Effectiveness of copper ions against *Mycobacterium avium* subsp. *paratuberculosis* and bacterial communities in naturally contaminated raw cow's milk

P. Steuer[1,3], C. Tejeda[1], O. Martinez[4], A. Ramirez-Reveco[2], N. González[1], I.R. Grant[5], A.C.G. Foddai[5], M.T. Collins[6], M. Salgado[1*]

[1]Instituto de Medicina Preventiva Veterinaria, [2]Instituto de Ciencia Animal, [3]Escuela de Graduados, Facultad de Ciencias Veterinarias, and [4]Instituto de Bioquímica y Microbiología, Universidad Austral de Chile, Valdivia, Chile; [5]Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast, Northern Ireland, United Kingdom, [6]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, USA.

Running headline: Copper to control MAP in milk

* Corresponding author: Miguel Salgado. Instituto de Medicina Preventiva Veterinaria – Facultad de Ciencias Veterinarias, Universidad Austral de Chile. Saelzer Building 5° Floor, Campus Isla Teja, PO Box 567, Valdivia, Chile. Phone: +56 63-2 444358. Fax: +56 63-293-233 (miguelsalgado@uach.cl).

Abstract

Aim

The focus of the present study was the evaluation of the copper ions treatment on the viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and other bacterial communities in cow's milk.

Methods and Results

A copper ions treatment was evaluated in naturally contaminated cow's milk to assay MAP load and/or viability; and relative abundance of other bacterial communities. In addition, physical-chemical analysis of the milk was also performed. All analyses were carried out before and after a copper ions treatment. After copper ions treatment, pH and copper concentration markedly increased in milk; the numbers of viable MAP significantly decreased. The relative abundance of the four target phyla decreased, with the phyla Bacteroidetes and Firmicutes surviving treatment in higher proportions (4% and 2.1 %, respectively). A progressively higher percentage of dead bacterial cells after 5 min and 20 min copper ions treatments was found (12% and 35%, respectively).

Conclusion

With the exception of some MAP tolerant strains, we have once again demonstrated that copper ions have a signifcant inactivating effect on MAP as well as certain other bacterial communities found in naturally contaminated cow's milk.

Significance and Impact of Study

This study showed a significant inactivation of both MAP and other bacteria by copper ions in raw cow's milk, information that could be useful as a tool for MAP control.

Keywords: milk, paratuberculosis, MAP, copper, treatment, bacterial communities

Introduction

Raw cow's milk contains a diverse microbial population and is an ideal environment for the growth of many microorganisms (Quigley *et al*. 2013). Microorganisms may enter milk from a variety of sources such as teat canal, surface of teat skin, feces, housing, bedding, feed, air, water, farm and milking equipment and farm personnel (Quigley *et al*. 2013, Velásquez-Ordoñez *et al*. 2019). At the phylum level, culture-dependent methods have revealed that the most frequent bacteria present on the bovine teat surface are Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (Verdier-Metz *et al*. 2012). In raw cow's milk, the genus present in significant proportions are lactic acid bacteria such as *Lactococcus* sp., *Lactobacillus* sp., *Streptococcus* sp., *Leuconostoc* and *Enterococcus* sp. (Quigley *et al*. 2013); and psychrotrophic microorganisms such as *Pseudomonas*, *Acinetobacter* and *Aeromonas* which flourish during cold storage (Raats *et al*. 2011). In addition, raw milk is a transmission pathway for pathogens, such as those causing mastitis (Velásquez-Ordoñez *et al*. 2019) as well as those responsible for chronic infections such as *Brucella abortus* (brucellosis), *Mycobacterium bovis* (bovine tuberculosis), and *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the cause of paratuberculosis (Skovgaard 2007).

