Antibacterial performance of a novel Cu-bearing stainless steel against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in whole milk

Li Nan[a*], Guogang Ren[b], Ke Yang[a]

[a]*Institute of Metal Research, Chinese Academy of Sciences, Shenyang 110016, China*
[b]*University of Hertfordshire, Hatfield AL10 9AB, UK*

Abstract

Pathogen microorganisms exist in various environments such as food/beverage processing facilities. They are not easily eliminated, and significantly raising the risk of bacterial cross-contamination. A novel 304 Cu-bearing stainless steel (304CuSS) with Cu-rich precipitates has shown excellent broad-spectrum antibacterial performance. This work introduced the inhibition ability of 304CuSS against bacteria or its antibacterial performance towards *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) in the whole milk. The experimental results demonstrated strongly that in comparison with 304SS, 304CuSS not only killed a high percentage of planktonic bacteria in the whole milk, but also inhibited sessile bacteria adhering to the steel surface, and therefore resisted the bacterial biofilm formation. The analysis concluded that the precipitated Cu-rich nano-particles from 304CuSS were the dominant antibacterial agent. The bacterial test methodologies employed were standard plate counting, bacterial epifluorescence microscopy observation, and supported by Transmission Electron Microscope (TEM) on the examination of the Cu-rich nano-particles embedded in the steel matrix. The bacteria microstructures on the stainless steel surfaces were also observed by Scanning Electron Microscope (SEM), and biofilm-stainless steel contact angles were analyzed through the surface free energies on the contaminated steel surfaces after contacting with *S. aureus* and *P. aeruginosa* in the whole milk. The research outcomes explicitly showed an application potential of this novel antibacterial stainless steel in the beverage and food processing industry.

Keywords: *Staphylococcus aureus*; *Pseudomonas aeruginosa;* whole milk; antibacterial; Cu-bearing stainless steel.

1. Introduction

Microorganisms are known for their detrimental effects on human health, especially in children and the elderly. They are also a source of increased social costs (Douwes et al., 2003; Browning et al., 1992; Wilson and Gifford, 2005). Milk is highly

nutritious and provides ideal an environment for the growth of pathogenic and spoilage organisms, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) (Seifu et al., 2004). These bacteria in the dairy or milk environment significantly increases the risk of food-borne illness because of the contaminated food or food products. The transfer or the passes of the microorganisms from food to equipment and food preparation utensils has been increasingly gaining concerns and studied in which, bacterial adhesion to the materials particularly stainless steel used in food industries has been reported (Gomes and Nitschke, 2012).
However, previous studies were more focused on the pathogens or biofilm formations on stainless steel without consideration of stainless steel surface composition and surface effects produced by variety of the composition changes. This study is intending to introduce an innovative Cu-bearing stainless steel (304CuSS) to the food and beverage industrial facilities, by providing stainless steel containing certain amount of Cu particles within the stainless steel reflected the existence of Cu element in which the nano-sized particles were formed in the 304CuSS during the thermal ageing treatment. 304CuSS was proved of functional in killing pathogen microorganisms such as *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (Nan, 2007/08/10/12).
The pathogenic bacteria existed in food/dairy processing facilities often include planktonic bacterial cells and their biofilms. Once a biofilm formed, the resistance of the bacteria to the disinfectants or antibacterial products would be enhanced. Bacterial biofilms can be vey tough and cause many serious engineering issues, such as mechanical blockage, and bio-related corrosion of the base materials. These biofilms are often more resisting to disinfection than planktonic organisms (Dat et al., 2012). Therefore, prevention of bacterial biofilm adhesion/formation on substrate material during transportation, storage and processing of food and dairy products is primary concern of the food / dairy industry.
Stainless steels, a class of popular materials for equipment and facilities available in almost all levels of the food / dairy processes are believed one of the most susceptible substrate materials for bacterial adhesions (Carpentier and Cerf, 1993) because of their biocompatibility nature. It is long been recognized that the copper (Cu) or copper alloys possessed antimicrobial property that reduces the infection. And recently on paper approved Cu reduction on horizontal transfer of antibiotic resistant genes on abiotic touch surface (Warnes, et al 2012). Therefore this study was investigating a novel austenitic Cu-bearing stainless steel (304CuSS) specifically targeting the antibacterial applications (Nan et al., 2007). Previous examinations of the 304CuSS have exhibited widely antibacterial ability to various microorganisms under the condition of physiological saline, i.e., *E. coli (*ATCC25922), *S. aureus* (ATCC25923), *Serratia marcescens (*ATCC8100), *Enterococcus faecalis* (ATCC29212) and *Pseudomonas aeruginosa (*ATCC9721) (Nan, et al., 2007/08/10). This material system (304CuSS) possesses long-lasting antibacterial ability since the copper ions (Cu^{2+}) can be continuously released from its surface (Nan and Yang, 2010).

However, it is crucial to understand the antibacterial mechanism of this material against multi-species of microorganisms. So far, there is no standard method existing

for evaluating the antibacterial performance of the products against a variety of bacteria in the actual environments (Bae et al., 2012; Chang et al., 2010; Enric et al., 2002).

The purpose of this study is to evaluate antibacterial ability of the 304CuSS on the whole milk in its processing conditions. The antibacterial testing methodology and contamination observation include heterotrophic plate counting (HPC), contact angle measurement and epifluorescence microscopy, which helps to show the adhesions of live bacteria to the steel.

