Technological Activities and Antibiofilm Effect of *Staphylococcus Equorum* Isolated from Fermented Sausage

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Abstract: The present study aimed at evaluating the technological properties and the ability of *Staphylococcus equorum* to inhibit biofilm formation by *Staphylococcus aureus* and *Escherichia coli* O157:H7. Seventeen *S. equorum* strains were isolated from fermented sausage and examined for their ability to grow at 10 or 15 ºC in the presence of 10 or 15% NaCl at pH values of 4.5, 5.0 or 5.5. Technological properties including catalases, superoxide dismutase, lipolytic and proteolytic activities of *S. equorum* strains were determined. All strains grew under the studied conditions and showed variable activities. The strain *S. equorum* HMA4 showed good characters of all the studied metabolic properties and had ability to grow under all studied conditions. The antagonistic effect of *S. equorum* HMA4 against *S. aureus* or *E. coli* O157:H7 biofilms was studied under the above conditions. There was a significant increasing in the reduction percentage of biofilm formation at pH 5.0 and 5.5 comparing with those obtained at pH 4.5. The antibiofilm effect of *S. equorum* HMA4 against *E. coli* O157:H7 was higher than its effect on biofilm of *S. aureus*. In conclusion, beside its role in the developing flavor and enhancing the color of the fermented foods, *S. equorum* HMA4 could be used as a protective starter against some foodborne pathogens in fermented foods.
Keywords: Biofilm; Protective starter; *S. equorum*; *S. aureus* and *E. coli* O157:H7.

1. Introduction

Microbial biofilms are defined as viable and nonviable microorganisms embedded in their polyanionic extracellular polymeric substances (EPS) that anchored to a biotic or abiotic surface [1,2]. Virtually all bacteria can grow as a biofilm [3]. Previous literatures indicated that approximately 80% of all bacterial infections either in human or animals are related to biofilms [1,4,5]. Antimicrobial biofilm resistance has become a global problem in the foodborne pathogens especially that grown on the surface of fermented foods [6-8]. Many strains of Shiga toxigenic *Escherichia coli* (STEC) are the reasons of foodborne disease, which recovered from meat products including the fermented sausages [9]. *E. coli* O157:H7 can grow in tryptone soy broth (TSB) containing 6.5% NaCl or at a pH of 4.5 to 9.0. Moreover, *E. coli* O157:H7 may represent a hazard in sausage batter, because when initially present at 10^4 CFU/g, this organism can survive fermentation, drying, and storage of fermented sausage regardless of whether an added starter culture was used [10]. The resistance of *E. coli* O157:H7 to NaCl concentration depends on the growth medium and temperature, where its growth at 37 °C was inhibited at 28% NaCl whereas at 10°C, growth was inhibited at 24% NaCl in TSB and at 26% NaCl in poultry extract broth [11]. Moreover, *E coli* O157:H7 has shown ability to attach to biotic and abiotic surfaces and to form biofilm [12]. *S. aureus* is able to grow in a wide range of temperatures (7 °C to 48.5 °C with an optimum of 30 to 37°C), pH (4.2 to 9.3, with an optimum of 7 to 7.5) and NaCl concentrations (up to 15% NaCl). These features enable *S. aureus* to grow in a wide variety of foods including fermented sausage [13]. Therefore, the high prevalence of these pathogens that resist high NaCl concentrations and low pH values, may promote health risk to the consumer of the fermented meat products.

In fermented meat products, Micrococcaceae participate in the development and stability of a good red color through nitrate reductase activity that leads to the formation of nitrosomyoglobin [14]. Coagulase-negative staphylococci (CNS) are a heterogenous group of Gram-positive cocci including *Staphylococcus xylosus*, *S. carnosus* and *S. equorum*. It is well known that, *S. equorum* is responsible for developing flavor of fermented sausage by reduction of nitrate to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by producing flavor and aroma compounds through proteolysis and lipolysis [15-20]. *S. equorum* represented 49% of staphylococcal isolates from French naturally fermented sausages [21].

Ten to forty percentage from southern Italian sausages [14] and 23.5% from fresh sausages [22]. Due to its ability to grow at 10 °C, *S. equorum* has been suggested for the development of starter preparations for curing of meat products [16]. Moreover, *S. equorum* is known as a bacteriocin producer such as micrococcin P1 [18,23].

Besides its technological activities for developing flavor and enhancement the color of fermented meat, the current study aimed to determine the antibiofilm effect of *S. equorum* against *S. aureus* and *E. coli* O157:H7.