Paratuberculosis (Johne's disease) is a chronic, contagious, infectious disease that affects domestic and wild ruminants causing chronic inflammation of the intestine (Fecteau 2018), caused by MAP. The disease causes significant economic losses mainly due to the decrease in milk production and early elimination ofthe infected animals (Lombard 2011). The organism has an extremely low metabolic activity and tends to form clusters or bacterial clumps (Harris and Barletta 2001). These characteristics make MAP highly resistant to adverse environmental conditions.

Recommendations to control paratuberculosis in dairy herds, such as surveillance and biosecurity measures, have been rather inefficient and thus new control approaches against this pathogen are necessary (Bastida and Juste 2011). Prior studies showed that MAP commonly contaminates raw milk, even when the milk originates from cows that are test-negative for paratuberculosis (Steuer *et al*. 2019).. Pasteurization has been used to reduce the MAP load in colostrum and milk. However, MAP is heat-resistant and multiple studies show that although several logs of MAP are killed, many MAP cells survive pasteurization (Grant *et al*. 1996; Grant *et al*. 2002; Hammer *et al*. 2002; Grant *et al*. 2005). MAP also has been recovered from powdered infant formula (Botsaris *et al*. 2016) and calf milk replacer (Grant *et al*. 2017), both of which are pasteurized products. Thus, there is a need for methods to inactivate MAP in raw milk.

The use of copper as an antimicrobial was officially approved for use in 2008 by the United States Environmental Protection Agency (EPA 2008).We previously showed that copper ions can cause a significant reduction of viable MAP in a liquid matrix (phosphate buffered saline, PBS), although some MAP strains tolerated copper ions (Steuer *et al*. 2018). Our study aim was to evaluate the effect of copper ions on the viability of MAP and other bacterial communities in naturally contaminated cow's milk.

Materials and methods

Study design

The milk was obtained from a dairy herd (Los Ríos Region, Chile) with a history of clinical cases of paratuberculosis and cows that had positive fecal culture results (54% of the adult herd).

The *in vitro* experiments were performed to evaluate the effect of copper ions on the viability of MAP and bacterial communities' survival in eight independent naturally contaminated

milk samples. A 1 l sub-sample of naturally contaminated milk intended for calf feeding was collected. From each milk sample, 1 ml was processed for cDNA extraction, in order to to confirme IS*900* target MAP gene presence, on the day of collection; the rest of it was frozen at -80°C for up to 2 weeks prior to the PMS-phage assay (50 ml from the original sample). Experiments were performed in duplicate.

Copper ions treatment of raw milk and ancillary study data

The copper treatment device consisted of a 0.5 l beaker glass in which two high purity copper plates were immersed. The setup of the copper treatment apparatus was the same as reported previously (Steuer *et al*. 2018). The copper plates were stimulated with a low voltage (24V) electrical current (3 Amperes) for 5 min to quickly release copper ions (i.e. complete treatment). For one experiment, the treatment time was extended to 20 min. The milk was mixed constantly during treatment.

In parallel to the main study, a complementary descriptive one, was performed. Firstly, the effect of copper treatment without the use of an electrical current was evaluated. Secondly, MAP was exposed to copper using copper sulphate ($CuSO_4$), at different concentrations (6.5 µM-100 mM). In addition, pH, conductivity, dissolved oxygen and copper concentrations were evaluated before and after the application of copper ions treatment: pH was measured by an electrode (Orion, model 420A); conductivity by an electrode (Hanna Instrumental, edge™); dissolved oxygen consumption by oximeter (Oxy 730, Inolab); and copper concentration by the atomic absorption spectrophotometry (GBC scientific equipment, SavantAA). Each determination was performed before and after treatment within the first 5 min, in triplicate, and the results were expressed as mean values.

Microbiological Analyses

Peptide mediated magnetic separation (PMS)-Phage amplification assay. This assay allowed us to quantitatively determine MAP viability in treated and non-treated milk samples. Fifty ml of milk was prepared and processed by PMS-Phage assay according to a published protocol (Foddai and Grant 2015). To confirm the identity of *Mycobacterium* sp. causing plaques, up to 10 plaques were cut from the agar and pooled before DNA was extracted and purified as described by Swift *et al*. (2013) and finally tested to confirm MAP by real-time IS*900* PCR (Salgado *et al*. 2014).

