

Perturbation of a Human Gut Ecosystem by Silver Chloride Colloids

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Abstract

The bactericidal and fungicidal properties of silver (Ag) and Ag salt colloids make them popular choices for a variety of applications including water purification, bio-medical therapies for burns, epilepsy, mental illness, gastroenteritis, syphilis and gonorrhoea, amongst other diseases. In addition, individuals deliberately drink colloidal Ag and Ag salts in the belief that this self-therapy will be beneficial. Despite this widespread usage, very little is known about the impact of this metal on human gut consortia. Here we investigated the impact of Ag chloride colloids, at several concentrations, on a defined anaerobic and facultative anaerobic gut bacterial community developed from the collected stool of a healthy donor. Defined microbial ecosystem therapeutic-1 (MET-1) consortia were exposed to different concentrations of colloidal Ag (25-200 mg/L) for 48 hrs and compared to unamended cultures. Carbon dioxide and nitrogen gases recovered from the headspace of anaerobic cultures were analyzed by an Agilent Technologies 7890B Gas Chromatograph. Fatty acid methyl ester profiles were extracted following the MIDI Sherlock Microbial Identification System protocol and detected by gas chromatography. Colloidal AgCl toxicity was also monitored by a suite of DNA analyses including polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and 16S ribosomal RNA gene fragment 454-pyrosequencing. Colloidal AgCl had an overall deleterious impact on MET-1 bacterial abundance and their metabolic activities as evidenced by the 20 - 78% reduction in CO₂ respiration and significant (p<0.01) changes in fatty acid profiles, including a greater reduction (15 - 100%) in most Gram negative signatures compared to controls. Multidimensional scaling and cluster analysis produced from PCR-DGGE profile-based DNA analysis, as well as gene sequencing also demonstrated the negative impact of colloidal Ag on MET-1, resulting in a shift in the community structure, including the apparent elimination of some beneficial species. Together, this research suggests that deliberate or inadvertent colloidal Ag ingestion could have a potential negative impact on our overall digestive health.

Keywords: Colloidal silver; Silver salt; Silver chloride; Microbial ecosystem therapeutic; Toxicity; Human intestinal microbiota

Abbreviations

AgCl: Silver chloride; MET-1: Microbial ecosystem therapeutic-1; CO₂: Carbon dioxide; N₂: Nitrogen; H₂: Hydrogen; GC: Gas chromatograph; FAME: Fatty acid methyl ester; PCR: Polymerase chain reaction; DGGE: Denaturing gradient gel electrophoresis; AgNPs: Silver nanoparticles; AgNO₃: Silver nitrate; NaCl: Sodium chloride; Ag⁺: Silver ion; Cl⁻: Chloride ion; ICP-MS: Inductively coupled plasma-mass spectrometry; NCBI: National center for biotechnology information; ANOVA: Analysis of variance; UPGMA: Unweighted pair group method with arithmetic mean; MDS: Multidimensional scaling

Introduction

Silver (Ag) is a xenobiotic element; it has not been identified as an essential trace element for mammals, nor is it known to fulfill any significant physiological role in tissues even after interaction with essential elements [1,2]. Nonetheless, because of their bactericidal and fungicidal properties, Ag or Ag salt colloids have been used in many applications including cosmetics and medical treatments such as wound infections, epilepsy, mental illness, venereal diseases, gastroenteritis, syphilis, gonorrhoea amongst others [3-7]. Ag has also been used in water purification systems in many developing countries and as well as to safeguard hospital hot water systems as a protection against *Legionella* infections [8,9]. Independent of such use, there is a wide range of ingested Ag (1-27 µg/day; [10-12]), but typically, low tissue levels (~0.05 µg/g) are reported [13]. These levels can be much higher in people exposed to Ag either inadvertently by occupation, or intentionally. Some individuals deliberately drink colloidal Ag and Ag salts in the belief that this self-treatment will be beneficial, and Ag

