Evaluation of Antibacterial Activity of Copper Sheets in the Germination of Alfalfa Seeds (*Medicago sativa* L.) within a Rotating Drum

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ABSTRACT— ***The consumption of sprouts in the human diet has grown during the last years, but great concern raised from public health institutions, food industry and consumers regarding their safety since foodborne diseases caused by microorganisms have been reported. Copper metal as a contact surface was studied during the germination of alfalfa seeds (Medicago sativa L.) inside a rotating drum on a laboratory scale and compared with a plastic surface of food-grade. A system of three rotating drums was used inside a thermo-regulated chamber to germinate seeds. To evaluate the antibacterial activity of copper sheets, alfalfa seeds were inoculated with 4.2 log cfu g^{-1} of Escherichia coli and after 84 hours of germination sprouts were evaluated for E. coli, mesophilic aerobic bacteria, the content of copper and other minerals (potassium, calcium, magnesium, sodium, iron, manganese, and zinc), total mass, unit mass and length, and color. The contact of alfalfa sprouts with copper sheets allowed to reduce the E. coli load from 6.54 to <0.1 log cfu g^{-1}. However, all sprouts exceeded in copper (> 10 ppm) according to Food Sanitary Regulations. Germinated mass and length decreased after copper treatments. No statistically significant differences were observed between treatments for the remaining quality parameters. Finally, it is concluded that copper was very efficient in reducing the microbial load of E. coli in alfalfa sprouts, complying with the regulations established by the Chilean Ministry of Health.***

Keywords— Sprouts, alfalfa, *Escherichia coli*, copper, food safety

1. INTRODUCTION

The consumption of minimally processed vegetables has grown rapidly in recent years, but great concern raised from institutions that deal with public health, the food industry and consumers regarding their safety, as a result of foodborne diseases caused by microorganisms [1]. Sprouts can be produced from a diverse range of seed types, which are monitored daily for humidity, temperature and luminosity conditions. Intrinsically they have a complicated food safety situation compared to other fresh products since possible microbial contaminants can multiply during the first days of germination, being able to reach levels being high enough to cause diseases [2]. Many cases of food sprouts from germinated seeds are associated with diseases caused by *Salmonella enterica* and *Escherichia coli* O157: H7 [3]. It has been shown that seeds are vectors for the transmission of a series of foodborne infection outbreaks. Among the seeds involved are alfalfa, fenugreek, clover, mung bean, and soybean. An increase is observed in recent years, being alfalfa sprouts the most common infectious vector [4]. The U.S. Food and Drug Administration recommends sanitizing seeds for sprouts with 20,000 ppm of calcium hypochlorite before germinating and testing of spent irrigation water for pathogens. Epidemiological evidence suggests that this sanitization protocol may prevent some outbreaks, but cannot completely prevent them. Experiments with radish and alfalfa sprouts grown from contaminated seeds have shown that human pathogens can be present within sprouts, even among plant cells, where they can resist decontamination treatments [5].

Alfalfa (*Medicago sativa* L.) is a plant that belongs to the legume family. Seeds in the mature state are approximately 1-2 mm long by 1-2 mm wide and 1 mm thick. It is considered the queen of foragers around the world since 32 million ha are cultivated [6]. Germination is the process by which a seed changes into a new plant. This process is carried out when the embryo swells and the cover of the seed are broken, which increases the bioavailability of nutrients and the palatability of the product [7]. The seed requires adequate conditions for germination, such as the presence of oxygen, light, heat, and humidity that determine the development of the characteristic smell and taste of sprouts. The seeds begin to germinate after absorbing about 125 % of their mass in water and swelling to break the seed cover. Alfalfa can germinate at a temperature above 3 °C, with an optimum germination temperature between 18 and 25 ° C [8].

Some technologies provide sanitization of sprouts, which are applied during pre and or post-harvest. These methods include physical, chemical, and microbiological treatments [9]. Physical methods include cold, heat, high pressure, irradiation, supercritical carbon dioxide and ultrasound treatments [10, 11, 12]. Chemical interventions consist of

sanitizers such as ozone, chlorine and electrolyzed water [13, 14, 15]. Biological interventions consist of the use of antagonistic microorganisms, antimicrobial metabolites, and bacteriophages. Combined methods that make use of these technologies should be used to carry out more efficient decontamination of seed and sprouted samples [2]. However, organoleptic parameters such as smell, color, taste, and turgor of the final product can be affected.