***MAP detection and bacterial load estimation using qPCR*.** To complement MAP detection and quantification by the phage assay, a real-time PCR protocol was carried out according to a published protocol (Salgado *et al*. 2014), before and after copper-treatment. Quantification was based on a standard curve for the estimation of MAP numbers by Roche 2.0 real-time IS*900* PCR (Steuer *et al*. 2018). Real-time PCR-derived copy numbers of the target region were expressed as MAP-specific bacterial cell equivalents (bce), according to Dzieciol *et al*. (2010).

Detection of viable bacterial communities in raw cow's milk. An RNA isolation, from raw milk, followed by cDNA synthesis was performed, using TRIzol method as recommended by the manufacturer (Invitrogen).

***Taxon-specific qPCR protocol*.** Primers targeting high taxonomic groups such as the phyla Bacteroidetes, Firmicutes, Actinobacteria and the γ subdivision of Proteobacteria, were used to amplify the milk-derived cDNA. The primers and qPCR conditions were those described by Bacchetti *et al*. (2011).

Relative quantification of raw milk bacterial communities taxon-specific level. The abundance of different bacterial communities present in raw milk samples before and after treatment with copper ions was compared by calculating the efficiency of each primer pair used (Mygind *et al*. 2002), and by normalizing the quantification to a control gene Bacchetti *et al*. (2011).

Live/Dead staining

A fluorescent live/dead staining technique was applied to milk samples before and after copper ions treatment for 5 and 20 min to differentiate cells treated with or without copper ions with undamaged and damaged permeable membranes. The samples were stained with Hoechst 33342/Propidium iodide, according to the manufacturer's protocol (Invitrogen). The slides were mounted, and stained cells were visualized (40x magnification) and evaluated using an inverted epifluorescence microscope (Leica DMI3000 B) coupled to a digital camera (Leica DFC 425 C). Images were processed with Adobe Photoshop 6.0. Viability percentages were estimated after examining at least 100 cells in different fields.

Statistical analysis

MAP plaque counts (PFU) and qPCR genome equivalents were measured before and after milk sample treatment. The Wilcoxon matched-pairs signed rank test was used to compare pre- and post-treatment PFU and qPCR results.

The Spearman correlation coefficient test was used to assess the relationship between numbers of MAP detected before copper treatment through PMS-phage assay and qPCR, with both counts expressed per ml of milk.

In addition, the calculated percentages of 16S taxon-specific copy numbers present in raw milk samples before and after treatment were plotted on a bar chart and differences evaluated

by a paired *t*-test. Differences in bacterial viability by live/dead staining between treatments were evaluated using the ANOVA Kruskal-Wallis test, followed by the multiple comparisons Dunn test. All data analyses were performed using GraphPad Prism 6 software, and for all analyzes, differences with $P < 0.05$ were considered significant.

Results

The application of the copper ions treatment together with an electrical current (complete copper ions treatment) resulted in a decrease, on average, of 2 $\log_{10}$ in MAP numbers in comparison to exposure to copper without an electrical current (Table 1). The complete treatment also, increased the copper concentration in milk from 0.61 to 15.0 mg l^{-1} , and the pH value in the milk sample from 7.5 before treatment to 12 (Table 1); slightly increased the electrical conductivity of the milk; and gradually decreased the oxygen concentration in the same sample (Table 1). No reduction in MAP load was observed after treating MAP-contaminated milk with copper sulphate, even at a concentration of 100 mM (Table 2).