species are then deposited into their tissues [14,15]. Indeed, a blue-grey discoloration of the human skin, argyria or 'blue man disease' is a well-known hallmark of oral colloidal Ag self-therapy [16,17]. The consequence of such exposure is not clear. Although, elemental silver in the colloid is inactive in presence of human tissues, there is the possibility of ionization in presence of body fluids [2,4]. The resulting biologically active Ag⁺ has a strong affinity for sulfhydryl groups, cell membranes and other anionic protein ligands, and finally poses a potential risk to the denaturation and inactivation of proteins and enzymes [2-4]. Disorders of the cardiovascular and reproductive systems have been detected in rats after exposure to Ag chloride (AgCl) in drinking water [18,19], and a neurological disorder has been observed after human oral ingestion of colloidal Ag [20]. Strikingly, we know very little about the impact of colloidal Ag on the human gut ecosystem.

The human gut microbiota is known to be a dense and very diverse ecosystem with more than 10¹⁴ gut bacteria playing an important role in host health [21,22]. Due to the intimate relationship between the gut

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microbiota and the host, changes in intestinal microbial communities can disrupt physiological functions with the potential for pathology [23,24]. A proxy for *in vivo* human studies can be achieved by using a synthetic stool mixture of dozens of distinct beneficial isolates, which not only mimics a healthy gut ecosystem but can be reproducibly and reliably cultured [25]. Using such a mixture, designated as microbial ecosystem therapeutic-1 (MET-1), we have recently observed that the community could be impacted by Ag, nanoparticle (AgNP) treatments [26]. As indicated, the use of Ag both in water purification systems and because of alternative health practices, appears to be increasing [5,8,9,16,27,28]. Therefore, we hypothesized that ingestion of Ag salt colloids, as aliquots of AgCl, could also perturb the human microbial community. Here we have evaluated the responses of MET-1 to AgCl colloids in three distinct toxicity assays including respiration, fatty acid methyl esters (FAME), and DNA sequence analysis using both denaturing gradient gel electrophoresis (DGGE) fingerprinting and 454-pyrosequencing.

Materials and Methods

Silver chloride colloid preparation

Aliquot of AgCl colloids were prepared from crystalline silver nitrate (AgNO₃; Fisher Scientific) and sodium chloride (NaCl; Fisher Scientific) as previously described [26]. Briefly, solutions of AgNO₃ (140 mM) and NaCl (280 mM) were made individually by dissolving in sterile MilliQ-water. The solutions were mixed (1:1) and simultaneously diluted 10-fold with sterile MilliQ-water by vortexing (~1000 rpm) to reach an AgCl concentration of ~1000 mg/L. The two-fold higher NaCl concentration ensured that no residual silver ion (Ag⁺) was present in the AgCl colloidal suspension. The final concentration of the AgCl working solution was determined by inductively coupled plasma-mass spectrometry (ICP-MS; XSeries II; Thermo Scientific, Germany), as described [29].

The human gut ecosystem culture and treatment groups

The human gut ecosystem used, microbial ecosystem therapeutic-1 (MET-1), is a synthetic stool mixture of 33 different bacterial species, formulated by extensive culture [25]. Briefly, each bacterial species was individually cultured on fastidious anaerobe agar under anaerobic conditions (90% N₂, 5% CO₂, 5% H₂), and then cultured together [25]. The MET-1 suspension was placed in an air-tight double-sealed container and a defined sterile growth medium [30] was used for the batch culture experiments [26] used in this study. The MET-1 bacterial suspension was added to defined sterile growth medium (1:10 dilution) in sterile glass serum bottles (50 mL; Sigma Aldrich) to a final volume of 5 mL for each Ag treatment including control with no Ag. The suspensions were then placed in a Ruskinn anaerobic chamber (The Baker Co., Sanford, USA) containing an appropriate atmosphere (90% N₂, 5% CO₂, 5% H₂) at 37 °C.

