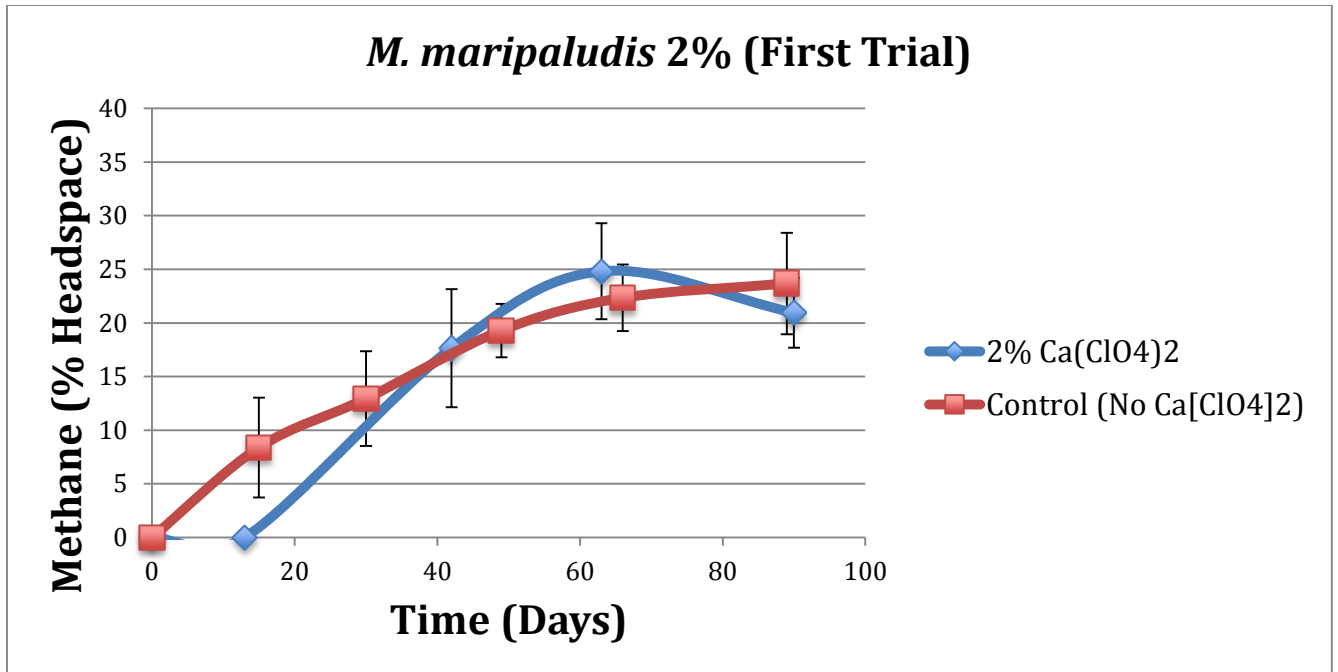


Figure 11: Methane production by *Methanococcus maripaludis* in the presence of 2% calcium perchlorate under anaerobic conditions – first trial.