Variable numbers of viable MAP (median $1.75x10^4$ PFU per 50 ml) were detected in raw milk by PMS-phage assay and this decreased significantly after copper ions treatment ($P= 0.0078$, mean 1.79 $\log_{10}$ reduction, Table 3). MAP numbers were also lower after copper treatment when IS*900* qPCR was employed to calculate bacterial cell equivalents (bce) ($P < 0.0063$). While IS*900* qPCR estimated 3.7 $x10^3$ MAP bce ml^{-1} before treatment, for 7 of 8 replicates, MAP was not detectable by IS*900* qPCR after copper treatment (Table 4). Spearman correlation coefficient test to assess the relationship between MAP numbers estimated by phage assay and qPCR showed a weak correlation ($\rho = -0.38$).

200 The estimated slope of the line and the efficiencies calculated for each pair of primers used in
201 this study (Universal and taxon-specific) are given in Table 5. Using 16S rRNA copy number
202 estimates, the phylum γ-proteobacteria, followed by the phyla Firmicutes and Bacteroidetes
203 dominated the bacterial communities in raw milk (Figure 1). The relative abundance of all the
204 bacterial communities in raw milk decreased after copper ions treatment (Figure 1).
205 Live/dead staining showed that copper ions treatment applied for 20 min, but not 5 min,
206 significantly reduced ($p<0.005$) the proportion viable bacterial cells compared to pre-
207 treatment controls (Figure 2).
208
209 **Discussion**
210 The focus of this research was on decreasing the number of viable MAP in milk fed to calves.
211 Numerous studies have confirmed the antimicrobial properties of copper and copper alloys
212 (Faundez *et al*. 2004; Wilks *et al*. 2005; Noyce *et al*. 2006a, 2006b; Methar *et al*. 2008; Grass
213 *et al*. 2011). In a previous study we demonstrated for the first time the ability of copper ions
214 to significantly decrease viable MAP numbers in a liquid matrix (PBS) (Steuer *et al*. 2018).
215
216 The present study included some variables (pH, conductivity, dissolved oxygen and copper
217 concentrations) that were not considered previously (Steuer *et al*. 2018). The ancillary study
218 data indicated that stimulation of the copper plates with an electrical current causes a faster
219 and larger release of copper ions from the plates compared to no application of electric
220 current. The latter is consistent with the high concentration of copper measured in milk after
221 treatment (20 times more than without electrical current application). Some studies suggest
222 that electrical current alone does not have a bactericidal effect; however, bacteria in biofilms
223 can synergistically be controlled when applying an electrical current together with biocides or
224 antimicrobial agents (Costerton *et al*. 1994; Jass *et al*. 1996; Versoza *et al*. 2019). Electrical

current may be less effective in milk since we observed that the amperage gradually decreased.

The minimal inhibitory concentration (MIC) of copper sulphate for *S. aureus*, *Salmonell*a and *E. coli* ranged from 2 - 12 mM, 20–28 mM and 16–20 mM, respectively (Aarestrup and Hasman 2004). Interestingly, we did not observe any reduction in MAP viability using copper sulphate at concentrations up to 100 mM.

Copper can act as an electron donor/acceptor by alternating between the redox states Cu^{+1} and Cu^{+2} (Karlin 1993). Therefore, the higher concentration of copper ions released, when treating milk with copper plates stimulated with an electrical current, may have reacted with the oxygen, decreasing its free concentration in milk. On the other hand, the redox properties of copper can also cause cellular damage (Grass *et al*. 2011). In addittion, the antimicrobial effects observed may have been due to the high alkalinity reached (pH 11.7) after 5 min treatment which could denature microbial structural proteins (De Benedictis *et al.* 2007) or saponify their cell wall (Katayama *et al.* 2001).

Although it is unclear the mechanism of action of the copper ions, significant inactivation of MAP ($p= 0.0063$) and the decreasing of other bacteria by the complete copper ions treatment was observed. The PMS-phage method (Foddai *et al.* 2010) revealed roughly a 2 $\log_{10}$ reduction in viable MAP counts after copper ions treatment (24 V x 5 min). This reduction is lower than that the 3 to 6 $\log_{10}$ MAP cells reported for pasteurization (Stabel *et al*. 2004; McDonald *et al*. 2005; Grant *et al*. 2005; Rademaker *et al*. 2007; Hammer *et al*. 2014).