Triplicate cultures of each of four treatment groups consisted of 25, 100 and 200 mg/L of AgCl colloids and triplicate control cultures not amended with AgCl. After AgCl colloid additions, the serum bottles (assuming a pressure of one atmosphere of the anaerobic gas mixture in each) were clinched with 20 mm sterilized rubber stoppers and secured using Wheaton™ aluminium seals (both Fisher Scientific), and then incubated in the dark room for 48 h at 37 °C in a sealed vessel containing a Gaspak™ EZ anaerobic gas pouch (Fisher Scientific) while being shaken (~10 rpm) on a Bigbill Thermolyne shaker (Dubuque, USA). As indicated, a range of concentrations was used, since, to our knowledge, there has been no study on the toxic effects of AgCl colloids

to human gut ecosystems. For the upper level, it is known that argyria is associated with a body burden of ~65 mg/L [31], and an individual who developed complications from self-administered 'therapeutic' Ag ions was estimated to have imbibed the equivalent of 150 mg/L in all fluids in a single day [32], which would result in up to 600 mg/L in the digestive tract, discounting any cellular absorption. Since these doses would represent extreme conditions, it seemed reasonable to use them as a guide for the highest concentration tested.

MET-1 gas generation analyses

MET-1 gas generation was sampled and analyzed as described [26]. Briefly, the gas was sampled *in situ* after 24 and 48 h incubation and immediately injected into an Agilent Technologies 7890B Gas Chromatograph (GC; Palo Alto, California, USA), equipped with a stainless steel column and packed with Agilent J&W PoraBOND Q (Palo Alto, California, USA). For gas production analyses, two major peak areas of CO₂ and N₂ were determined using Agilent Technologies Chem Station Integration Software (Palo Alto, California, USA).

Fatty acid analyses

Phospholipid fatty acids were extracted from triplicate pellets, prepared from a subsample (1 mL) of each bottle by centrifugation at 2000 xg for 10 min, and subsequently using the MIDI Sherlock Microbial Identification System (Microbial ID Inc., Newark, DE, USA) as described [33] (Keystone Labs; Edmonton, Canada). Briefly, each pellet was saponified (30 min at 100 °C using 1 mL of NaOH in aqueous methanol, 15% w/v), methylated (10 min at 80 °C with 2 mL HCl in aqueous methanol, 54% w/v), extracted (10 min using 1.25 mL of hexane/methyl-tert-butyl ether, 50% v/v), washed (5 min using 3 mL of 0.3 M NaOH containing a few drops of saturated NaCl), and the organic phase analyzed with a GC equipped with flame ionization detector (Agilent). The chromatographic peak areas were converted to mol% of total fatty acids for each sample. All abundant fatty acids (>0.15% mol%) were then further analyzed and sub-grouped into Gram positive, Gram negative and saturated categories, as stated [34].

DNA extraction and PCR-DGGE

Each culture was sampled (200 µL) at the end of the 48 h incubation period and genomic DNA extracted using a QIAamp DNA stool mini kit (Qiagen Sciences, LLC, MD, USA) following the manufacturer's protocol. The extracted DNA was stored (-20 °C) until used as a template for polymerase chain reaction (PCR). Genomic DNA was PCR amplified in triplicate in a Veriti® 96 Well Thermal Cycler (Applied Biosystems, Burlington, Canada) with the primer pairs: 341F (5'-CCTACGGGAGGCAGCAG) with an additional 40-nucleotide GC-rich sequence on the 5' end (GC clamp) and 518R (5'-ATTACCGCGTCTGCTGG; [35]). The PCR conditions included denaturation for 5 min at 94 °C, followed by 30 amplification cycles (1 min at 94 °C, 1 min at 64 °C, 3 min at 72 °C) with a final extension for 7 min at 72 °C. After size confirmation by electrophoresis on 1% (wt/vol) agarose gels and visualization with an ultraviolet (uv) light transilluminator, the PCR-amplified gene fragments were then analyzed by DGGE as described [26]. Subsequently, the DGGE-generated images were analyzed by Syngene Genetools software (version 4.03.03, Synoptics Ltd.).