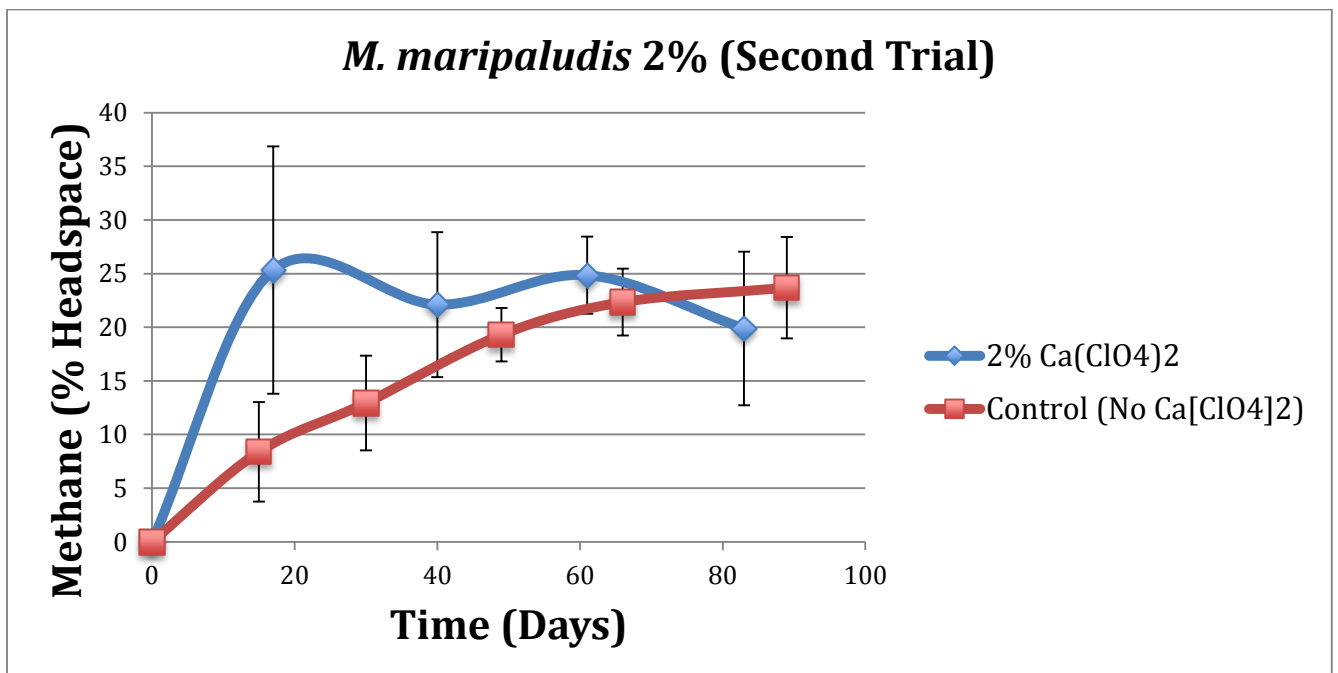


Figure 12: Methane production by *Methanococcus maripaludis* in the presence of 2% calcium perchlorate under anaerobic conditions – second trial.

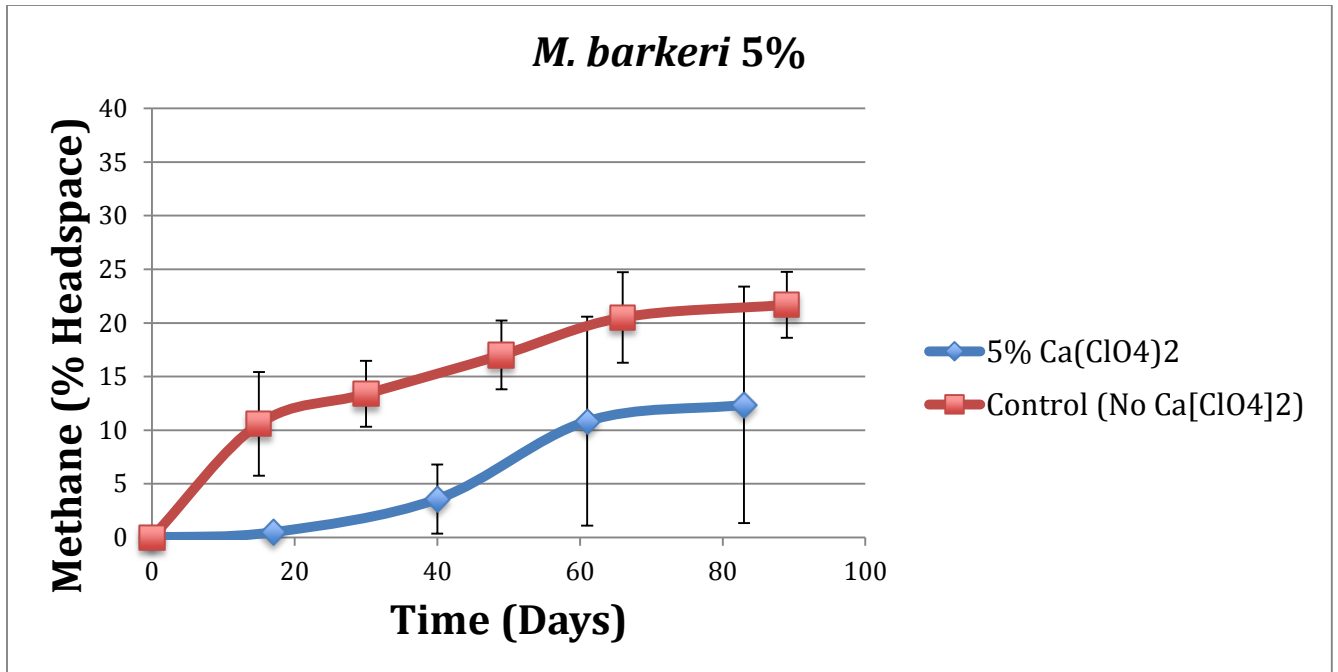


Figure 13: Methane production by *Methanosarcina barkeri* in the presence of 5% calcium perchlorate under anaerobic conditions.