Staining of bacterial cells with Hoechst 33342/Propidium iodide (live-dead) stain confirmed that copper ions cause a loss in the bacterial cell viability in milk, but this was only significant after a 20 min treatment (Figure 2).

MAP counts before copper ions treatment estimated by IS*900* qPCR and PMS-phage assay were weakly correlated ($\rho = -0.38$), where a slightly higher number of MAP per ml was reported by IS*900* qPCR. The lower PFU counts per ml may be explained by counting of clumps of cells rather than just single cells, although milk samples were declumped by ultrasonication, maybe different degrees of declumping were achieved (Foddai and Grant 2017). The higher estimation of MAP per ml by IS*900* qPCR could be because this technique would not have discriminated between viable cells and nonviable MAP cells. However, after copper ions treatment MAP was not detected. These results strongly suggest that copper ions cause direct DNA damage in MAP cells, as has been reported elsewhere for other bacteria (Warnes *et al*. 2010).

The phylum γ-proteocbacteria, followed by the phyla Firmicutes and Bacteroidetes, were the dominant bacterial communities detected in raw cow's milk employed in this study. Firmicutes and Bacteroidetes bacteria best tolerated copper ions treatment which suggests that copper ions may not be as effective in killing bacterial communities that resist adverse environmental conditions by sporulation. Those copper tolerant MAP strains may use a similar survival strategy since it can produce spore-like structures (Lamont *et al*. 2012), as do many members of the Firmicutes group (Filippidou *et al*. 2015).

MAP and the most copper tolerant bacterial communities may use one or more heavy metal resistance mechanisms that have been described for bacteria in general. These mechanisms include exclusion by permeability barriers, intra- and extra-cellular sequestration, active

transport membrane efflux pumps, enzymatic detoxification, and reduction in the sensitivity of cellular targets to metal ions (Borkow and Gabbay 2005). Alternatively, this apparent MAP tolerance to copper treatment could simply be a function of its thick, lipid-rich and relatively impermeable cell wall (Whan *et al*. 2001; Grant *et al*. 2002). Also, MAP in raw milk is primarily located inside somatic cells which provides additional protection (Gerrard *et al*. 2018).

Although some MAP and bacterial communities strains were observed to survive copper ions treatment, we have again demonstrated that copper ions had an significant inactivating effect on MAP, as well as other bacterial communities, occurring in milk. Whether such a treatment represents an effective and practical decontamination method for inactivating MAP in milk being fed to calves on farms requires further study.

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Conflict of interest: The authors declare no conflict of interest

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Table 1 Mean of triplicates values for MAP load, copper concentration, pH, electrical conductivity, and oxygen concentration estimated in MAP naturally contaminated milk after the application of two strategies of copper ions treatment for exposure times ranging from 0-5 min.

	MAP load		Cu [ ]		pH		E C		O_2 concentration	
Cu exposure time (min)	Complete Cu TT[o]	Cu TT w/o E	No TT	Complete Cu TT	No TT	Complete Cu TT	No TT	Complete Cu TT	No TT	Complete Cu TT
0	$4.1x10^4$	$4.2x10^4$	0.60	0.69	7.5	7.5	4.6	4.6	4.6	4.6
1	$2.2x10^4$	$3.8x10^4$	0.61	1.9	7.5	7.7	4.5	4.6	4.6	4
2	$1.0x10^4$	$3.2x10^4$	0.60	3.5	7.5	7.8	4.6	4.6	4.5	3.4
3	$6.3x10^3$	$2.8x10^4$	0.62	5.7	7.5	9.1	4.5	4.7	4.5	3.1
4	$1.8x10^3$	$2.1x10^4$	0.60	9.38	7.5	10.8	4.6	4.9	4.5	3
5	$4.2x10^2$	$1.1x10^4$	0.61	15.6	7.5	11.7	4.5	5.1	4.6	2.8