Several studies have demonstrated the antimicrobial capacity of copper and its alloys to bacteria, fungi, and viruses [16, 17, 18, 19, 20]. The mechanisms by which this occurs are not fully understood, but some studies indicate that the copper surface eliminates bacteria by an attack consisting of three mechanisms: bacterial membrane damage, DNA degradation and intracellular damage inside the membrane plasma [21,22, 20]. The survival of *E. coli* O157 on seven 1 cm x 1 cm copper plates with different degrees of purity has recently been evaluated and compared with stainless steel. Culture plate counts, as well as those performed by microscopy, showed that three alloys, 95 % (C87300), 93 % (C83300), and 85 % (C83600) of copper percentage could completely eliminate bacterial inoculums when they were held at 22 ° C for 6 hours. It was also revealed that when the number of bacteria at the beginning of the experiment was lower than 10^3 colony forming units (cfu), all copper alloys were highly active and completely eliminated contamination. Noyce et al. (2006) [17] concluded that the viability of the *E. coli* O157 pathogen could be significantly affected by three factors: the composition of the substrate or surface alloy, ambient temperature, and the presence of beef juice. Copper due to its antimicrobial properties could be a valuable tool to contribute to the safety of sprouts against *E. coli* [23]. Different concentrations of dissolved copper cause metabolic disorders and growth inhibition for most plant species [24]. Rice and wheat have been studied, having a low tolerance to high concentrations of copper sulfate pentahydrate [25, 26]. Also in alfalfa and corn, an increase in its germination rates has been found at low concentrations of copper sulfate pentahydrate [27, 28], where it has been shown that plants can not only survive at low concentrations, but also prefer low concentrations of dissolved copper for better development. Commercial production of sprouts generally involves the use of rotating drums, bins, trays or within pack. In general, small sprout producers tend to use only trays, while large producers use just rotary drums or use trays and drums. For commercial reasons, the trend in the industry seems to shift towards the use of rotating drums [29]. Handmade production of sprouts implies the germination of seeds in previously sanitized glass jars. The protocol described by Fu et al. (2001) [30] refers to how experiments and different sprouting operations influence microbial growth, water irrigation and rinses with sterile tap water, to determine the effect of the frequency of irrigation on the growth of *Salmonella* and *E. coli*.

The hypothesis of this experimental work postulates that the contact area of a copper surface between alfalfa seeds and water is able to reduce the burst of *E. coli*, without affecting the quality and mass of sprouts. The general objective was to implement a laboratory-scale rotary drum system, which allows evaluating the antibacterial capacity of metallic copper sheets during the germination process of alfalfa seeds (*Medicago sativa* L.).

2. MATERIALS AND METHODS

2.1 Germination equipment

The characteristics of the design and construction of the germinator were based on the recommendations described by Fu et al. (2008) [29], which describe a reference model of equipment consisting of a rotary drum germination chamber connected to a motor, a controller and a solenoid valve sprinkler system connected to a controller (Figure 1). The construction of the equipment and its automation was carried out in the Robotics Laboratory of the Faculty of Agricultural Engineering, Universidad de Concepción. The germination chamber was built with plywood and glass wool as an insulating material, with a wall thickness of 5 cm, and dimensions of 163 cm × 53 cm × 112 cm. The interior was painted with enamel resistant to ultraviolet radiation and fungi. An air replacement system was installed inside the chamber, which consists of an air inlet and an axial extractor. Inside the cabinet, three rotary drums were installed, made of hydraulic PVC pipes of 300 mm diameter, 200 mm long and a wall thickness of 7 mm. Each drum was divided into four equal quadrants by means of food-grade PE-MHW plastic plates to introduce seeds for germination. In each compartment, two conical type micro-sprinklers were installed, for seed irrigation. The drums were equipped with plastic covers with windows and a mesh to aerate the sprouts. The three germinators were installed on a drive roller in parallel with a driven roller. The drive roller was driven by a 12 V DC motor with 10 rpm gearbox, coupled to an axle and mounted on bearing support (Figure 2.). This design allows a load capacity of 125 grams of dried seeds per drum, equivalent to 300 grams of soaked seeds. A rotation speed of 1 rpm, the ambient temperature of 30 ° C and an irrigation frequency of 200 mL of water sprayed for 75 s every 20 min were established as germination conditions. Water was