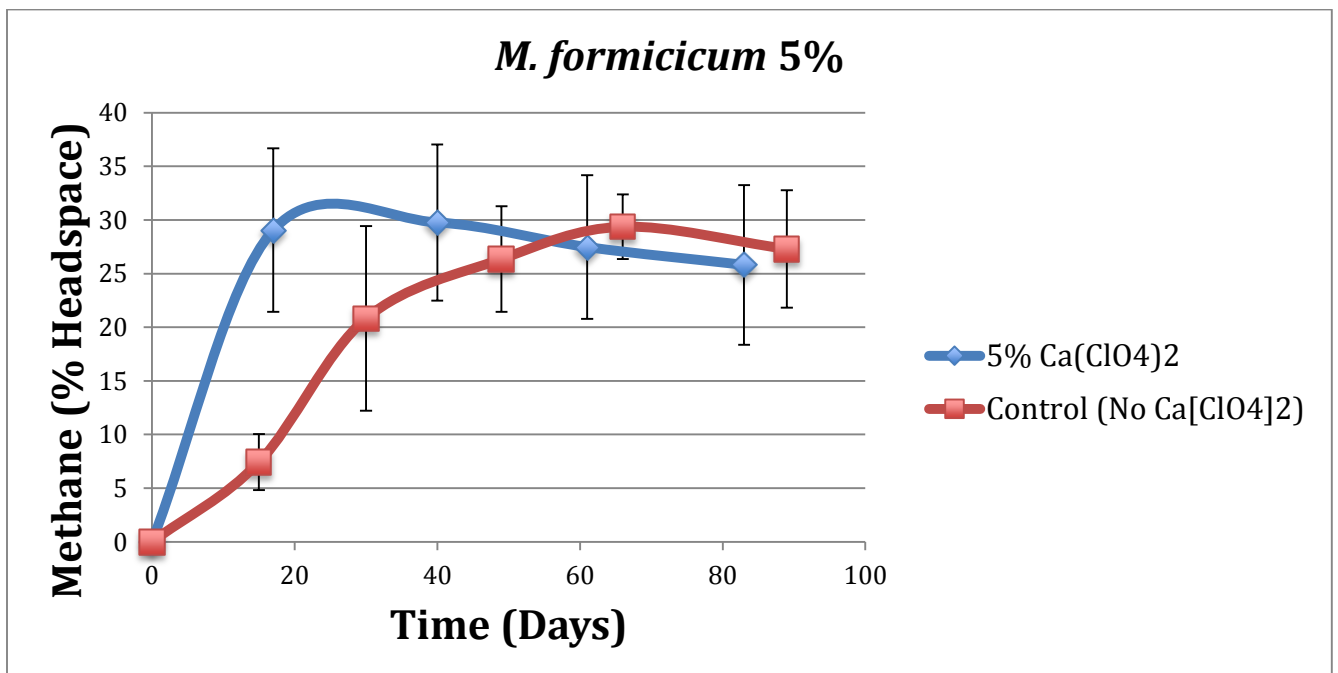


Figure 14: Methane production by *Methanobacterium formicicum* in the presence of 5% calcium perchlorate under anaerobic conditions.