DNA sequence analyses

Bacterial tag-encoded pyrosequencing of MET-1 community (in triplicate treatment groups) was performed on a Genome Sequencer Roche 454 FLX Titanium platform (Roche, Nutley, NJ) by the

Research and Testing Laboratory (MR DNA; Shallowater, TX, USA) following standard protocols. Details of the sequence data processing and analysis have been described [26]. Concisely, after amplification of the 500 bp V1 to V3 region with 16S rRNA primers (Gray 28f: 5'-TTTGATCNTGGCTCAG and Gray 519r: 5'-GTNTTACNGCGGCK GCTG) [36], sequencing reads were subjected to a distributed MegaBLAST.NET algorithm and outputs evaluated by the .NET and C# analysis pipeline. Bacterial identity was based on the percentage of the total length of each sequence aligned with 16S sequences of the National Center for Biotechnology Information (NCBI) database library RDP ver 9 and also in comparison with the known MET-1 sequences. Finally, the 454 pyrosequencing distributions in each treatment group were then normalized using the corresponding total mol% fatty acids profiles [26].

Statistical analyses

The impact of AgCl colloids on MET-1 community gas production, FAME analysis and DNA sequence were assessed with two-way analysis of variance (ANOVA) using SPSS (SPSS Inc., Illinois). The significant differences between any two pairs of means were evaluated by Tukey's HSD *post-hoc* test. Dendrogram plots of DGGE bands profiles were based on the banding patterns with intensity assessed using the Pearson correlation coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) methods. Multidimensional scaling (MDS) of human gut bacterial community composition from the phylogenetic sequences of equal length was used to compare multivariate responses to the concentrations of AgCl colloids including control treatments (no Ag) using SAS Ver 9.4 (SAS Institute, Inc.).

Results

Effects on MET-1 gas production

Total gas volumes (CO₂, N₂ and unidentified gas) in the head space of the MET-1 cultures were significantly reduced by ~20 - 36% ($p < 0.05$) in the 25 - 200 mg/L AgCl treatment groups compared to control treatments. This reduction was correlated with a lower production of CO₂, which was significantly reduced ($p < 0.05$) by 20 - 78% at AgCl concentrations in a dose dependent manner (Figure 1). Consequently, the proportion of MET-1 generated N₂ gas was raised at increasing AgCl concentrations and significantly different ($p < 0.05$) at 100 and 200 mg/L AgCl treatment groups compared to the control.

Effects on MET-1 fatty acid profiles

Phospholipid fatty acid signatures were significantly influenced ($p < 0.05$) after colloid Ag treatment (Figure 2). Of 31 different fatty acid chromatographic peaks, the dominant 23 (>0.15 mol%) were selected for comparative analysis. Most Gram negative signatures were reduced by 15 - 100% in the AgCl treatment groups, whereas a few Gram positive and most saturated signature fatty acids (e.g. 18:1 ω 9C, 16:0) were increased by 13 - 45%. However, saturated capric (10:0) and stearic (18:0) fatty acids were also reduced by 65 - 90% and 30 - 35%, respectively, in AgCl treatments compared to control. Regardless of any increase or decrease, however, the total fatty acids profiles were positively correlated ($r = 0.682$; $p \leq 0.01$) with the reduction in the total gas volume after 48 h exposure. Together these independent assays indicated a colloidal AgCl-mediated toxicity for the MET-1 community.

Effects on DNA analyses

The UPGMA based hierarchical cluster analysis of DGGE banding profiles showed that after treatment with colloidal AgCl, the MET-1

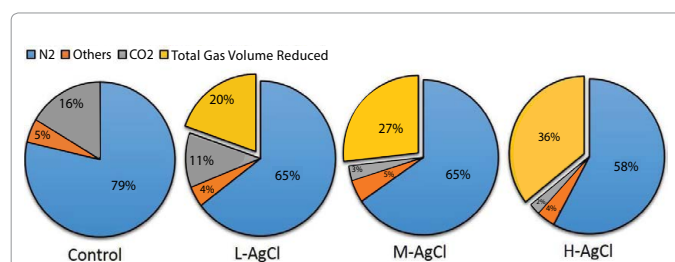


Figure 1: Carbon dioxide (CO₂) and nitrogen (N₂) gas recovered from the total gas generated by the MET-1 bacterial communities after 48 h exposure to AgCl and control (no Ag), where L-, M- and H- prefixes on AgCl represent 25, 100 and 200 mg/L concentrations, respectively. The percentage of the total gas volume reduced after exposure to AgCl colloids, compared to the control, is shown in the separated pie slice of the AgCl treatment groups.