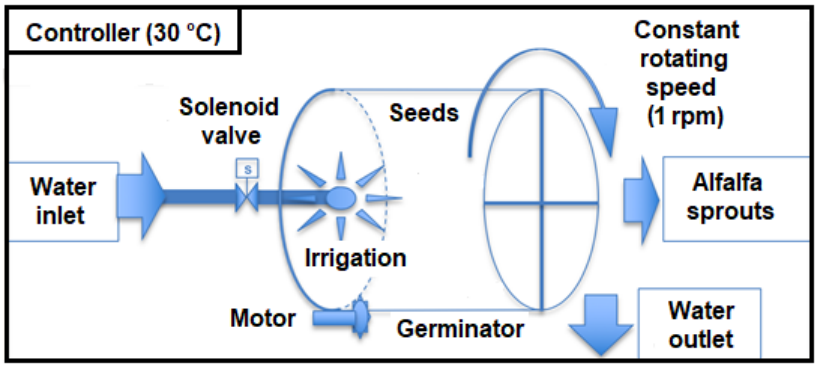

Figure 1: Schematics of the rotating germinator

Figure 2: Rotating germinator

introduced to each drum by hoses, pipes and connectors using a mesh filter, to ensure that irrigation water will be purified. Alfalfa seeds were irrigated inside the drums by means of spray nozzles. The excess of water was collected in a container located at the bottom of the germination chamber. For environmental control of the system, a REX-C100 controller equipped with a K type thermocouple was used to measure internal temperature of the cabinet. This micro-controller operated the following devices: three heaters of 220 V AC, an air fan of 24 V DC for temperature control, a 24 V DC exhaust fan, a solenoid valve, 24 V DC to control irrigation of the drums, a 12 V DC hydraulic pump to extract water excess and a 12 V DC reduction motor for rotation of the drums. The fan allowed recirculation of air from inside the chamber to homogenize the temperature in the three drums while the extractor allowed renewing of air every 15 minutes inside the chamber. For monitoring and data acquisition, an arduino UNO micro-controller shield for arduino UNO, with a SD card socket driver (bridge H) MONSTER MOTO SHIELD, with a temperature measuring probe, model DS18B20 was used. This micro-controller was responsible for recording the temperature inside the container into the SD card. Temperature was recorded every 5 s with date and time register. The frequency of irrigation remained fixed while the system was operating.

2.1 Operation of germination equipment

For the operation tests of the equipment, a batch of 10 kg of alfalfa seeds (variety Q31) provided by Seeds Generation 2000 Ltda., Chillán Viejo, Ñuble, Chile, was used as raw material without any previous pelletizing treatment. For germination, 0.5 kg of seeds were washed three times with potable water at constant agitation for 1 min, until a transparent wash water was obtained, followed by a hydration process prior to germination by immersion in 4.5 L of drink water for 3 h. After hydration, drainage of water excess was performed. From the previous process, 1.2 kg of hydrated seeds were obtained, whereby 0.9 kg were used in three rotary drums previously sanitized with 2000 ppm of chlorine (0.3 kg each) and the remaining 0.3 kg were discharged. A germination temperature of 30 ° C, a rotating speed of 1 rpm, and an irrigation frequency of 200 mL of unsterilized drink water was applied for 75 s every 20 min during germination. The water excess was drained to a collection tray located at the bottom of the germination drums. Germination time was 84 h with previous soak of 3 h. The flowchart of the seed treatment process is shown in Figure 3.