MAP load: expressed in bce ml^{-1}:

Cu [ ]: copper concentration expressed in mg l^{-1}

EC: electrical conductivity expressed in mS cm^{-1}

O_2: oxygen concentration expressed in mg l^{-1}

Complete Cu TT: copper plates immersed in the milk samples and stimulated with a low voltage (24V) electrical current (3 Amperes) for 5 min

Cu TT w/o E: copper plates immersed in the milk samples without the application of the electrical current

No TT: no treatment with copper ions on the MAP contaminated milk sample

507 **Table 2** Mean MAP load (expressed as bce ml^{-1}) after treatment of naturally contaminated
508 milk (in triplicate) with different concentrations of copper sulphate ($CuSO_4$) for 1 and 7 days
509 of exposure.

	MAP load (bce ml-1)	
$CuSO_4$ concentration	**Day 1**	**Day 7**
6.5 µM	$4.2x10^4$	$4.3x10^4$
12.5µM	$4.1x10^4$	$2.2x10^4$
25 µM	$3.1x10^4$	$2.0x10^4$
50 µM	$2.5x10^4$	$3.8x10^4$
100 µM	$2.0x10^4$	$5.2x10^4$
250 µM	$3.3x10^4$	$4.1x10^4$
500 µM	$4.5x10^4$	$6.2x10^4$
750 µM	$3.2x10^4$	$8.1x10^4$
1.0 mM	$8.9x10^3$	$5.1x10^4$
1.5 mM	$9.2x10^3$	$5.3x10^4$
2.0 mM	$7.6x10^3$	$6.2x10^4$
2.5 mM	$1.7x10^4$	$4.8x10^4$
3.0 mM	$1.5x10^4$	$4.x10^4$
3.5 mM	$2.3x10^4$	$4.2x10^4$
4.0 mM	$9.5x10^3$	$3.1x10^4$
4.5 mM	$8.5x10^3$	$1.2x10^4$
5.0 mM	$1.0x10^4$	$3.3x10^4$
10.0 mM	$1.5x10^4$	$6.2x10^4$
50.0 mM	$2.2x10^4$	$3.7x10^4$
100.0 mM	$1.1x10^4$	$2.4x10^4$

510 **Table 3** MAP counts (mean PFU per 50 ml of the duplicates) in raw milk samples determined

511 by PMS-phage assay before and after treatment with copper ions at 24V for 5 min

Milk sampling	**Untreated milk samples**	**Copper-treated milk samples**	**Log_{10} reduction observed**
I	$1.0x10^5$	$1.5x10^4$	0.82
II	$3.3x10^4$	$4.0x10^2$	1.91
III	$1.8x10^1$	3.0	0.78
IV	$1.1x10^2$	<1.0	2.04
V	$3.3x10^2$	<1.0	2.51
VI	$5.0x10^4$	$4.0x10^2$	2.09
VII	$2.0x10^3$	$1.5x10^1$	2.12
VIII	$4.0x10^4$	$3.8x10^2$	2.02
Median value (range)	**$1.75x10^4$ ($1.8x10^1$-$1.0x10^5$)**	**$1.97x10^2$**	**1.79**

512 *Only experimental results validated with proper positive and negative controls have been

513 included in this table.