2. Materials and methods

2.1 Isolation and identification of *S. equorum*

S. equorum isolated from traditional fermented sausages from the local market of Giza City, Egypt was used in this study. The strains were isolated on Mannitol Salt Agar (MSA, Oxoid) after 48 h at 30 °C and identified as reported by Mauriello *et al.,* [14]. Briefly, colonies from countable plates were initially tested for morphology, Gram-stain and catalase production. Gram-positive and catalase-positive cocci were purified by streaking on MSA. They were subjected to the oxidation/fermentation (OF) test in OF medium and to the anaerobic growth in semisolid thioglycollate medium [24]. Sensitivity to furazolidone, bacitracin and lysostaphin was determined as described by Kloos and Bannerman [25]. Production of pigment was observed on P-agar. Staphylococci were assayed for coagulase activity using the test tube with coagulase plasma (Becton, Dickinson & Company, NJ, USA) and for novobiocin sensitivity. Other biochemical properties were studied using API Staph identification strips and API LAB Plus software according to the manufacturer's instructions (API, Biome´rieux System). Identification of the most potent strain was confirmed by partial sequencing of the 16S rRNA gene using fD1 (5′-agagtttgatcctggctcag-3′) and rD1 (5′-aaggaggtgatccagcc-3′) (*E. coli* positions 8–17 and 1540–1524, respectively) primer [26]. The nucleotide sequence data obtained in the current work were submitted to the EMBL Nucleotide Sequence Database.

2.2 Microbial growth

The isolated *S. equorum* strains were tested for growth ability in yeast tryptone broth (YTB) at different temperatures (10, 15, 20, 30 and 37 °C). The effect of pH on the ability of growth was examined in YTB at pH values of 4.5, 5.0 and 5.5 (adjusted with 0.1 M HCl) at temperatures usually used for meat fermentation (10 and 15 °C). The effect of NaCl concentrations (10% and 15%) was also studied at 10 or 15 °C [14]. Ten microlitres of an overnight culture of each strain were inoculated into the 250 mL of different media described above under static conditions for 48 h. The growth response was followed by determining the optical density at 600 nm (OD_{600}), and registered, whether positive or negative, after 48 h.

2.3 Technological properties

2.3.1 Catalase assay

Catalase activity (CA) was measured on resting cells according to Aebi's method [14,19]. Results were expressed in arbitrary units (AU): μ mol of degraded H_2O_2/min/ml of cells with $OD_{600} = 1.0$.

2.3.2 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by the method described by Mauriello *et al* [14]. Briefly, 2 mL overnight culture of the isolated strain was centrifuged at 13000g for 5 min. The cell pellet of each strain was washed once in 50 mM K_2HPO_4, pH 7.8. For SOD extraction 100 mg cell pellet (wet weight) was suspended in 1 mL of the same buffer. Pellets were disrupted using glass beads (~ 0.1 mm) on a vortex mixer for 5 min. Afterwards, the mixture was centrifuged and the supernatant was used for determination of SOD activity. Ten μL of cell extract were added to 1.0 ml of 150 μ M nitroblue tetrazolium (NBT), 10 mM methionine, 1.2 μ M riboflavin, 50 mM K_2HPO_4 pH 7.8 and incubated at room temperature in a light chamber with a 60 W bulb lamp for 8 min. Results were expressed as percentage OD_{560} according to the following equation: SOD activity = (1-S/C) · 100; where S is the OD_{560} of the sample and C is the OD_{560} of the control. Control is consisting of a solution without adding cell extract and incubated under the same conditions.

2.3.3 Nitrate reductase assay

Nitrate reductase (NR) activity was measured as described by Mauriello *et al* [14]. 2 mL overnight culture of the isolated strain was centrifuged at 13000g for 5 min. The cell pellet of each strain was resuspended in equal volume of 50 mM phosphate buffer pH 7.0. Yeast tryptone agar (YTA, tryptone 1%, yeast extract 0.5%, NaCl 0.4%, agar 1.5%, pH 7.0) plates supplemented with KNO_3 (1 g/L) were prepared and wells were created using cork borers with 6 mm diameter. Thirty micrograms of the resuspended culture were loaded into wells. After incubation at 30 °C for 7 h the plates were flooded with 1 mL of a 1:1 solution of NIT1 (0.8 g sulphanilic acid in

100 mL of acetic acid 5 N) and NIT2 (0.6 g N-N-dimethyl-1-Naphthylamine in 100 ml of acetic acid 5 N) for the detection of nitrite. The appearance of red haloes (expressed in mm) surrounding the wells indicated the presence of nitrate reductase activity.

2.3.4 Lipolytic activity

Lipolytic activity (LA) was measured according to the method described by Mauriello *et al* [14]. Briefly, one ml of an overnight culture of each strain was inoculated into 10 ml of a broth containing 1% tryptone, 0.5% yeast extract, 3% NaCl, pH 7.0, supplemented with 4% (w/v) beef fat. For the preparation of media, beef fat was homogenized by vigorous shaking. After incubation at 30 ºC for seven days, the samples were used for the determination of free fatty acids [14]. Lipolytic activity was expressed as a percentage of oleic acid.

2.3.5 Proteolytic activity

Proteolytic activity (PA) was measured according to the method described by Mauriello *et al* [14,19]. A clear zone surrounding the inoculated wells indicated proteolytic activity and its diameter was measured in mm.