communities grouped separately from the control group, differing by 17% - 42% depending on the concentration (Figure 3, Left Panel). PCR-DGGE analysis showed differences in banding patterns and intensities in the colloidal AgCl and control treatment groups with a few bands completely disappearing at the higher treatment concentrations (not shown).

DNA sequencing from each of the triplicate treatment groups was consistent with the PCR-DGGE results in that there was a significant reduction ($p < 0.01$) in diversity, which ranged from 14 - 36% at increasing colloidal AgCl concentrations compared to the controls (Figure 3, Panels A-D). Only relatively abundant sequences (reads $\geq 1\%$) were used in this analysis, however, it must be noted that a significant difference ($p < 0.05$) was also observed between exposure groups when the minor contributors of MET-1 community (defined as 'Others' group) were considered. For example, no sequences corresponding to the low abundant species *Collinsella aerofaciens* (~0.16%), *Dorea longicatena* (~0.2%), *Eubacterium desmolans* (~0.13%) and *Ruminococcus obeum* (~0.1%) were recovered after colloidal AgCl exposure. In addition, even at the lowest colloidal AgCl exposure, there were shifts in the evenness of the consortium. For example, the strict Gram negative anaerobe, *Bacteroides ovatus* (~35%) was significantly ($p < 0.001$) reduced by ~84% after 25 mg/L colloidal AgCl exposure compared to the control, but appeared to be less impacted at higher AgCl treatments. *Ruminococcus torques* (~2%) showed a similar reduction (80%) at the lowest concentration. Depending on the species a different effect was also observed; the proportion of Gram negative *Raoultella sp.* (~23%) and *Escherichia coli* (6%) were significantly increased ($p < 0.01$) in the lowest AgCl treatment groups, but decreased by 64% and 65%, respectively, at the highest AgCl exposures relative to the controls. The balance of the anaerobes such as *Roseburia faecalis* (~5.5%), *Eubacterium rectale* (~5%), *Roseburia intestinalis* (4%) and *Acidaminococcus intestinalis* (~2.3%) were also significantly decreased ($p < 0.01$) by 91 - 95%, 82 - 96%, 76 - 92% and 84 - 92%, respectively, after AgCl treatments (Figure 3). MDS analysis confirmed these observations and showed that the phylogenetic compositions in the treatment groups were clearly distinct from the controls (Figure 3, Panel E). Samples from the lowest AgCl exposure group were shifted away from those of the two higher concentrations as well as the controls, suggesting some variable toxicity at different AgCl concentrations.

Discussion

Research with a variety of animal models, as well as case studies in humans, have shown that health concerns associated with colloidal Ag are low, notwithstanding the dramatic change in the