2. Materials and methodology

2.1. Stainless steel samples, medium and bacteria

Stainless steels:

Standard 304SS sheet sample was purchased from Taiyuan Steel Co. and the 304CuSS was produced by Institute of Metal Research, Chinese Academy of Sciences. All samples were prepared with the size of 10×10×1 mm^3 for the antibacterial tests. Their chemical compositions are listed in Table 1.

All samples were heated at 1040 °C for 30 min followed by quenching in water, and then they were aged at 700 °C for 6 h to produce Cu-rich precipitates in 304CuSS, in order to provide the steel with excellent antibacterial ability. This was followed by successively milling using 600 to 1000 SiC papers, followed by acetone rinsing, ultrasonic cleaning for 15 min in ethanol, and then dried in air. The samples were sterilized under UV light for 30 min prior to use.

Table 1 Chemical composition of experimental 304CuSS and 304SS (wt. %).

Stainless steels	Cr	Ni	Cu	C	Si	Fe
304CuSS	18.66	9.78	3.88	0.026	0.048	Balance
304SS	18.39	10.12	—	0.028	0.052	Balance

Medium:

In this study, the whole milk was chosen to investigate the antibacterial performance of 304CuSS up to 72h. Close to the actual environments, the whole milk (Pure milk, Deyatür lnc, Germany) was pasteurized in the dairy production processes prior to the market. The nutrient compositions (/100ml) of the solution was reported as following: energy 267 kJ, protein 3.3 g, fat 3.5 g, Carbohydrate 4.8 g, sodium 50 mg, calcium 120 mg. Experimental temperature was set at 25±1.0˚C to prevent the mediums deteriorated.

Bacteria:

S. aureus (ATCC25923) and *P. aeruginosa* (ATCC21328) were purchased from Guangdong Microbiology Culture Center, Guangzhou, China. The bacteria were incubated and cultured overnight in the shaker with constant temperature of 37˚C

before the test. The concentration of bacteria was adjusted to (2~5) × 10^3 CFU/ml, respectively.

2.2. Microstructure observation

After heat treatment, the slices in 0.4 mm thickness were cut from the 304CuSS for Cu-rich precipitates observation on a high-resolution TEM (JEM-2010, JEOL, Japan) with point resolution of 0.19 nm. At first, the slices were mechanically ground from both sides to about 30 μm and then thinned by standard twin jet polishing or ion milling. After that, the thin foils were examined on the equipment. Fine structure investigation, energy dispersive X-ray spectrum (EDXS) analysis with high-angle annular dark field (HAADF) and electron energy loss spectrum (EELS) nano-probe analysis were conducted on a FEG-Tecnai30 electron microscope.

2.3. Direct plate-counting assay

Standard direct plate counting method in analog to World Health Organization (WHO) heterotrophic plate-counting (HPC), was used to determine the number of planktonic bacteria in this study (Huq, et al, 2008;Figueras, et al, 2010; Lautenschlager, et al, 2010; Nan et al, 2007; Nan and Yang, 2010; Nan et al., 2008). 0.9 % NaCl (physiological saline) (w/w) was adopted as the diluent, which was sterilized by an autoclave for 20 min before use. In order to examine the antibacterial abilities of steel samples against pathogen bacteria in the whole milk, both samples of 304CuSS and 304SS were placed into the sterilized 24-well plates, and then 1ml of the solutions were separately poured into the plates with samples. These plates were co-cultured at 25±1.0°C in an incubator for 3h, 6h, 12h and 24h successively. After dilution in physiological saline, serial dilutions of bacteria were plated onto the LB plates (Nan et al., 2008). Colonies were counted and results were expressed in log (CFU/ml). Five groups of the same samples were used for the testing results.

Please check following papers to find out the WHO standard bacterial testing method for milk:

4. Lautenschlager, K., et al., *Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition.* Water Res, 2010. **44**(17): p. 4868-77.

5. Figueras, M.J. and J.J. Borrego, *New perspectives in monitoring drinking water microbial quality.* Int J Environ Res Public Health, 2010. **7**(12): p. 4179-202.

6. Huq, A., et al., *Biofilms in water, its role and impact in human disease transmission.* Curr Opin Biotechnol, 2008. **19**(3): p. 244-7.

2.4. Contact angle measurement

Contact angles were obtained using the sessile drop method by a contact angle analyzer (OCA-20, Dataphysics, Germany). After the samples were exposed to the whole milk with bacteria for 24h, removing the planktonic bacteria on the plates using 10 ml of sterile distilled water for three times, the mean±SD of contact angle of four points on the surface was regarded as the contact angle of each tested sample at 25°C (Zhao and Liu, 2006).

2.5. Morphologies observation

To examine the sessile multi-species microorganisms on a sample surface after they contacted with steels for 24h, the samples were taken out of the milk and fixed in 4% (v/v) glutaraldehyde for 4 h. It was then dehydrated by 25%, 50%, 75% and 100% (v/v) ethanol successively for 30 min. The steel surface was finally sputter-coated with gold to provide conductive surface for field emission SEM (SUPRA 55, ZEISS, Germany) observation according to the standard SEM operation procedure (Xu et al., 2012). The chemical compositions of samples were analyzed on the energy-dispersive spectrometry (EDS).