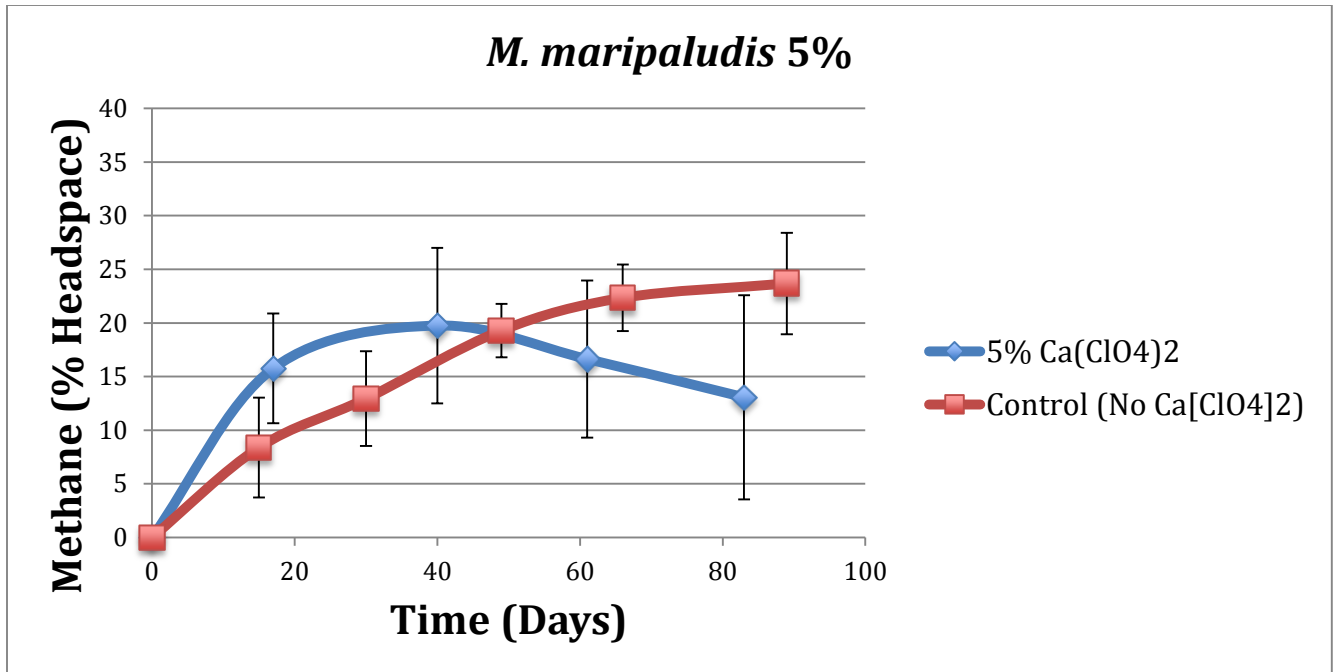


Figure 15: Methane production by *Methanococcus maripaludis* in the presence of 5% calcium perchlorate under anaerobic conditions.

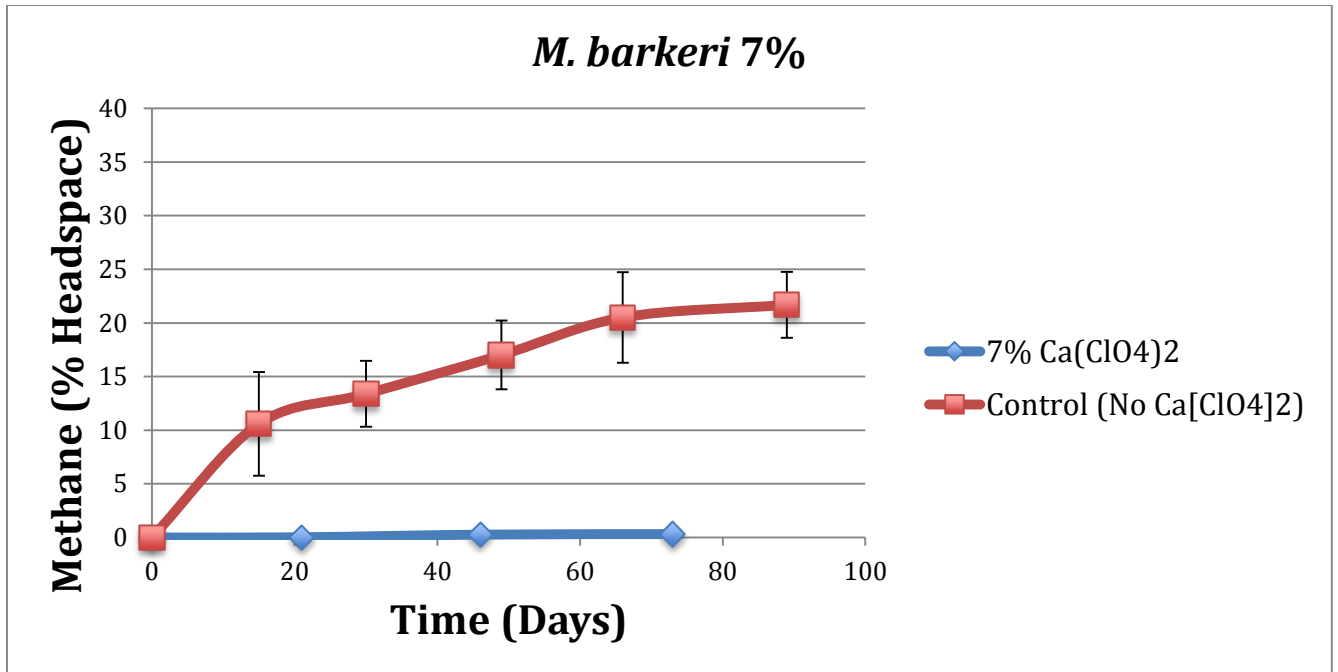


Figure 16: Methane production by *Methanosarcina barkeri* in the presence of 7% calcium perchlorate under anaerobic conditions.