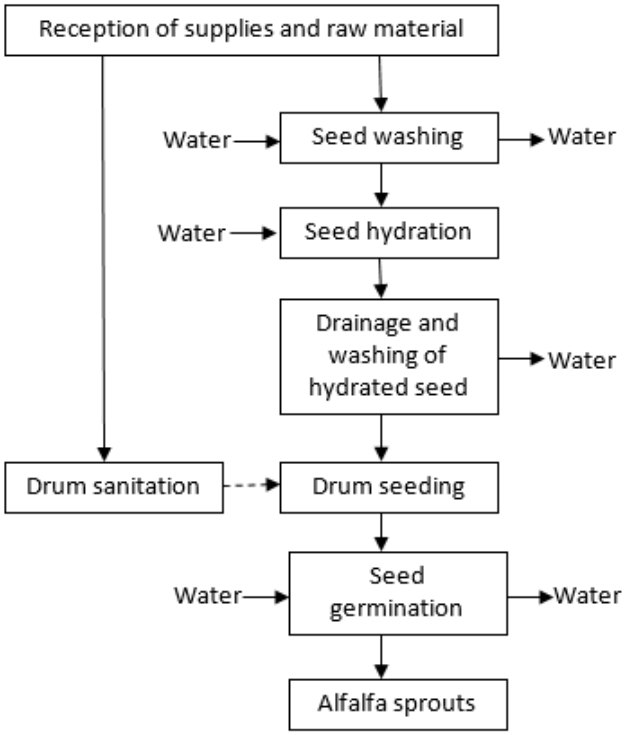

Figure 3: Flowsheet of alfalfa germination process

2.2 Microbiological analysis in alfalfa sprouts

For the preparation of the inoculum of *Escherichia coli* (*E. coli* ATCC 25922) and seed inoculation, microbiological analyses were performed in the Laboratory of Microbiology and Applied Mycology of the Faculty of Agricultural Engineering, Universidad de Concepción. Cells were grown in trypticase soy broth (DIFCO) at 37 °C for 18 - 24 h until reaching $1.5 \cdot 10^4$ cfu g^{-1}. Subsequently, the inoculum of *E. coli* was sprinkled on the alfalfa seeds by means of a manual spraying diffuser, and after 30 minutes of fixation, introduced in the drums. From the sprouts obtained after 84 h, a sample of 10 g of product per treatment was taken at random. Samples were mixed with 90 mL of 1 % peptonized water (Merck) for 1 min in a sterile bag and homogenized in a mixer (IUL, Classic Masticator). Dilutions were made in 9 mL of 1 % peptonized water. Regarding the mesophilic aerobic (MA) microorganisms, samples were inoculated in the middle of CASO agar (casein agar, soybean meal and peptone) plates and incubated at 37 °C for 48 h. For the determination of *E. coli*, samples were added to the middle of EMB Levine agar (Merck). Plates were incubated at 37 °C for 48 h, observing metallic green colony formation in case of the presence of *E. coli*. Microbiological counts were expressed in logarithmic units of colony-forming units per gram of product (log cfu g^{-1}).

2.3 Determination of minerals in alfalfa sprouts

Samples were collected from the germinator in transparent plastic bags, after being labeled, frozen at -5 °C until sent to the laboratory for mineral determination. The concentrations of copper, magnesium, iron, calcium, zinc, manganese, potassium and sodium were determined only for the sprouted samples manufactured in the rotating drum without copper sheets and without inoculation of *E. coli*. However, copper content was determined in all samples. These measurements were done in the Nutritional Soil and Plant Laboratory of INIA – Quilamapu (Chillán, Chile). Atomic absorption spectrometry was used as described by Sadzawka et al. (2007) [31]. Results were expressed on the basis of dry mass.

2.4 Quality of alfalfa sprouts

After manual drainage, the total mass of sprouts was measured in triplicate by means of an electronic scale (Shimadzu, model BL-320H). In addition, ten alfalfa sprouts were randomly selected and the mass of ten units was determined in triplicate using an electronic scale (Shimadzu, model BL-320H). The color was measured in sprouts harvested after a refrigeration treatment for 9 h at 2 °C. To assess the most representative values of each treatment, three measurements were made. Color comparisons were done at daylight using the Munsell table. These values were converted by using the Munsell Conversion software and expressed as CIELab parameters, lightness (L), redness or

greenness (± a) and yellowness or blueness (± b). Length of ten units of sprouts was measured by means of a digital Vernier caliper (Caldi-6MP, Truper).

2.5 Experimental design and statistical analysis

A completely randomized, independent experimental design was used for each trial, with four treatments and three repetitions per treatment. Data obtained were evaluated by analysis of variance (ANOVA) and in the case of significant differences between treatments at 5 %, the Tukey multiple comparisons test was applied. All results were statistically analyzed using Statgraphics statistical software, version XVII.