2.6. Epifluorescence microscopic observation

After removing planktonic bacteria from the milk contacted with samples, the sessile bacteria or biofilms on steel surfaces were stained with 4',6-diamidino-2-phenylindole (DAPI) (0.5μg/ml) for 10 min prior to microscopic examination and then rinsed with PBS for three times (Lafleur et al., 2013). Epifluorescence microscopy (Carl Zeiss Inc., Oberkochen, Germany) was used to evaluate the growth of bacteria on surfaces of different samples for 24h.

2.7. Statistical analysis

All data in this study represented the mean ± SD of five experimental replicates.

3. Results and discussion

3.1. Material characterization

Previous studies demonstrated that the existence of ε-Cu phase in the 304CuSS matrix provided strong antibacterial performance compared to 304SS (Nan et al., 2007). The observation using TEM revealed the morphologies of precipitates of the Cu nanoparticles or clusters were in a good conjugation with the stainless steel matrix in the present 304CuSS that was aged at 973 K for 6h as shown in Fig. 1. Accordingly these nano-scaled particles presented in Fig. 1 should be prevailingly the Cu-rich precipitates (Gagliano and Fine, 2004). These rod-like shape Cu-rich precipitates in 304CuSS showed their average size around 80 nm, (Fig. 1a). In order to confirm their Cu-rich precipitation prevalence in the steel matrix, further TEM examination was carried out and the result was shown in Fig. 1b, that an HAADF image proved the Cu-rich precipitates. Meanwhile, the corresponding composition analyses made by SEM-EDXS also confirmed the Cu-rich precipitation particles as listed in Table 2. The results illustrated that these particles within the matrix had higher concentration of copper element (19.6wt.%) in comparison of average matrix Cu content (3.8wt.%), The Cu elemental micro-structure in these Cu rich precipitates are identified to be in the form of ε-Cu.

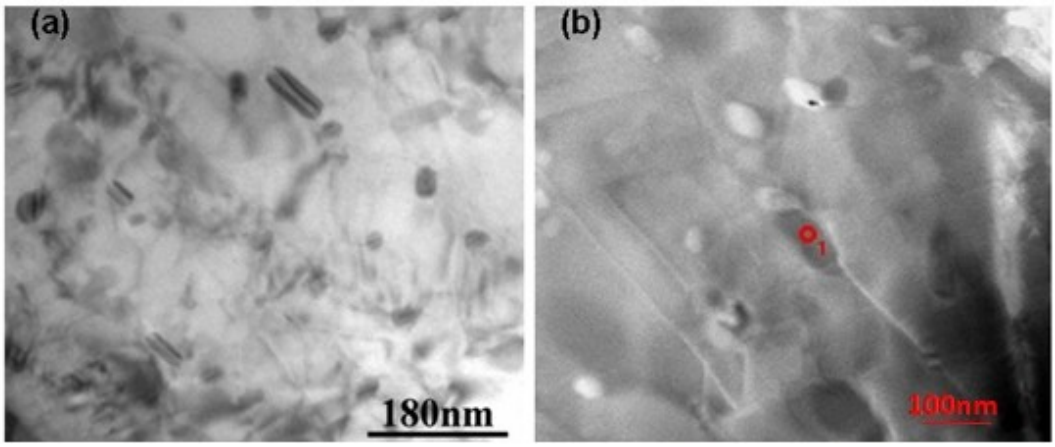

Fig. 1 Morphologies of the ε-Cu-rich precipitates in the 304CuSS steel aged at 973K for 6h, (a) Cu-rich precipitates shown as nano scaled particles (dark) and (b) HAADF image showing the ε-Cu rich nanoparticles (dark) of the precipitates.

Table 2 EDXS analyses of the precipitate and matrix in the 304CuSS (wt.%).

Element	Precipitate in matrix	Matrix
Cr	8.2	18.66
Ni	1.8	9.78
Fe	70.4	67.76
Cu	19.6	3.8
Total	100.0	100.0

3.2. Planktonic bacteria counts (could the contents in this section be combined?)

The test results demonstrated that the number of planktonic bacteria was growing gradually, and increased with extension of the contact time. After contact with 304SS, the bacteria in the whole milks significantly increased.

Whole milk: The bacterial increment was over 9 times, as observed from the bacterial counts on plates and shown in Fig. 2. The growth of the number of planktonic bacteria after the milk contacted with the 304SS for 3, 6, 12 and 24h. A modest increase of bacteria was observed in the whole milk when contacted with the 304CuSS for 24h contact. After 24 h exposure to the milk, the inhibition rate of 304CuSS against the planktonic bacteria was from 59.2±1.3% (3h) to 66.9±2.0% (24h) in comparison with 304SS, indicating that the conventional stainless steel has no ability on inhibiting the bacterial growth in this medium.