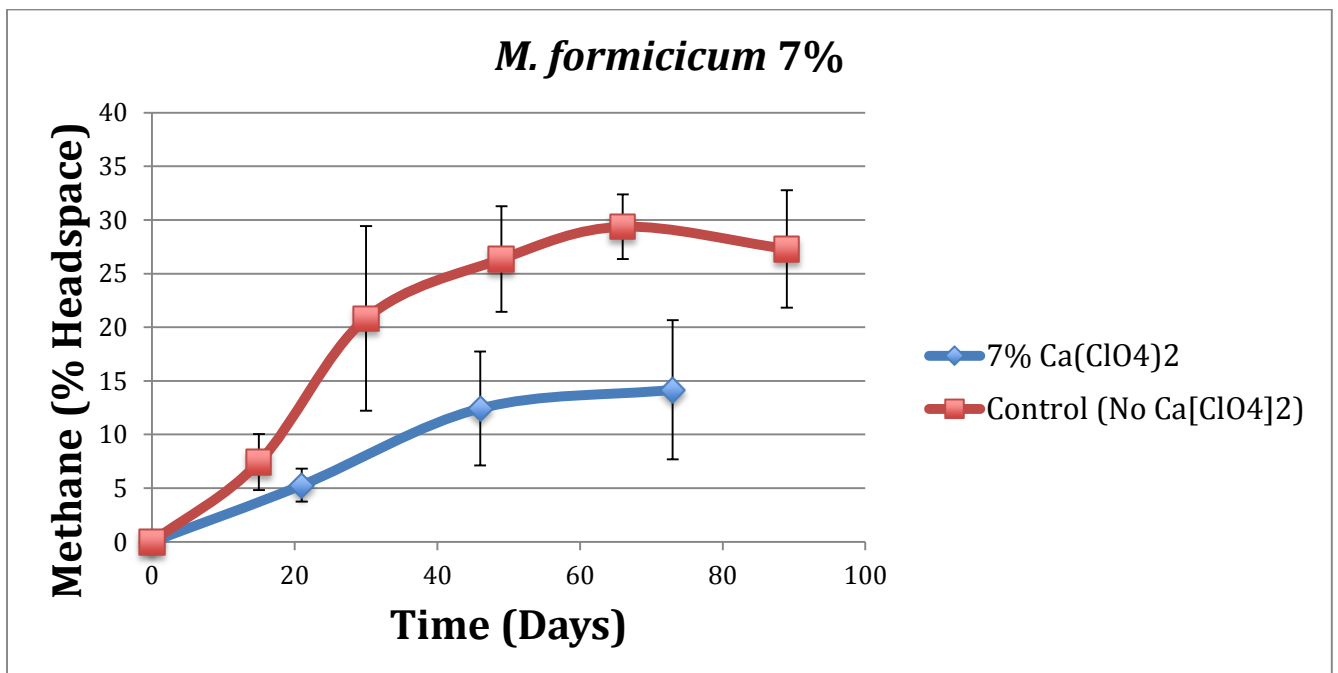


Figure 17: Methane production by *Methanobacterium formicicum* in the presence of 7% calcium perchlorate under anaerobic conditions.

