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Apple pectin-derived oligosaccharides produce carbon dioxide radical anion in Fenton reaction and prevent growth of*Escherichia coli* and *Staphylococcus aureus*

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Abstract

Pectin is the main soluble fiber in apples or citruses. It may be fermented by gut microbiota to metabolites showing local intestinal and systemic effects. A wide range of beneficial effects of dietary pectin includes impacts on the redox milieu and microbiota profile. We prepared pectin-derived oligosaccharides (apple (APDO) and citrus) and polygalacturonic acid-derived oligosaccharides, using alkaline hydrolysis by hydrogen peroxide, and analysed them by Fourier Transform Infrared spectrometry. Furthermore, we analyzed the effects of pectin-derived oligosaccharides on hydroxyl radical ($HO^{\bullet}$)-generating Fenton reaction using electron paramagnetic resonance spin-trapping spectroscopy, and the effects on the growth of *Escherichia coli* and *Staphylococcus aureus* in the presence of dietary-relevant $HO^{\bullet}$-generating system (iron + ascorbate). The oligosaccharides react with $HO^{\bullet}$ radical to produce carbon dioxide radical anion ($CO_2^{\bullet-}$). A comparative analysis showed that APDO has the most prominent bacteriostatic effect. This might be at least partially related to the higher capacity of APDO to produce $CO_2^{\bullet-}$, which specifically targets proteins and appears to have a longer lifetime and larger diffusion radius in biological systems compared to $HO^{\bullet}$.

Keywords: Pectin-derived oligosaccharides, Hydroxyl radical, Carbon dioxide radical anion, *E. coli*, *S. aureus*.

1. Introduction

Intestinal tract appears to be the main site of beneficial actions of dietary pectin and pectin derivatives. These may work against pathogenic flora (El-Nakeeb & Yousef, 1970; Song, Wang, & Zhang, 2014; Parsons et al., 2014) and cancer cells (Zhang et al., 2014). For example, a recent study has shown that the supplementation of apple pectin to rats with diet-induced obesity leads

to suppressed metabolic endotoxemia (Jiang et al., 2016). Some of these actions may be related to the redox activity of pectin. Pectin is known to affect the activity of antioxidative enzymes and the stability of small redox-active molecules, such as ascorbate (Asc) (Pérez, Fissore, Gerschenson, Cameron, & Rojas, 2012; Sanders et al., 2004), and to efficiently scavenge free radicals (Bogdanović Pristov, Jovanović, Mitrović, & Spasojević, 2013).

A number of hydroxyl and carboxyl groups distributed along the backbone, as well as a certain amount of neutral sugars in side chains, make pectin a good starting material for the preparation of a broad spectrum of derivatives (Chen, Liang, & Liu, 2013). It has been documented that pectin derivatives may inhibit the proliferation of intestinal pathogens and stimulate beneficial microbiota growth (Holck, Lorentzen, Vigsnæs, Licht, Mikkelsen, & Meyer, 2011; Ebersbach, Andersen, Bergström, Hutkins, & Licht, 2012; Wang, Wang, Mou, Luo, & Jiang, 2015). The beneficial physiological effects of pectin-derived oligogalacturonides have been related to redox activity (Li, Li, Dong, Zhu, & Liu, 2014). Various methods have been applied for pectin modification, including depolymerization (chemical, physical and enzymatic degradation), chain elongation (cross-linking and grafting), and substitution (alkylation, amidation, quaternization, thiolation, sulfation, and oxidation, etc) (Chen et al., 2014). In addition to different processing methods, structure and biological activity of derivatives depends on the source of pectin. It has been shown recently that the structure of pectin isolated from different sources shows huge diversity (Müller-Maatsch et al., 2016).

In this study, we processed pectin and polygalacturonic acid using alkaline hydrolysis by hydrogen peroxide (H_2O_2) to yield water-soluble oligosaccharides. The methodhas been applied previously on peach gum polysaccharides (Yao, Cao, & Pan, 2013), and the product has shown high antioxidative capacity and bacteriostatic effects against *E. coli* and *S. aureus* (Yao, Cao, &

Wu, 2013). Pectin (apple and citrus) and polygalacturonic acid-derived oligosaccharides (APDO, CPDO, and PGDO, respectively) were examined for their redox activity in hydroxil radical ($OH^{\bullet}$)-generating system and for the effects on proliferation of *E. coli* and *S. aureus*.