Whole milk with *S. aureus*:, When the whole milk containing *S. aureus* contacted with steel samples, the number of planktonic bacteria increased much faster than that in the pure whole milk, as also shown in Fig. 2. Bacterial colonies increased between 2.51±0.3 log (CFU/ml) and 4.27±0.9 log (CFU/ml) after contact with 304CuSS from 3h to 24h. There were more colonies on 304SS between2.81±0.3 log (CFU/ml) and 6.36±1.3 log (CFU/ml) after contact with bacterial for the same periods. The bacteria was allowed much more easily to be survived and propagated after the whole milk contacted with 304SS, and in contrast, the antibacterial rate of 304CuSS reached

99.2% after 24h exposure to the medium.
Whole milk with *P. aeruginosa*: After 304CuSS was immersed in the milk with *P. aeruginosa* for different lengths of time, the number of planktonic bacteria almost

The increment of planktonic bacteria count was 43 times for 304SS and the bacteria reached to 6.55±1.2 log (CFU/ml) in 24h). By contrary, only 4.35±1.1 log (CFU/ml) of bacteria appeared on the 304CuSS, and its inhibition rate against the planktonic bacteria in the milk medium was 99.3%. Therefore, it can be concluded that 304CuSS shows a better antibacterial performance against planktonic bacteria in the whole milk with and without bacteria in comparison to 304SS, which can significantly lower the risk of bacterial bio-contamination.

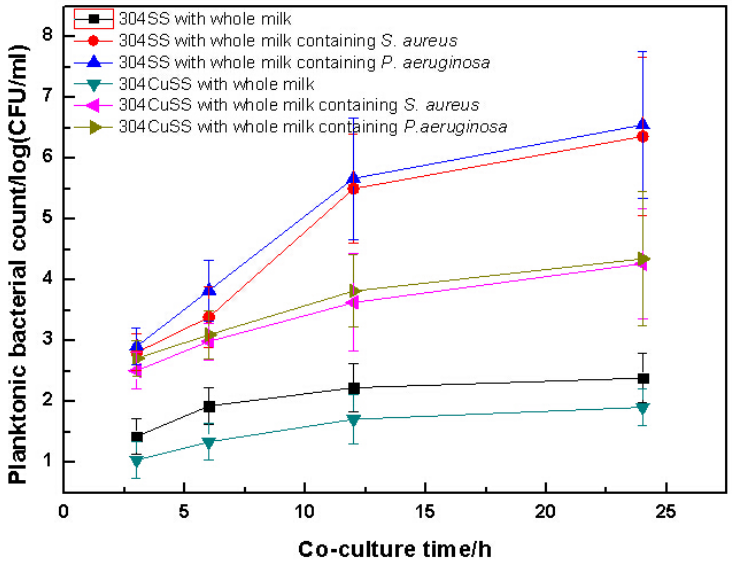

Fig. 2 Planktonic bacterial counts on surfaces of 304CuSS and 304SS samples exposure to the whole milk with and without bacteria for different time lengths.

3.3. Sessile bacteria observation

Morphologies of sessile bacteria in the whole milk with and without bacteria after contact with steel samples for 24h are shown in Fig. 3. The results show that after contact with steel samples, not only single sessile bacterium but also clustered ones in the form of biofilms appeared on the surfaces of 304SS, while only a few scattered ones observed on the 304CuSS surface, indicating that the sessile bacteria were much easier adhesive to 304SS than to 304CuSS. In addition, it is observed from Fig. 3 that the island-like thick biofilms were found on the 304SS steel surfaces after contacted with the whole milk containing bacteria, while only long rod-like and thin clusters appeared on the same steel substrates exposed in the milk without bacteria. The morphology results demonstrated that once the whole milk was contaminated with bacteria, the bacteria were developed into the biofilms and not easily to be removed from the 304SS surfaces. Meanwhile as a contrast, the chemical compositions of the

304CuSS surfaces after contacting with different mediums for 24h are listed in Table 3. Certain amount of Cu concentration on the stainless steel surface demonstrated that the exposure of the 304CuSS to different mediums would guarantee the release of Cu^{2+} into the surrounding environments.

Live sessile bacteria on the steel surfaces were also observed by DAPI staining under epifluorescence microscopy, as shown in Fig. 4. A series of images of bacterial adhesion on the steel samples presented different functions of 304CuSS and 304SS after 24h contacts with the whole milk with and without bacteria. The images show the variations of sessile bacteria growth after contact with steel samples in terms of different solutions, while the number of sessile bacteria spots was also much varied, indicating a dynamic activity process in bacteria formation and succession on two types of steel samples. As shown in Fig. 4, a large amount of live sessile creatures were accumulated and clustered on the 304SS surface, while by contrary, only a few of scattered ones were observed on the 304CuSS surface.