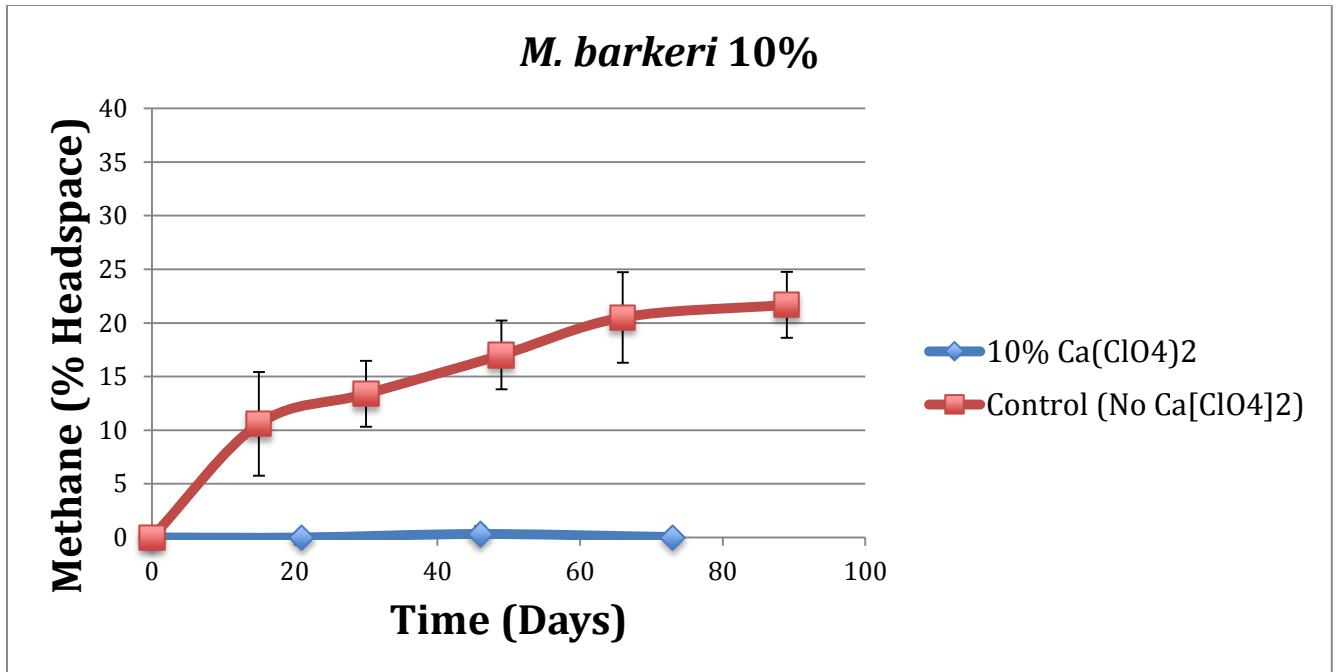


Figure 18: Methane production by *Methanosarcina barkeri* in the presence of 10% calcium perchlorate under anaerobic conditions.

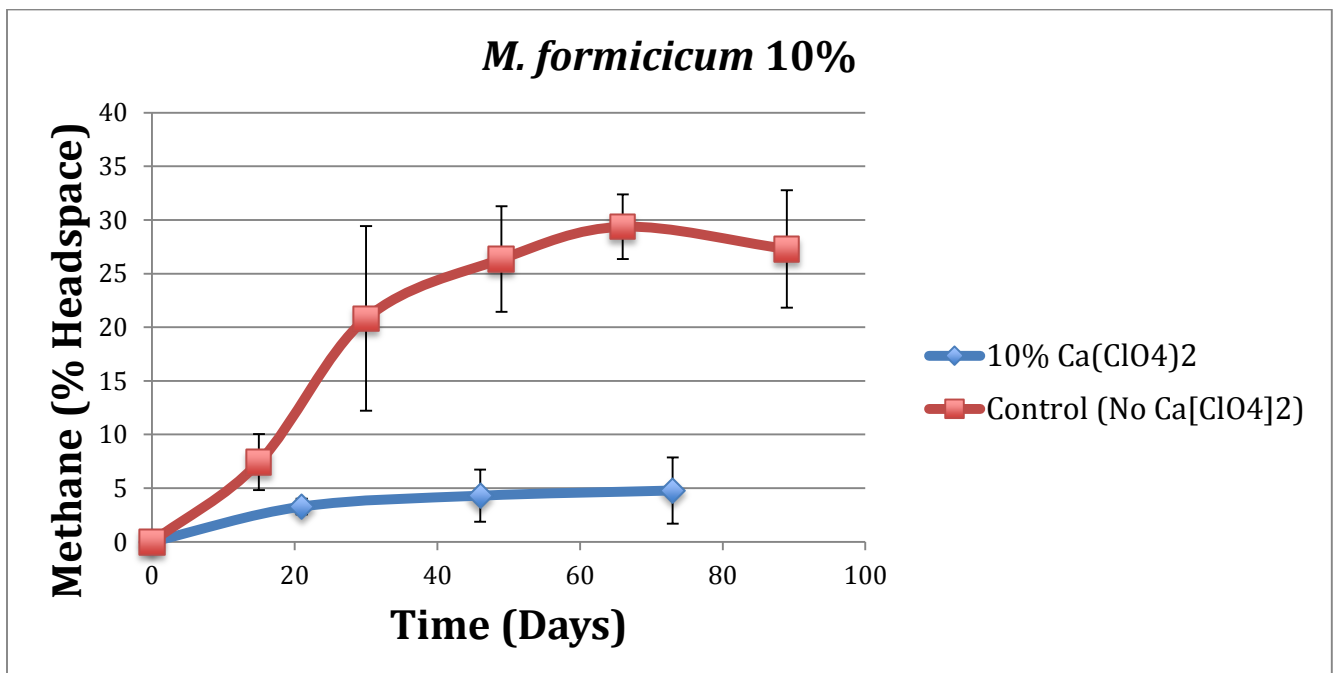


Figure 19: Methane production by *Methanobacterium formicum* in the presence of 10% calcium perchlorate under anaerobic conditions.