2. **Materials and methods**

2.1. Derivation of oligosaccharides

APDO, CPDO, and PGDO were prepared via hydrolysis using H_2O_2 under following conditions: time, 8h; temperature, 55°C; H_2O_2 concentration, 4% (v/v); and NaOH concentration, 2 M (Yao, Cao & Wu, 2013a**)**. The hydrolysates were filtered, neutralised with HCl, concentrated to aprox. 20% (w/v), precipitated with 5 volumes of ethanol, and freeze-dried to yield a water-soluble white powder. The Fourier Transform Infrared (FTIR) spectra were obtained in solid state using attenuated total reflectance – ATR sampling technique on Thermo-Nicolet 6700 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer at wave numbers between 400 and 4000 cm^{-1} and spectral resolution of 4.0 cm^{-1} in transmission mode. Spectra were analyzed using Omnic 7.3 software.

All chemicals were obtained from commercial providers: H_2O_2 (Carlo Erba Reagents, Milano, Italy); polygalacturonic acid (extracted from orange (P3889)), pectin extracted from citrus fruit (P9135), and pectin extracted from apple (76282) (Sigma–Aldrich, St. Louis, MO, USA).

2.2. EPR spin-trapping spectroscopy

Hydroxyl radical was generated via Fenton reaction. Samples were prepared by adding $FeSO_4$ (0.2 mM) to solutions containing spin-trap DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide, Enzo Life Sciences International, Plymouth Meeting, PA, USA; 5 mM), and H_2O_2 (1 mM), with or without APDO, CPDO, or PGDO (15 mg/mL). Following 2 min of incubation, samples were drawn into 10-cm long, gas-permeable Teflon tubes (wall thickness

0.025 mm and internal diameter 0.6 mm; Zeus industries, Orangeburg, SC, USA), which were placed into quartz capillaries. EPR spectra were recorded at room temperature using Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) with EW software (Scientific Software Inc., Bloomington, IL USA) and the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 20 mW; scanning time, 2 min. Simulations were performed using computer program WINEPR SimFonia (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) with the following parameters: the adduct of DEPMPO with $HO^{\bullet}$ radical (DEPMPO/OH): a_N = 14 G, a_H = 13.2 G, a_H^{γ} = 0.3 G (3H), a_P = 47.3 G; the adduct of DEPMPO with carbon dioxide radical anion ($CO_2^{\bullet-}$; DEPMPO/CO_2): a_N = 14.5 G, a_H = 17.3 G, and a_P = 51.6 G. DEPMPO was purified twice and tested for hydroxylamine impurities according to previously described procedure (Jackson, Liu, & Liu, 2002). All experiments were performed in phosphate buffer saline (pH 7.4) that was prepared using ultrapure MilliQ (18 MΩ) water.

2.3. Effects on E. coli and S. aureus

Bacterial strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were obtained from American Type Culture Collection and grown in a standardized medium, without (control) or with APDO, CPDO, or PGDO (100 μg/mL). In order to mimic the gut environment, we applied Fe^{3+}/Asc/O_2 system to produce $HO^{\bullet}$ radical (Mojić et al., 2014). All samples were checked for pH, which was not below 7.2. Samples were incubated at 37°C for 5h under aerobic conditions. Following the incubation, the samples were diluted with 0.9% NaCl and plated on endo-agar (Torlak, Belgrade, Serbia). The number of bacteria was determined after an overnight aerobic incubation at 28°C. The number of living bacteria was determined using serial dilution method.

The dilutions were designed to provide an inoculum in the range of 50–200 CFU and the number of CFU in the inoculum was determined by plating on endo-agar.

2.4. Statistical analysis

All experiments were performed in quadruplicate on two experimental days. Statistical significance was tested by one–way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison *post-hoc* test. Results from the experiments on bacteria were presented as means ± S.E. (standard error), and were taken to be statistically different if $p<0.05$.

3. Results and Discussion

FTIR spectra of apple pectin and APDO exhibited intense broad band of stretching vibrations of hydrogen bonded hydroxyl groups (3600-3200 cm^{-1}) (Fig. 1). Pectin showed a very strong band at 1737 cm^{-1} that is assigned to C=O stretching of COOH group and a band at 1229 cm^{-1} that corresponds to in-plane bending vibrations of OH groups from COOH. Similar bands are almost absent in the spectrum of pectin derivative, where dominant bands at 1434 cm^{-1} and 1632 cm^{-1} indicate symmetric and asymmetric carboxylate stretching vibrations. In-plane deformation band of water (1628 cm^{-1}) is well observed in spectrum of pectine, while it is overlapped with carboxylate stretching band in APDO.