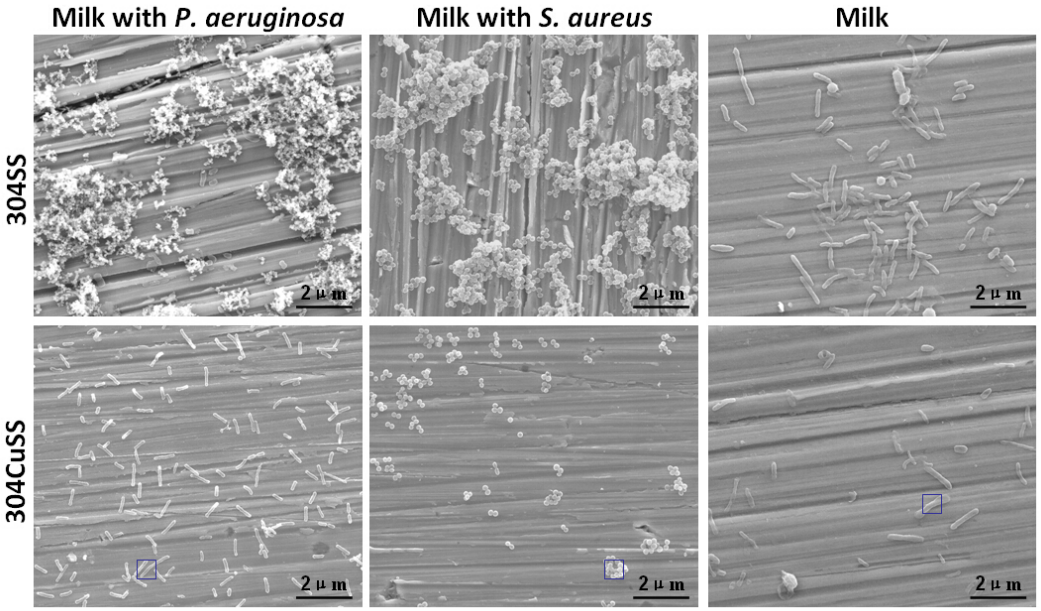

Fig. 3 Morphologies of bacteria in milk after contact with steel samples for 24h.

Table 3 EDS analyses on chemical compositions of the 304CuSS surface after contacting with whole milk mediums for 24h (squares in Fig. 3).

Chemical composition (wt. %)	Fe	Cr (18.66)	Ni (9.78)	Cu (3.88)
Whole milk with *P. aeruginosa*	balance	17.26	0.87	4.58
Whole milk with *S. aureus*	balance	17.18	0.66	4.21
Whole milk	balance	17.43	1.22	4.10

Please add one line of the orginal 304CuSS composition for comparison in the table.

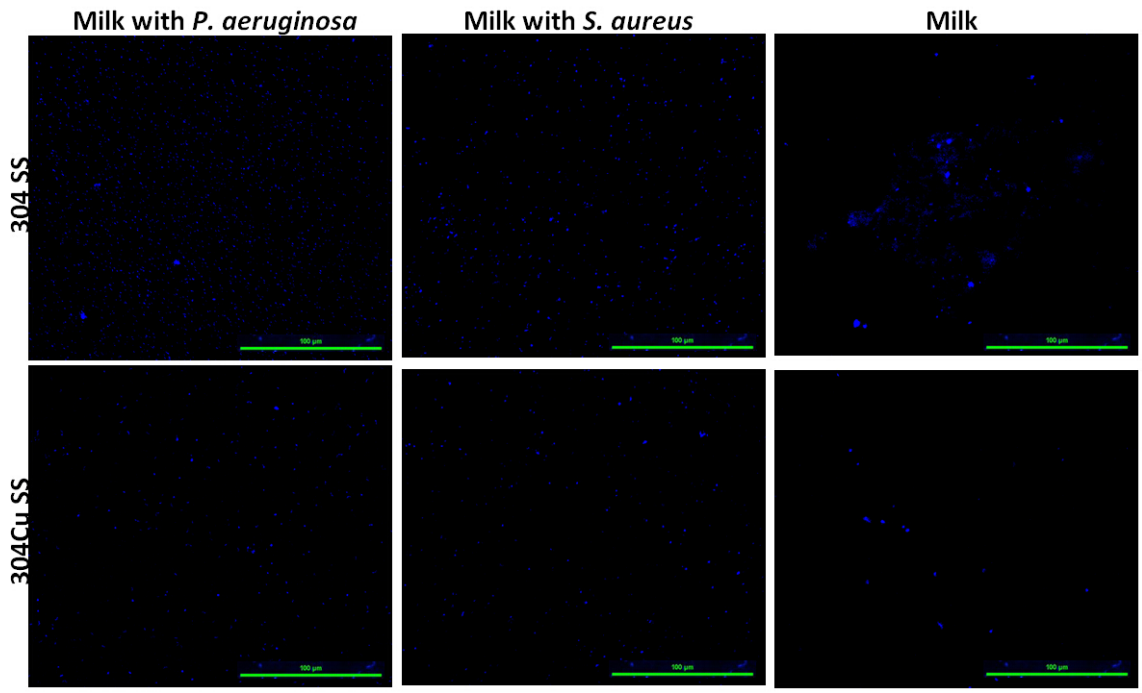

Fig. 4 Images of bacteria in drinks stained with DAPI under epifluorescence microscopy after contact with 304SS and 304CuSS samples for 24h, magnification × 40.

3.4. Surface analysis

The formation of biofilms is a process containing two successive steps, beginning with the initial attachment to a contact surface followed by accumulation of multi-layer cell clusters – intercellular adhesion (Michu et al., 2011). Once sessile bacteria are fixed / adsorbed on the substrate, they will evolve easily into biofilms by gathering together. In this study, after removing the planktonic bacteria on steel surfaces, the contact angle was measured to characterize the physiological performance of the substrate materials after contact with while milk for 24h, as shown in Fig. 5. Surface free energy (SFE) of the substrate was calculated by using the following standard equation (Zhang et al., 2008):

$$\gamma_L(1+\cos\theta) = 2\sqrt{\gamma_S^d \gamma_L^d} + 2\sqrt{\gamma_S^p \gamma_L^p} \quad (1)$$

where γ_L is the experimentally determined surface tension of the liquid; θ is the contact angle; γ_S^d is the dispersion component of the solid steel surface free energy; γ_L^d is the dispersion component of the liquid surface free energy; γ_S^p is the polar component of the surface free energy of the solid; γ_L^p is the polar component of the surface free energy of the liquid. Fig. 5(a) represents the variations of contact angles after samples contaminated by bacteria in the whole milk for 24h.