3. RESULTS AND DISCUSSION

3.1 Germinator operation

The rotary germination equipment was installed in the Bioprocess Laboratory of the Faculty of Agricultural Engineering, Universidad de Concepción. The manufactured equipment operated correctly during all the stages of the process. During a whole operation period of 84 h, time and temperature data were obtained by the microcontroller. Germination temperature ranged between 26 and 31 °C, being optimum for alfalfa germination between 18 to 25 °C. Experiments were carried out at a temperature higher than the optimum germination temperature in order to favor microbial growth during all treatments. In parallel, the temperature in the germination chamber varied between 20 and 22.5 °C. The temperature differences between the germination chamber and the inside of the rotating drum were due to the orientation of heating and forced air exchange. A difference of approximately 2-3 °C was recorded between the actual temperature and that of the system. The above may be due to the use of two different types of thermocouples since a type K thermocouple was used in the control and a DS18B20 temperature sensor in the register. This fact added to the error associated to the instruments could explain this difference.

3.2 Mineral contents

A considerable difference in copper content was clearly observed for those treatments in which copper sheets were used, compared to those in which a plastic sheet was used, as shown in Table 1. The copper contents for sprouts obtained by means of the germinator with a plastic sheet, with and without the presence of *E. coli*, were 44.2 and 32.9 ppm, respectively. However, the copper content in sprouts produced in the germinator with copper sheet surface increased considerably, reaching values between 721 and 728 ppm.

Table 1: Copper concentration in alfalfa sprouts

Treatment	**Copper concentration (ppm)**
Plastic without *E. coli*	32.9 ± 7.5 A
Copper without *E. coli*	721 ± 105 B
Plastic with *E. coli*	44.2 ± 11.9 A
Copper with *E. coli*	728 ± 169 B

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

The increase in copper content in alfalfa sprouts in the presence of a copper-enriched medium has been previously reported in the literature [23]. The copper concentration found in commercial sprouts according to the study carried out by Plaza et al. (2003) [32] was 17.77 ppm, which is slightly lower than the results found in this study. The Chilean Sanitary Regulations of Foods, article No. 160 [33], do not establish a specific maximum limit of copper for alfalfa sprouts; however, this food can be included in "other products", whose value is 10.0 ppm. Therefore, all the sprouts in this study were exceeded in copper according to this standard. The high copper content for sprouts without any contact with a copper surface could be related to the origin of the water used in this study, which was purchased by means of an independent water purification system but extracted from a well. It is recommended to evaluate the use of copper alloys with other materials to avoid an excess of copper in the final product. The copper concentration or contact time with copper could be decreased to reduce residues in sprouts. The mineral content in alfalfa sprouts is very dependent on germination conditions. In general, the mineral content found in this study was in accordance with the data found in the

literature [32], as illustrated in Table 2 that shows the content of minerals present in alfalfa sprouts produced in the absence of copper and *E. coli*.

Table 2: Mineral content in a sample of sprouts in absence of copper and *E. coli*

Mineral	Concentration (ppm)	Reference concentration [32] (ppm)
Potassium	4100	8680
Calcium	3500	3320
Magnesium	2.50	2,32
Sodium	1567	4720
Copper	29.90	17.77
Iron	117	153.73
Manganese	15.30	17.77
Zinc	127	53.32

3.3 Microbiological counts

With respect to the results of the count of mesophilic aerobic (MA) microorganisms in the sprouts for the different treatments, it was observed that the MA was affected by the presence of *E. coli* (Table 3). In treatments with plastic sheets MA increased from 9.20 to 10.22 log cfu g^{-1} and for copper treatments it increased from 8.71 to 10.25 log cfu g^{-1}. The contact of seeds with copper metal did not significantly change this parameter during germination. The *E. coli* count showed a significant reduction in the presence of a copper surface, from a value of 6.54 to 0.1 log cfu g^{-1} (Table 4). This result is comparable to those found by Noyce et al. (2006) [17], where *E. coli* bacteria were completely removed, when kept in contact with a copper surface for 6 hours at 22 ° C. Hygienic conditions during the germination process were correct, since *E. coli* was not observed in treatments without the inoculum of *E. coli*. According to the Chilean Ministry of Health, for fruits and other pre-processed edible vegetables ready for consumption, the maximum limit allowed for MA is 4.7 log cfu g^{-1}, while for *E. coli* it is 10 cfu g^{-1} [33].

Table 3: Number of aerobic mesophilic bacteria in alfalfa sprouts

Treatment	RAM (Log ufc g^{-1})
Plastic without *E. coli*	9.20 AB
Copper without *E. coli*	8.71 A
Plastic with *E. coli*	10.22 BC
Copper with *E. coli*	10.25 C

Logarithm of the average value. Different letters in the same column indicate significant difference (p = 0.05).