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516 **Table 4** MAP load estimations (mean MAP bce ml^{-1} of the duplicates) determined by qPCR

517 for untreated and copper-treated (24V for 5 min) raw milk samples

Milk Sampling	Untreated milk samples	Copper-treated milk samples	Log_{10} reduction observed
I	$3.83x10^3$	ND	3.58
II	$3.56x10^3$	ND	3.55
III	$5.24x10^3$	ND	3.72
IV	$4.49x10^3$	$2.3x10^1$	2.29
V	$1.16x10^3$	ND	3.06
VI	$4.75x10^2$	ND	2.67
VII	$3.38x10^4$	ND	4.52
VIII	$6.23x10^2$	ND	2.79
Median value (range)	**$3.69\ x10^3$ ($4.75x10^2$-$3.38x10^4$)**		**3.27**

518 ND: no MAP DNA detected.

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530 **Table 5** Calculated primer pair efficiencies used in the taxon-specific qPCR assay

Target taxon	Primer pair	Slope of the line	Efficiency (%)
Universal	907F+106R	-3.42	96.06
Actinobacteria	Act920F3+act1200R	-3.5	93.1
γ-Proteobacteria	1080μF+μ1202R	-3.3	100.9
Bacteroidetes	798cfbF+cbf967R	-3.47	94.1
Firmicutes	928firmF+1040firmR	-3.63	88.5

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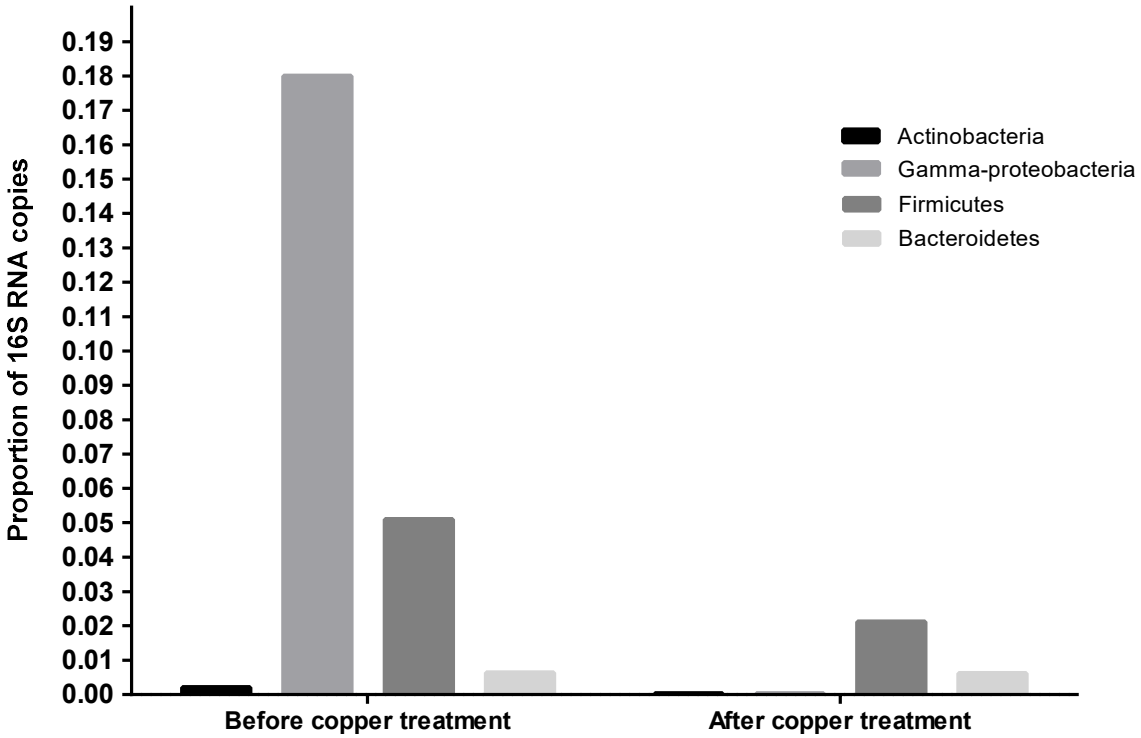