For the fresh whole milk:

After diiodomethane was dropped onto both steels, It was found that the contact angles of them were not significantly changed. While dropping the deionized water onto the steels, the contact angles for 304CuSS were much higher than that of 304SS. It has been referred that the effect of deionized water to the substrate surface was

more prevalent compared to diiodomethane. The relevant polar component showed the obvious function for the surface free energy (SFE) calculation, as shown in Fig. 5(b). After contact with the whole milk for 24h a large difference on SFE was presented between two steels, the mean value of SFE (63.9±0.08 $mJ \cdot m^{-2}$) for 304SS was obviously higher than that of 304CuSS (52.6±0.12 $mJ \cdot m^{-2}$).

For whole milk contaminated by *S. aureus:*

Likewise, the mean values of SFE for 304SS contacted with milk containing *S. aureus* (69.8±0.29 $mJ \cdot m^{-2}$) and *P. aeruginosa* (74.7±0.15 $mJ \cdot m^{-2}$) were also higher in comparison to those for 304CuSS (63.5±0.42 $mJ \cdot m^{-2}$ and 68.8±0.82 $mJ \cdot m^{-2}$).

Comparison between the whole milks contaminated by *S. aureus and P. aeruginosa*, in addition, the values of SFE for the steels contacted with the medium with *P. aeruginosa* and *S. aureus* were much higher than those contacted with the fresh whole milk, indicating that the adhesion affinity of sessile bacteria on the steel samples were stronger in the mediums with bacteria. The excellent antibacterial abilities of 304CuSS against multi-species microorganisms could inhibit the sessile bacteria adhesion and succession on its surface, which is in agreement with the epifluorescence observation shown in Fig. 4. These results demonstrate that the risk of bacterial bio-contamination on the 304SS surface is obviously higher than that on the 304CuSS, regardless if it is fresh whole milk, or milks with or without bacteria. Especially for a longer duration of contamination, 304CuSS provided much better capabilities than those of 304SS in terms of their antibacterial and anti-biofilm formation.

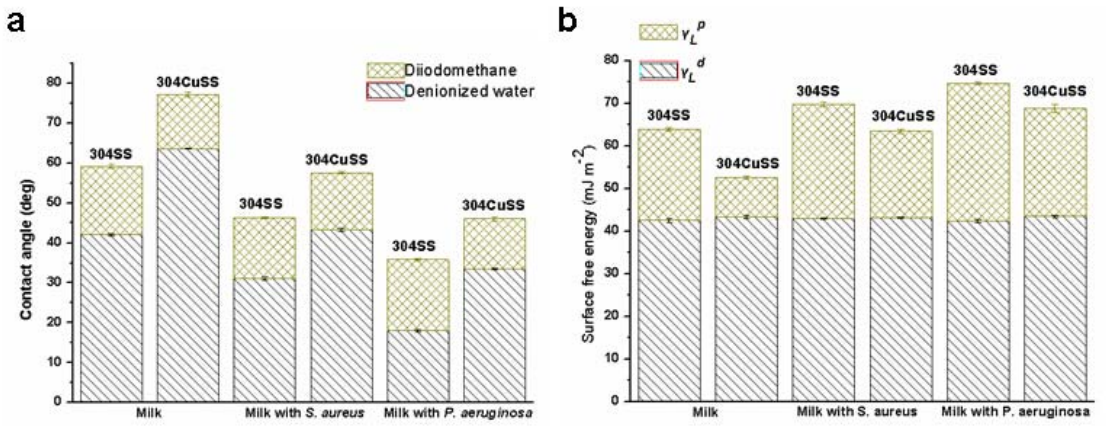

Fig. 5 Surface analyses of stainless steel samples after contact with different bacteria in the whole milk for 24h, (a) contact angle; (b) surface free energy (SFE).

As shown in Fig. 5 Fresh milk contains relatively fewer bacteria, but contamination during handling can rapidly multiply the bacterial numbers. Multi-species microorganisms, such as *Escherichia coli*, *Pseudomonas*, *Staphylococci* and *Bacillus cereus* are normally present in milk (Rendueles and Ghigo, 2012), which can cause the deterioration of milk, and even possibly lead to cross–contaminations to

human, animals as well as environments.

3.5. Biofilm formation and food safety

Bacterial adhesion is a physicochemical phenomenon controlled by properties of the receptive surface, the liquid medium and the microorganism (Meylheuca et al., 2006). Once the biofilms are adsorbed (attached/adhered) on material surfaces, they are hard to be removed in which a series of problems can be easily brought about to cause a serious food contamination and safety issue. Especially, the extracellular polymeric substances (EPS) can gradually grow with a metabolism proceeding, as being generally considered vital in cementing bacterial cells together to form the biofilm structure (Stoodley et al., 2002). In this study, the number of sessile bacteria on the 304CuSS was far fewer than that on the 304SS, as shown in Fig. 4, reasonably indicating that less EPSs existed on the 304CuSS surface.