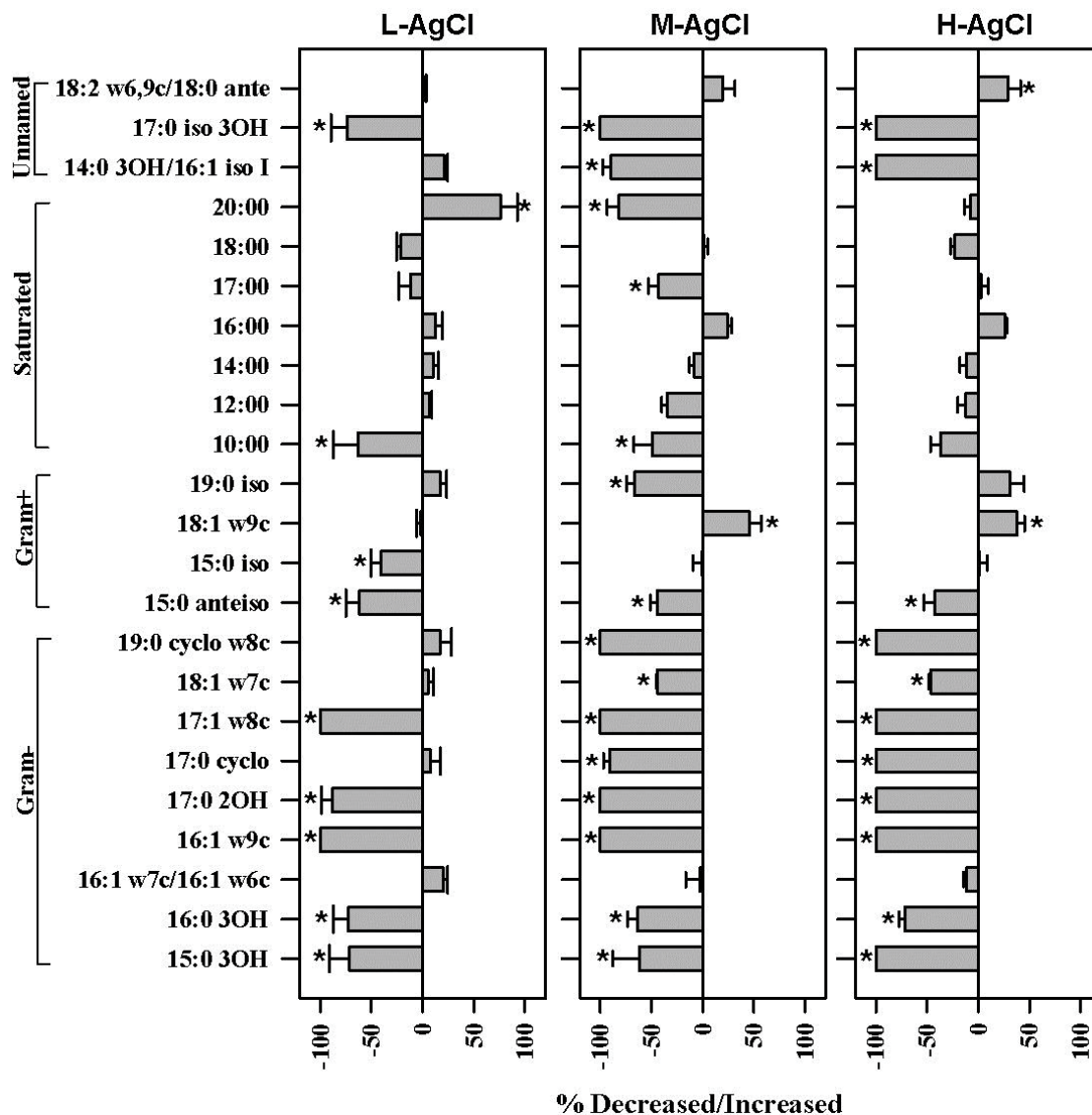


Figure 2: The percentage, either decreased or increased, in MET-1 fatty acid profiles of AgCl treatments at 25 (L-AgCl), 100 (M-AgCl) and 200 (H-AgCl) mg/L concentrations, respectively, compared to controls (no AgCl colloids). Bars represent the means of three independent fatty acid assessments and standard errors. Fatty acids are grouped according to their most-frequently associated categories. Unknown lipid indicators are indicated as 'Unnamed'. The symbol "*" represents significant differences ($p < 0.05$) compared to controls.

appearance of individuals with argyria [31]. However, the impact on the gut microbiome has not been previously explored. Here we show that colloidal Ag in the range of concentrations achieved after self-administration or inadvertent exposure can shift the microbial community significantly. Even at 25 mg/L colloidal AgCl, there was a 20% decline in respiration, fatty acid signatures of certain bacterial groups were reduced by 15 - 100%, and there was a distinct shift in the phylogenetic diversity and evenness as assessed by both the PCR-DGGE and pyrosequence analysis. The impact to the bacterial community was concentration-dependent; and at the two highest dosages used, the consortium no longer resembled the initial inoculum. It is important to recall that MET-1 was designed as a therapeutic consortium in order to treat patients suffering from pathogenic bacteria and other conditions [25,37], and thus the species used in its formulation could be defined as 'probiotic'. Therefore, the loss of any of these species may be significant. For example, *Dorea longicatena* although only representing 0.2% of the

original consortium, was completely eliminated in all of the Ag colloid treatment groups. Importantly, this species is thought to be essential for healthy microbiota homeostasis [38].