Figure 2A shows characteristic signal of DEPMPO adduct with $HO^{\bullet}$ that is produced via Fenton reaction. In the presence of APDO, CPDO, and PGDO, the formation of DEPMPO/OH adduct in Fenton reaction was completely inhibited, whereas the adduct with $CO_2^{\bullet-}$ (Fig. 2). In other words, APDO, CPDO and PGDO produced $CO_2^{\bullet-}$ when exposed to $HO^{\bullet}$. The production of $CO_2^{\bullet-}$ by APDO was significantly higher (by approximately 60%) compared to CPDO or PGDO. It is noteworthy that APDO, CPDO and PDGO did not decrease the intensity of EPR signals of

the resulting adducts, but rather increased it. This can be explained by a higher stability of $CO_2^{\bullet-}$ compared to $HO^{\bullet}$ (Michelson & Maral, 1983), resulting in higher yield of adducts.

The exposure of *E. coli* to pro-oxidative conditions (Asc + $FeCl_3$) reduced the number of living bacteria ($p<0.05$).APDO ($p<0.001$) and CPDO ($p<0.001$) further reduced the number of *E. coli* (Fig. 3). APDO showed the most prominent effects. The number of *E. coli* colonies was decreased by 65% by the addition of APDO, whereas CPDO provoked a decrease of about 45%.

Results were slightly different for *S. aureus*. The exposure to pro-oxidative conditions did not changed the number of living *S. aureus* ($p=0.09$). However, in the presence of pectin-derived oligosaccharides the number of living bacteria was significantly reduced ($p<0.001$). APDO and PGDO showed similar bacteriostatic effects, whereas CPDO was comparatively less efficient (Fig. 4). It is worth mentioning that the effects of PGDO on *S. aureus* were less pronounced compared to the effects on *E. coli*. Pectin has an enormous potential for modifying gut microbiota, but these modifications occur at the level of individual strains and species and are not easily predicted *a priori* **(**Chung et al., 2016). It has been shown that pectin exert a selective pressure on bacteria, and that their survival depends on the expression of pectin-related enzymes and enzyme systems that are yet to be fully elucidated (Ndeh et al., 2017).

Pectins, a family of complex polysaccharides, and their degradation products such as polygalacturonic acid – an intact form of pectic-poly/oligosaccharide, show significant biological activity **(**Delphi, Sepehri, Khorranizadel, & Monsoori, 2015**)**. Our results imply that APDO, CPDO and PGDO show strong inhibitory effects on pathogenic bacteria. The production of $CO_2^{\bullet-}$, which was promoted by chemically processed pectin from apple by approximately 65% in comparison to processed polygalacturonic acid and citrus pectin, might be the main cause of the antimicrobial activity of the apple pectin derivative. It has been previously observed that

unprocessed pectin and polygalacturonic acid react with $HO^{\bullet}$ to produce superoxide radical anion ($O_2^{\bullet-}$) **(**Bogdanović Pristov, Jovanović, Mitrović, & Spasojević, 2013; Spasojević & Bogdanović Pristov, 2010**)**. It has been documented that pectin and $HO^{\bullet}$ react to produce the pectin C(5) radical, which further reacts with O_2, thus forming a C(5) peroxyl radical. This radical is unstable and is further decomposed **(**Spasojević & Bogdanović Pristov, 2010). It appears that structural alterations provoked by chemical processing of pectin that were applied here promote decomposition to $CO_2^{\bullet-}$ and some other EPR-silent fragments, and prevent the production of $O_2^{\bullet}$. Knowing redox properties of pectin is important for understanding some other health-protective effects. For example. recent results suggest that dietary pectin is a radio-protective agent **(**Sureban et al., 2015). Radiation generates highly-damaging $HO^{\bullet}$ radical via hydrolysis, so the capacity of pectin derivatives to remove $HO^{\bullet}$ might be a part of protective mechanisms.