During biofilm formation, some trace organism is firstly adhering to the material surface to form a conditioning layer, directly leading to a formation of SFE. The testing results of SFE on the steel samples revealed that the sessile bacteria were much more affinity to the surface of 304SS than that of 304CuSS. This suggested that a better adhesive binding between bacteria and 304SS where the EPS supported the biofilms on the surface and considered to be highly integrated in comparison to those towards 304CuSS.

All these results and the nature of biofilm formations were reported with similar work done on the changes regarding to the bacterial-substrate contact angle and SFE values, which were contributed to those changes of surface functionalities of the fresh substrate stainless steels as well as well their surface treatment (Tang et al., 2006). When the surface energy on stainless steel becomes lower, bacterial adhesion becomes weaker to bind to the steel surface (Abu-Tarboush et al., 1996). The present results also indicate that 304CuSS provided lower surface energy, leading to the inhibition of the biofilm formation and stopping of the biofilm propagation by killing most of the planktonic bacteria, which significantly restricted the further EPS adhesion towards the steel surface. It is also worthy to mention that the fast growth of bacteria will result in milk contamination withdrew based on the industrial quality control standard. All these might be explained by the increased secretion of substances from the bacterial metabolites, causing the composition and physiological characteristic of the general dairy products to be destroyed and their physical stabilities to be deteriorated (Espitia et al., 2014).

The tests on 304CuSS against planktonic bacteria in the milk were carried out for a 24hr period and a strong antibacterial performance was demonstrated in comparison to that of 304SS. The antibacterial function is mainly came from a continuous release of Cu^{2+} ions from the 304CuSS surface. It has also been proved that the released Cu^{2+} ions from the metal may lead to the collapse of the LPS patches of the bacterial cell surface, consequently alter the permeability and functionality of the outer cell membrane, finally cause the death of planktonic bacteria (Nan et al., 2008). As of the major target, this work evaluated the advantages of innovative 304CuSS against multi-species microorganisms. This antibacterial functionality was all reflected in

tests on the fluidic medium containing fresh milk with and without bacteria, respectively.

4. Conclusions

This work was concentrated on the antibacterial performance of a novel 304CuSS against *S. aureus* and *P. aeruginosa* in the fresh whole milk. Through analyzing the microstructure and antibacterial performance of 304CuSS, the conclusion was that the Cu-rich nano-particles within 304CuSS were identified as the key factor of killing bacteria and inhibiting biofilm formation agent. The bacterial test results showed that most of the planktonic bacteria were killed by the strong antibacterial ability of 304CuSS through continuous release of Cu^{2+} ions steel matrix, which also effectively inhibit the sessile bacteria adhesion and bio-film formation on the Cu-steel surface. This paper proved that the bacteria were more easily adhered to the surface of 304SS compared to that of the 304CuSS. These results give an implication that 304CuSS can reduce the deterioration of the dairy product from bacterial contamination..

5. Acknowledges

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References

Abu-Tarboush, H.M., Al-Saiady, M.Y., El-Din, A.H., 1996. Evaluation of diet containing Lactobacilli on performance, Fecal Coliform, and Lactobacilli of young dairy calves. Animal Feed Science and Technology 57, 39-49.

Bae, Y.M., Baek, S.Y., Lee, S.Y., 2012. Resistance of pathogenic bacteria on the surface of stainless steel depending on attachment form and efficacy of chemical sanitizers. International Journal of Food Microbiology 153, 465-473.

Browning, G.L., Holland, W.R., Kreiss, H.O., 1992. A comparison of differential systems and numerical methods for the computation of smooth oceanographic flows. Dynamics of Atmospheres and Oceans 6, 499-526.

Carpentier, B., Cerf, O., 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. Journal of Applied Bacteriology 75, 499-511.

Chang, Y.J., Lee, J.W., Lin, C.H., Chang, C.Y., Lee, Y.C., Hwa, M.Y., 2010. Photocatalytic deactivation of airborne microbial cells by the stainless steel sieves with surface coated TiO2 thin films. Surface & Coatings Technology 205, 5328-5332.

Dat, N.M., Hamanaka, D., Tanaka, F., Uchino, T., 2012. Control of milk pH reduces biofilm formation of *Bacillus licheniformis* and *Lactobacillus paracasei* on stainless steel. Food Control 23, 215-220.

Douwes, K.E., Schmalzbauer, E., Linde, H.J., Reisberger, E.M., Fleischer, K., Lehn, N., Landthaler, M., Vogt, T., 2003. Branched filaments no fungus, ovoid bodies no bacteria: two unusual cases of mycetoma. Journal of the American Academy of Dermatology 2, 170-173.

Enric, R., Laurence, B.P., Dominique, D., 2002. Assessing bactericidal properties of materials: the case of metallic surfaces in contact with air. Journal of Microbiological Methods 49, 225-234.

Espitia, P.J.P., Du, W.X., Avena-Bustillos, R.J., Soares, N.F.F., McHugh, T.H., 2014. Edible films from pectin: Physical-mechanical and antimicrobial properties - A review. Food Hydrocolloids 35, 287-296.

Gagliano, M.S., Fine, M.S., 2004. Characterization of the nucleation and growth behavior of copper precipitates in low-carbon steels. Metallurgical and Materials Transaction A 35, 2323-2329.

Gomes, M.Z. Nitschke, M., 2012. Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. Food Control 25, 441-447.