Other bacteria are central for nutrient utilization. The commensal *Bacteroides ovatus*, for example, is valued for its enzymatic contribution to carbohydrate metabolism [39]. In the presence of 25 mg/L colloidal AgCl, its abundance dropped from 35 - 6%. Under these conditions then, it is perhaps not surprising that *Escherichia* and *Raoultella*, two genera from the Enterobacteriaceae, increased in abundance since they would likely be able to utilize available carbohydrate substrates [40]. At still higher concentrations of colloidal AgCl, these genera decreased, as it is known that AgCl is highly toxic to these Gram negatives [41]. In addition, two other butyrate-producing anaerobes, *Faecalibacterium prausnitzii* and *Roseburia spp.*, which are considered to be essential due to their anti-inflammatory and immune-modulatory properties, were

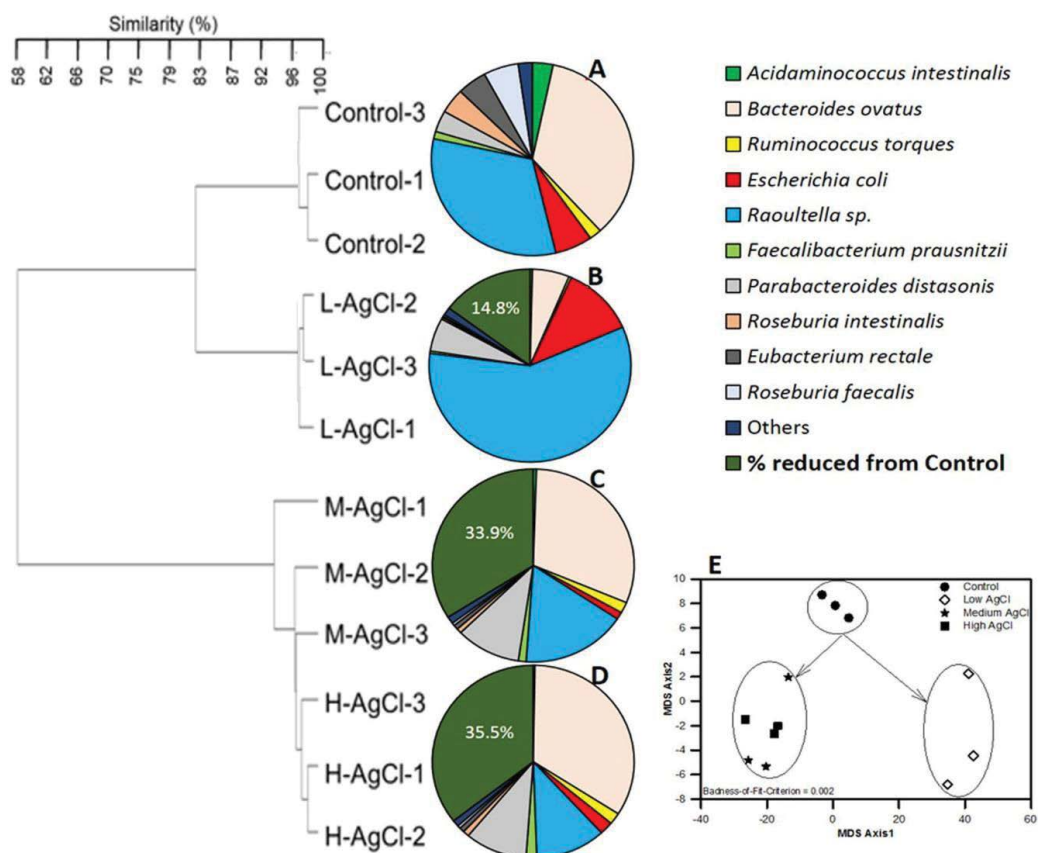


Figure 3: The impact of AgCl colloids on MET-1 DNA analyses. *Left panel* shows the UPGMA clustering analysis (dendrogram) using the observed PCR-DGGE band patterns generated after exposure to AgCl and control (no Ag), where the L-, M- or H- prefix before AgCl represents 25, 100 and 200 mg/L concentrations, respectively. Numbers at the end of the descriptors (1, 2, 3) indicate each of the replicate samples. *Mid panel* shows the human gut bacterial phylogenetic composition (species level) normalized to the total fatty acids after 48 h exposure to A) 0 (Control), B) 25 (L-AgCl), C) 100 (M-AgCl), and D) 200 (H-AgCl) mg/L AgCl colloids, respectively. Means of the triplicate samples in each treatment (with >1% relative abundance) were used for sequence identity, whereas less abundant species are shown as 'Others'. The quantity of the total phylogenetic composition reduced in different AgCl treatment groups from control (with no Ag) is shown (%) in the mid panels B, C and D. *Right Panel (Panel E)* shows multidimensional scaling (MDS) plots of human gut bacterial community composition after 48 h exposure to 0 (Control), 25 (Low), 100 (Medium) and 200 (High) mg/L AgCl, respectively. Arrows indicate the distance of the AgCl-treatment groups from the control with no Ag.

also significantly ($p < 0.01$) reduced by 68 - 92% in the colloidal AgCl treatment groups. Taken together, these observations suggest that there could be a considerable impact on the metabolic activities in our gut ecosystem after colloidal Ag ingestion.