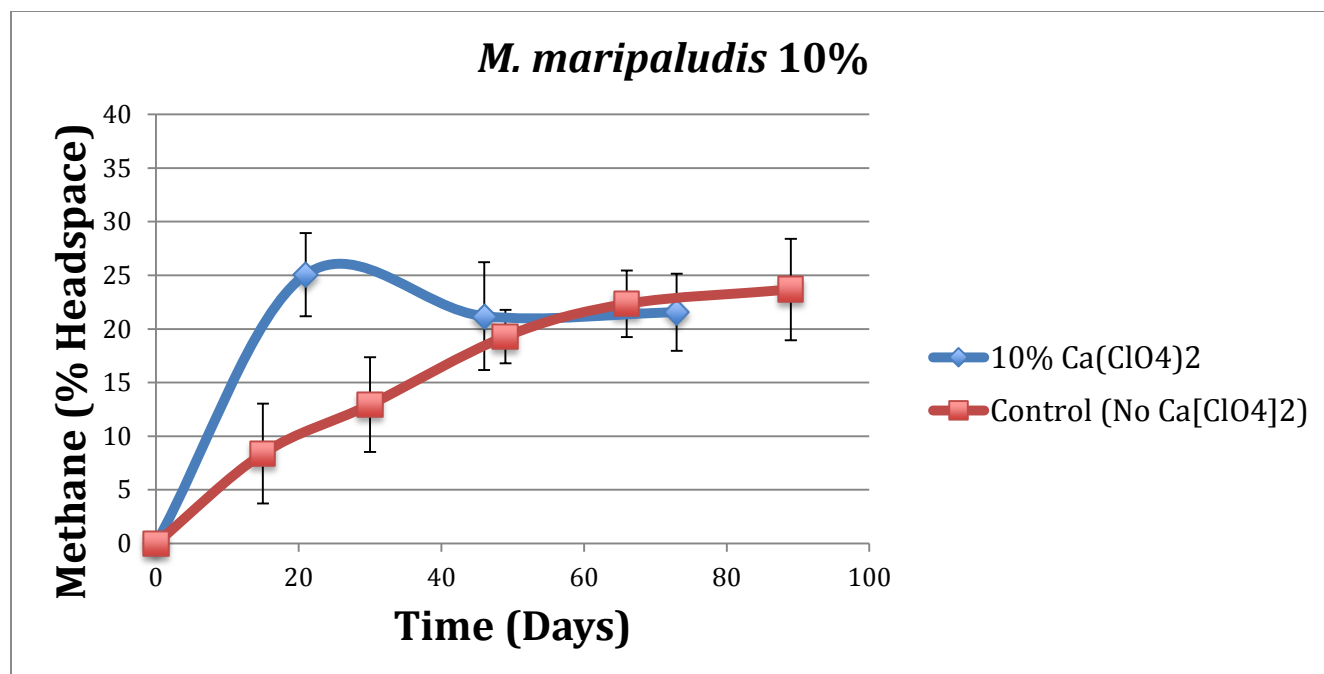


Figure 20: Methane production by *Methanococcus maripaludis* in the presence of 10% calcium perchlorate under anaerobic conditions.

Discussion

An interesting observation can be made regarding the media of these organisms in light of the results discovered in this project. Organisms that performed the strongest (*M. formicicum* and *M. maripaludis*) also grow under media conditions in which salt concentrations are the highest relative to the other media conditions for *M. wolfeii* and *M. barkeri*. MM medium has the lowest salt concentrations while MSH medium has the highest salt concentration (Appendix A). Therefore, if an organism prefers a hypersaline environment, it may be more likely to grow in the presence of perchlorate salts as well. It is unclear whether this phenomenon is due to environmental conditions, organism-specific conditions, or a combination of the two. *M. maripaludis* is a halophile, which may give it an advantage in the presence of perchlorates. The

prequel to this project sought an explanation for this trend, and it was found that in media with more salt components (e.g. MSH), perchlorate concentration was decreased. MM medium was measured and found to have little change in the originally measured perchlorate concentrations. However, MSH perchlorate concentrations decreased in some cases 20-50% [8]. Therefore, we believe that ongoing interactions with media components could be leading to a reduction in the perchlorate concentrations, which explains why the halophile performed so strongly.