Lafleur, J., Yasuda, M., Shiaris, M., 2013. Induction of resistance to *Staphylococcus aureus* in an environmental marine biofilm. Journal of Microbiological Methods 93, 68-71.

Martínez-Gonzales, N.E., Hernandez-Herrera, A., Martine z-Chavez, L., Rodriguez-Garcia, M.O., Torres-vitela, M.R., Mota de la Garza, L., Castillo, A., 2003. Spread of Bacterial Pathogens during Preparation of Freshly Squeezed Orange Juice. Journal of Food Protection 8, 1343-1527.

Meylheuca, T., Methivier, C., Renaulta, M., Herrya, J.M., Pradierb, C.P., Bellon-Fontaine, M.N., 2006. Adsorption on stainless steel surfaces of biosurfactants produced by gram-negative and gram-positive bacteria: Consequence on the bioadhesive behavior of Listeria monocytogenes. Colloids and Surfaces B: Biointerfaces 52, 128-137.

Michu, E., Cervinkova, D., Babak, V., Kyrova, K., Jaglic, Z., 2011. Biofilm formation on stainless steel by *Staphylococcus epidermidis* in milk and influence of glucose and sodium chloride on the development of *ica*-mediated biofilms. International Dairy Journal 21, 179-184.

Nan, L., Liu, Y.Q., Lu, M.Q., Yang, K., 2008. Study on antibacterial mechanism of copper-bearing austenitic antibacterial stainless steel by atomic force microscopy. Journal of Materials Science: Materials in Medicine 19, 3057-3062.

Nan, L., Liu, Y.Q., Yang, W.C., Xu, H., Li, Y., Lu, M.Q., Yang, K., 2007. Study on Antibacterial Properties of Copper-containing Antibacterial Stainless Steels. Acta Metallurgica Sinica 10, 1065-1070.

Nan, L., Yang, K., 2010. Cu ions dissolution from Cu-bearing antibacterial stainless steel. Journal of Materials Science & Technology 26, 941-944.

Nan, L., Yang, W.C., Liu, Y.Q., Xu, H., Li, Y., Lu, M.Q., Yang, K., 2008. Preliminary Study on Antibacterial Mechanism of Copper-bearing Antibacterial Stainless Steel against *E.coli*. Journal of Materials Science & Technology 24, 197-201.

Nan, L., Cheng, J.L., Yang, K., 2012. Antibacterial behavior of a Cu-bearing type 200 stainless steel. *Journal of Materials Science & Technology* 28(11), 1067-1070.

Rendueles, O., Ghigo, J.M., 2012. Multi-species biofilms : how to avoid unfriendly neighbors. FEMS Microbiology Reviews 36, 972-989.

Seifu, E., Buys, E.M., Donkin, E.F., Petzer, I.M., 2004. Antibacterial activity of the lactoperoxidase system against food-borne pathogens in Saanen and South African Indigenous goat milk. Food Control 15, 447-452.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Annual Review of Microbiology 56, 187-209.

Tang, S., Lu, N., Myung, S.W., Choi, H.S., 2006. Enhancement of adhesion strength between two AISI 316L stainless steel plates through atmospheric pressure plasma treatment. Surface and Coatings Technology 200, 5220-5228.

Warnes, Sarah L., Highmore, Callum J. and C. William Keevil 2012. Horizontal Transfer of Antibiotic Resistance Genes on Abiotic Touch Surfaces. Implications for Public Health. mBio 3(6): doi:10.1128/mBio.00489-12.Wilson, G.S., Gifford, R., 2005. Biosensors for real-time in vivo measurements. Biosensors and Bioelectronics 12, 2388-2403.

Xu, D., Wen, J., Gu, Xu, T., Raad, I., 2012. Biocide cocktail consisting of glutaraldehyde, ethylene diamine disuccinate (EDDS), and methanol for the mitigation of souring and biocorrosion. Corrosion 68, 994-1002.

Zhang, W., Luo, Y.J., Wang, H.Y., Jiang, J., Pu, S.H., Chu, P.K., 2008. Ag and Ag/N_2 plasma modification of polyethylene for the enhancement of antibacterial properties and cell growth/proliferation. Acta Biomaterialia 4, 2028-2036.

Zhao, Q., Liu, Y., 2006. Modification of stainless steel surfaces by electroless Ni-P and small amount of PTFE to minimize bacterial adhesion. Journal of Food Engineering 72, 266-272.

Table captions

Table 1 Chemical composition of experimental stainless steel samples (wt. %).

Table 2 EDXS analyses on compositions of the precipitate and matrix in the experimental steel (wt. %).

Table 3 EDS analyses on chemical compositions of the 304CuSS surface after contacting with mediums for 24h (squares in Fig. 3)

Figure captions

Fig. 1 Morphologies of the Cu-rich precipitates in the steel aged at 973K for 6h, (a) Cu-rich precipitates and (b) HAADF image of the precipitates.

Fig. 2 Planktonic bacterial counts on surfaces of steel samples after contacted with the mediums for different times.

Fig. 3 Morphologies of bacteria in drinks after contact with steel samples for 24h.

Fig. 4 Images of bacteria in drinks stained with DAPI under epifluorescence microscopy after contact with steel samples for 24h, magnification × 40.

Fig. 5 Surface analyses of steel samples after contact with different drinks for 24h, (a) contact angle; (b) Surface free energy (SFE).