Table 4: Number of *E. coli* bacteria in alfalfa sprouts.

Treatment	*E. coli* (Log ufc g^{-1})
Plastic without *E. coli*	< 0.1 A
Copper without *E. coli*	< 0.1 A
Plastic with *E. coli*	6.54 B
Copper with *E. coli*	< 0.1 A

Logarithm of the average value. Different letters in the same column indicate significant difference (p = 0.05).

3.4 Quality parameters

A slight decrease in the total mass of sprouts was observed for the treatment that included copper in the absence of *E. coli*, using 75 g of seeds per compartment or 300 g of seeds per drum as initial raw material, and a copper contact area of 0.64 m^2 (Table 5). However, this trend was not repeated in the presence of *E. coli*. In case of wheat germination, copper caused a decrease in seed germination [34]. The mass obtained from ten units of alfalfa sprouts did not show statistically significant differences between treatments (Table 6). The length of sprouts showed a significant decrease in those treatments in which the copper sheet was introduced, with respect to those performed using a plastic surface. Data illustrating this trend are shown in Table 7.

Table 5. Total mass of sprouts per drum.

Treatment	Mass (g)
Plastic without *E. coli*	1299.1 ± 32.9 C
Copper without *E. coli*	985.7 ± 37.7 A
Plastic with *E. coli*	1098.5 ± 193.0 AB
Copper with *E. coli*	1188.8 ± 11.7 BC

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

Table 6. Mass of ten alfalfa sprouts.

Treatment	Mass (g)
Plastic without *E. coli*	0.303 ± 0.024 A
Copper without *E. coli*	0.305 ± 0.016 A
Plastic with *E. coli*	0.315 ± 0.010 A
Copper with *E. coli*	0.289 ± 0.011 A

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

Table 7. Length of alfalfa sprouts.

Treatment	Length (mm)
Plastic without *E. coli*	54.35 ± 11.05 B
Copper without *E. coli*	46.34 ± 7.40 A
Plastic with *E. coli*	60.42 ± 10.63 C
Copper with *E. coli*	41.80 ± 10.20 A

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

With respect to the level of brightness (L) of sprouts, Table 8 shows that sprouts from the treatment with plastic and *E. coli* were opaquer than the remaining treatments. This indicator could be influenced by microbiological counts of *E. coli*, and a possible bio-film formation. The degree of redness or greenness (±a) of the sprouts did not show significant differences between the treatments (Table 9). Neither there was any significant difference between treatments with respect to the degree of yellowness or blueness (±b) of sprouts according to Table 10.

Table 8: Lightness level (L) of alfalfa sprouts

Treatment	L
Plastic without *E. coli*	86.21 ± 0.00 B
Copper without *E. coli*	86.21 ± 0.00 B
Plastic with *E. coli*	81.35 ± 0.00 A
Copper with *E. coli*	84.59 ± 2.81 B

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

Table 9: Degree of redness or greenness (±a) of alfalfa sprouts

Treatment	a
Plastic without *E. coli*	-6.48 ± 3.28 A
Copper without *E. coli*	-4.80 ± 0,15 A
Plastic with *E. coli*	-5.85 ± 2.96 A
Copper with *E. coli*	-4.60 ± 0.31 A

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

Table 10: Degree of yellowness or blueness (±b) of alfalfa sprouts

Treatment	b
Plastic without *E. coli*	87.19 ± 0.77 A
Copper without *E. coli*	91.34 ± 8.12 A
Plastic with *E. coli*	76.89 ± 8.13 A
Copper with *E. coli*	86.35 ± 14.52 A

Average value ± standard deviation. Different letters in the same column indicate significant difference (p = 0.05).

4. CONCLUSION

The copper sheet coated rotary drum system proved to be a suitable device for the germination of alfalfa seeds. The copper surface showed high efficiency to reduce the microbial load of *E. coli* in alfalfa sprouts, complying with the regulations established by the Ministry of Health of Chile. However, the sprouts of all treatments exceeded in copper content allowed for consumption. The presence of copper in the germination system did not significantly affect the count of mesophilic aerobic bacteria in alfalfa sprouts. Likewise, in general the quality parameters of the sprouts were not affected. More studies are needed on other seed species tolerant to environments saturated with copper and surfaces that use copper or alloys in contact with sprouts.

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