Several unforeseen challenges have led to sources of error in this project. Inaccurate measurements are an inevitable source of error in this project, whether due to human error or equipment calibration error. After autoclaving the experimental media containing calcium perchlorate, precipitate was observed at the bottom of every test tube. We believe this precipitate is calcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$]. The calcium came from $\text{Ca}(\text{ClO}_4)_2$, and the phosphate comes from K_2HPO_4 contained in Solution B. It is unclear if the presence of this precipitate had an effect on the results obtained in this project. Additionally, since methanogens are strict anaerobes, they lack the enzyme superoxide dismutase. As a result of lacking this enzyme, methanogens perish in the presence of oxygen [O_2]. Several replicates experienced contamination from the aerobic environment, which limited the number of replicates in certain trials. Oxygen contamination occurs for a variety of reasons including poor technique when using a syringe and permeable equipment.

Conclusion

M. wolfeii, *M. barkeri*, *M. formicicum*, and *M. maripaludis* all grew at 1% concentrations of $\text{Ca}(\text{ClO}_4)_2$. In the 2% $\text{Ca}(\text{ClO}_4)_2$ trials, *M. wolfeii* failed to grow, revealing it had reached its apparent threshold. As the 5%, 7%, and 10% trials continued, *M. barkeri* and *M. formicicum* decreased their methane production as $\text{Ca}(\text{ClO}_4)_2$ concentrations increased. *M. barkeri* grew at 5%, but did not grow at 7% or 10% $\text{Ca}(\text{ClO}_4)_2$. *M. maripaludis* remained relatively consistent as $\text{Ca}(\text{ClO}_4)_2$ concentrations increased. This is most likely explained by the fact that *M. maripaludis* is a halophile, and potential interactions with media components may be decreasing $\text{Ca}(\text{ClO}_4)_2$ concentrations [8]. It seems that as the level of salt in the media increases, the microorganism's tolerance for $\text{Ca}(\text{ClO}_4)_2$ increases as well. Since all four species grew at 1% and three of the tested species grew at conditions up to 5% $\text{Ca}(\text{ClO}_4)_2$, the results of this project cannot reject the possibility that methanogenic Archaea may be living on Mars in the presence of $\text{Ca}(\text{ClO}_4)_2$ in the soil.

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Appendix A: Media

Methanogen Medium (50 mL)

| <u>Ingredient</u> | <u>MM</u> | <u>MS</u> | <u>MSF</u> | <u>MSH</u> |
|---|------------------|------------------|-------------------|-------------------|
| 500 μ L Solution A | X | X | X | X |
| 100 μ L Solution B | X | X | X | X |
| 100 μ L Solution C | X | X | X | X |
| 50 μ L Solution D | X | X | X | X |
| 0.1g Yeast Extract | | X | X | X |
| 0.1g Trypticase Peptone | | X | X | X |
| 0.025g Mercaptoethane Sulfonic Acid | | X | X | X |
| 500 μ L Sodium Formate | | | X | |
| 1.475g NaCl | | | | X |
| 0.085g MgCl ₂ | | | | X |
| 0.025g KCl | | | | X |

Solution A:100g/L NH₄Cl100g/L MgCl₂ + 6H₂O40g/L CaCl₂ + 2H₂O**Solution B:**200g/L K₂HPO₄ + 3H₂O**Solution C:**

0.5g/L Resazurin

Solution D:500 mg/L Na₂ + EDTA + 2H₂O150 mg/L CoCl₂ + 6H₂O100mg/L MnCl₂ + 4H₂O100mg/L FeSO₄ + 7H₂O100mg/L ZnCl₂

40mg/L $\text{AlCl}_3 + 6\text{H}_2\text{O}$
30mg/L $\text{Na}_2\text{WO}_4 + 2\text{H}_2\text{O}$
20mg/L $\text{CuCl}_2 + 2\text{H}_2\text{O}$
20mg/L $\text{NiSO}_4 + 6\text{H}_2\text{O}$
10mg/L H_2SeO_3
10mg/L H_3BO_3
10mg/L $\text{Na}_2\text{MoO}_4 + 2\text{H}_2\text{O}$