Colloidal Ag toxicity to bacteria is reported to be a function of particle size and the release of Ag^+ in the suspension [42,43]. Similar to Choi et al. [42], the particle size used here ($\sim 2 \mu m$) was the same for every treatment group and thus the observed dose-dependent toxicity is assumed to be related to the dissociation of colloidal AgCl into Ag^+ and Cl^- . Ag^+ is known to interact with sulfhydryl groups, replacing H atoms, resulting in S-Ag bonds in the bacterial membrane, inhibiting respiration and collapsing the proton motive force, and ultimately resulting in cell death [44,45]. The two highest AgCl concentrations (100 and 200 mg/L) would have exerted high toxicity due to the dissociated Ag^+ . It is also possible that the bacteria were more sensitive to Ag^+ -mediated toxicity in the presence of the halide [46,47]. The effects of Ag^+ can be reduced in the presence of moderate concentrations of Cl^- , however, at higher levels of Cl^- , bioavailable Ag^+

increased due to the formation of dichloro-silver ion ($AgCl_2^-$). Since we used a two-fold concentration of Cl^- (as NaCl) to make colloidal AgCl in these experiments, this is the probable source of Ag^+ toxicity at the high concentrations tested.

Regardless of the mechanism, we have shown that in this model system, significant changes in human gut microbial diversity and evenness can result from colloidal AgCl exposure. Thus a possible negative impact on digestive health is a cautionary note for those that are exposed, either by self-inflicted or accidental oral ingestion.

Conclusion

The bactericidal and fungicidal properties of Ag or aliquot Ag salt colloids make them popular in a number of commercial and medical applications and have also encouraged sales of colloidal Ag as a dietary supplement. Based on our investigations of a defined anaerobic gut microbial ecosystem (MET-1), however, Ag colloid treatments (25 - 200 mg/L) had an overall deleterious impact on bacterial abundance and metabolic activity. This was evidenced by significant changes in

bacterial respiration, fatty acid profiles and community structure. Some 'probiotic' bacteria appeared to be eliminated by the Ag treatment even at the lowest concentrations tested, suggesting to us that colloidal Ag ingestion could have a potential negative impact on our overall digestive health.

Competing Interests

All authors declare that they have no competing interests.

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