Today, about 71% of apples are consumed as fresh apples while about 20% is processed into value-added products, of which 65% are processed into apple juice concentrate and other products. The by-product, apple pomace, is a rich and potentially commercially-viable source of pectin. It has been shown that apple pectin derivatives can alter the profile of human gut microbiota in a manner that could have benefits to health (Koutsos et al., 2017). The results presented here add to this, and elucidate potential mechanisms as well as the importance of the pectin source and processing method.

Conflict of interest

The authors declare no competing financial interest

Acknowledgement

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Abbreviations:

APDO Apple pectin derived oligosaccharides;

Asc Ascorbate

CPDO Citrus pectin derived oligosaccharides;

$CO_2^{\bullet-}$ Carbon dioxide radical anion;

DEPMPO 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide

EPR Electron paramagnetic resonance spin-trapping spectroscopy

FTIR Fourier Transform Infrared

$HO^{\bullet}$ Hydroxyl radical;

H_2O_2 Hydrogen peroxide;

$^{\bullet}O_2^-$ Superoxide radical anion

PGDO Polygalacturonic acid-derived oligosaccharides;

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FIGURE LEGENDS

Fig. 1. FTIR spectra of apple pectin and apple pectin-derived oligosaccharides.

Fig. 2. EPR spectra of DEPMPO spin-adducts. A: Fenton reaction: Fe^{2+} (0.2 mM) + H_2O_2 (1 mM). Gray: Simulation of signal of DEPMPO/OH adduct; B: Fenton system + APDO (15 mg/mL), Gray: Simulation of signal of DEPMPO/CO_2 adduct; C: Fenton system + CPDO; D: Fenton system + PGDO.

Fig. 3. Effects of APDO, CPDO or PGDO in the presence of acorbate (Asc) and Fe^{3+} on the growth of *E. coli*. Results are presented as mean ± S.E. The effects of oligosaccharides are compared to system with only Asc + Fe^{3+} (*** $p<0.001$).

Fig. 4. Effects of APDO, CPDO or PGDO with acorbate (Asc) + Fe^{3+} on the growth of *S. aureus*. Results are presented as mean ± S.E. and compared to the system with only Asc + Fe^{3+}.. Statistical analysis showed significant reduction on growth of *S. aureus* exposed to APDO, CPDO or PGDO with Asc + Fe^{3+} (*** $p<0.001$).