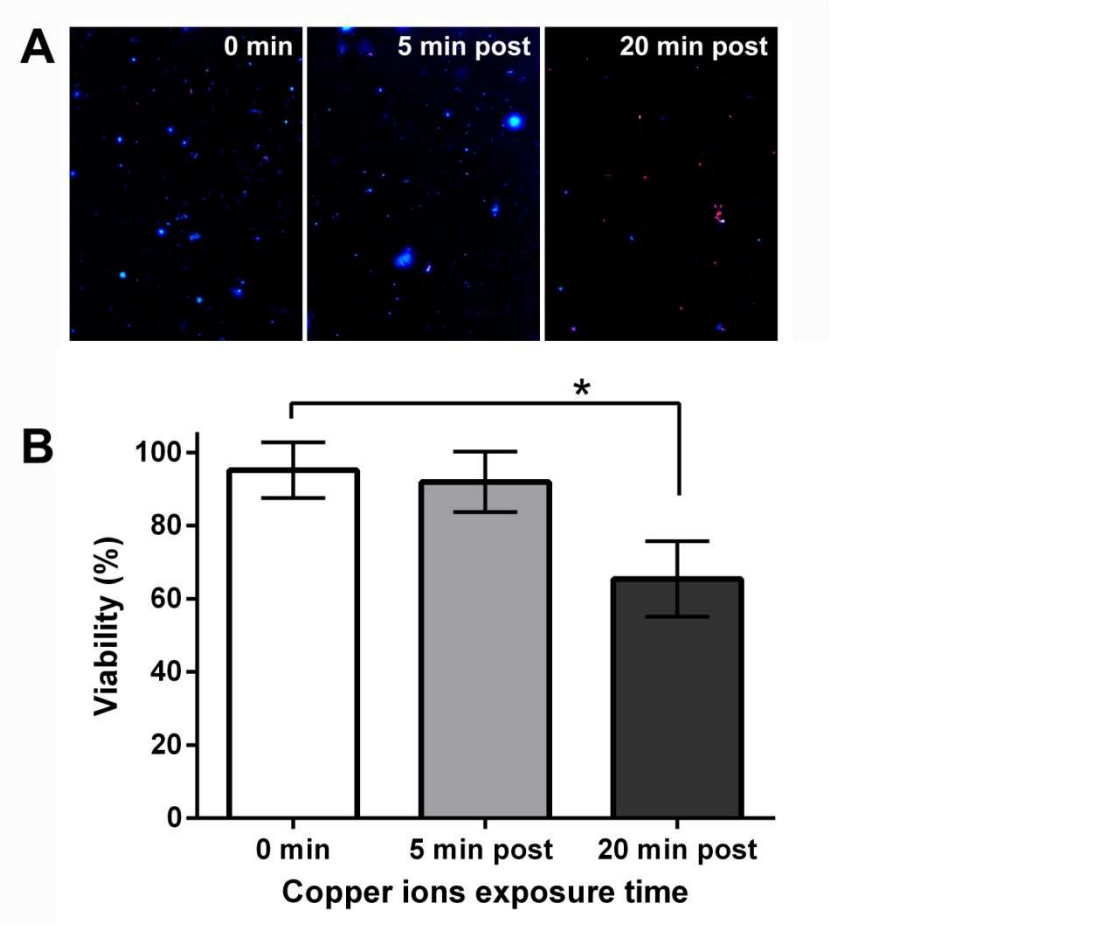


Figure 1. Relative quantification of 16S rRNA copies belonging to each phylum present in raw milk samples before and after treatment with copper ions.



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550 **Figure 2.** Plasma membrane integrity or viability (%) analysis by Hoechst 33342/Propidium
551 iodide stain. A: Representative field captures for plasma membrane integrity analysis by,
552 blue green or red fluorescents marks, corresponding to bacterial cells recognized as live or
553 dead, respectively, after different copper ions exposure times (0, 5 and 20 min) B:
554 Quantification of the cell viability loss after the same copper ions treatments. A minimum of
555 100 cells were counted for each sample. Each bar represents the mean ± SD of a total of three
556 independent experiments. Significant differences between exposure times are shown one-way
557 ANOVA Kruskal-Wallis / Dunn tests (*: $p < 0.05$).

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