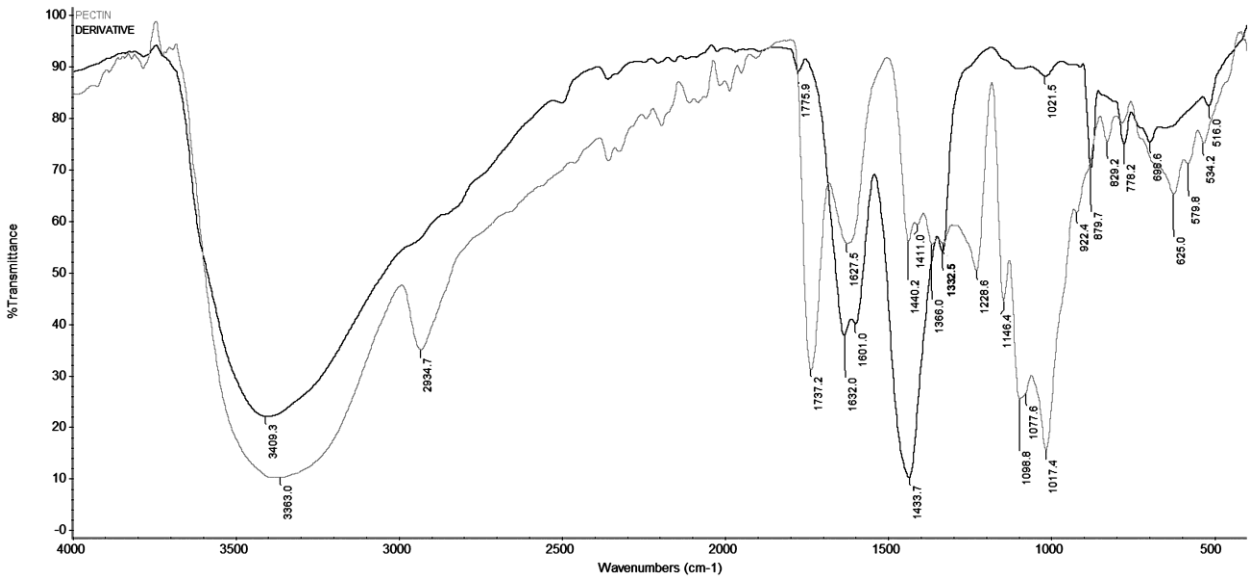

Fig. 1

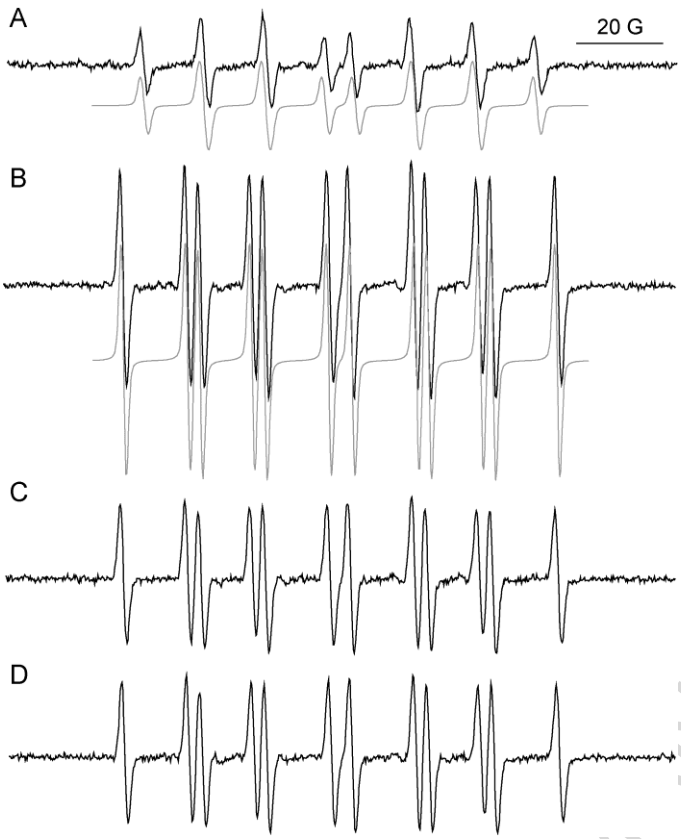

Fig. 2

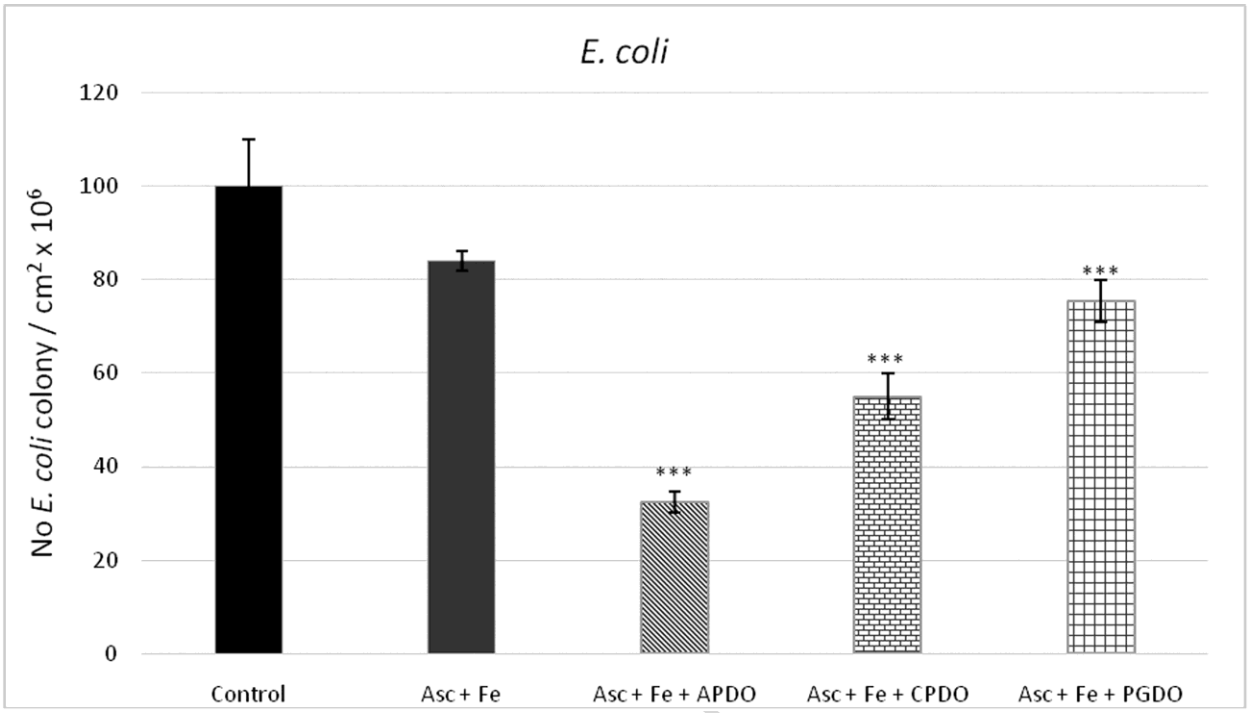

Fig. 3

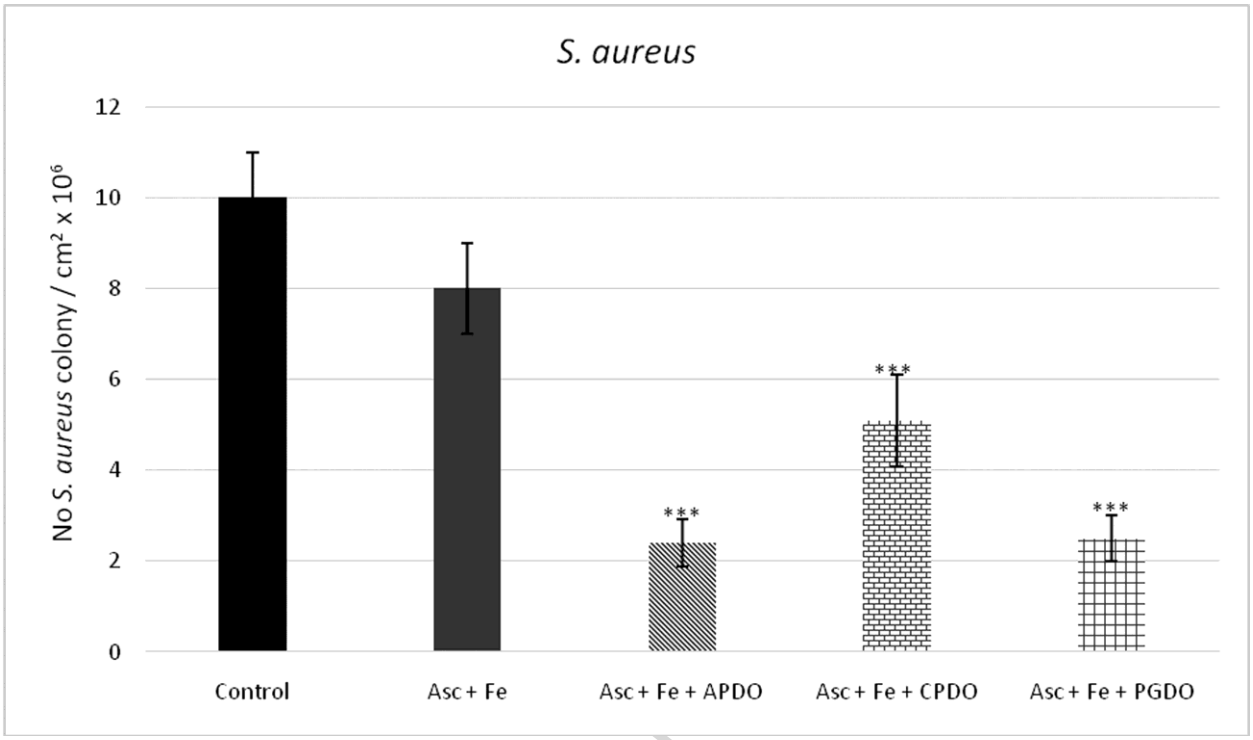

Fig. 4

Graphical abstract

Highlights

- Pectine derived oligosaccharides react with $HO^{\bullet}$ radical to produce $CO_2^{\bullet-}$.
- Apple pectin derived oligosaccharides has the most bacteriostatic effect.
- Bacteriostatic effect is related with oligosaccharides ability to produce $CO_2^{\bullet}$.