2.4 Anti-biofilm effect of *S. equorum* against foodborne pathogens

Two foodborne pathogens; *S. aureus* and *E coli* O157:H7, were obtained from the culture collection of Food Science Department, Faculty of Agriculture, Ain Shams University. The antagonistic effect of *S. equorum* HMA4 against biofilm formation by the studied foodborne pathogens were tested by a colorimetric method adapted from previous work [27-29]. *S. equorum* and the tested pathogens were cultivated overnight at 37°C separately in BHI broths. After incubation, the three bacterial suspensions were washed with the saline solution, diluted in BHI (pH 7.4), and mixed in order to obtain the final suspension containing 5×10^7 CFU/mL of each tested pathogen. With each tested pathogen a quantity of 100 µL of *S. equorum* were deposited per well in 96-well (Minitek, USA), that were incubated at tested temperatures (10 or 15 °C). After 24 h, the wells were washed twice with a saline solution (NaCl 0.9%), and then 100 µL of crystal violet (0.25%) were added in each well for biofilm colorization. After 10 min, wells were washed twice again with the saline solution and the remaining crystal violet was released by addition of 100 µL of acetic acid (33%).

The Optical density (OD) of each well was measured at 570 nm using a UV/Visible spectrophotometer (6105-Jenway, U.K.). The positive control was the amount of biofilm formed by the pure culture of the tested pathogens, whereas the negative control was sterile BHI.

To study the anti-biofilm effect of *S. equorum* at different conditions including NaCl and pH, the above-mentioned experiment was carried out in the presence of 10 or 15% NaCl at pH 4.5, 5.0 or 5.5.

Reduction percentage of biofilm formation was expressed using the following equation:

% Reduction = [(OD_{570} of tested pathogen - OD_{570} of tested pathogen and *S. equorum*)/ OD_{570} of tested pathogen]x100.

2.5 Statistical analysis

The data obtained from three replicates were analyzed by a two-way ANOVA using 'Proc Mixed' (SAS 8.2, Cary, NC) for interaction effects of the strains, produce and sampling period. In all cases, the level of statistical significance was of $P<0.05$.

3. Results and discussion

3.1 Technological properties

Seventeen *Staphylococcus* strains were isolated from sausage and identified as *S. equorum* according to the morphological and biochemical tests and API-Staph results. All isolated strains have grown well at temperature range of 10 to 37 ºC (data not shown). The ability of the isolated strains to grow at temperatures usually used for meat fermentation (10 and 15 ºC) at different pH values and NaCl concentrations was determined (Table 1). All isolated strains grew at pH 5.0 and 5.5, however, at pH 4.5 only 23.5 and 41.1% of the tested strains had ability to grow at 10 and 15 ºC, respectively. On the other hand, all strains grew in the presence of 10% NaCl at 10 and 15 ºC. Inhibitory effect on the ability of some *S. equorum* strains (47.1 and 29.4% at 10 and 15 ºC, respectively) was recorded when 15% NaCl was added to the growth medium (Table 2).

These results indicated that not all *S. equorum* strains can grow under the main factors (pH and salt stress) encountered during production of fermented meat. These results were in accordance with those reported by Sondergaard and Stahnke [20]. They found that increasing salt concentration from 5% to 15% w/v decreased growth of *S. carnosus*, *S. equorum* and *S. xylosus*, but the effect of pH and temperature was much stronger than the effect of salt. Moreover, Carvalho *et al*., [30] stated that *S. equorum* showed to grow under all the studied conditions being more effective at 15°C, 20°C and pH 5.5 at 20°C, and NaCl concentration of 10%; therefore, can

be guaranteed their application in technological processes with varying temperatures. Recently, Lee *et al.,* [17] demonstrated that, *S. equorum* KS1039 could be used as a potential starter culture for the fermentation of high-salt foods.

Table 1. Effect of pH on the growth ability of *Staphylococcus equorum* strains

Strains	Growth at 10 ℃			Growth at 15 ℃		
	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5
HMA 1	-	+	+	+	+	+
HMA 2	-	+	+	-	+	+
HMA 3	+	+	+	+	+	+
HMA 4	+	+	+	+	+	+
HMA 5	-	+	+	-	+	+
HMA 6	-	+	+	-	+	+
HMA 7	+	+	+	+	+	+
HMA 8	-	+	+	-	+	+
HMA 9	-	+	+	-	+	+
HMA 10	-	+	+	+	+	+
HMA 11	-	+	+	-	+	+
HMA 12	-	+	+	-	+	+
HMA 13	+	+	+	+	+	+
HMA 14	-	+	+	-	+	+
HMA 15	-	+	+	-	+	+
HMA 16	-	+	+	-	+	+
HMA 17	-	+	+	+	+	+

Table 2. Effect of NaCl concentration on the growth ability of *Staphylococcus equorum* strains

Strains	Growth at 10 ℃		Growth at 15 ℃	
	10% NaCl	15% NaCl	10% NaCl	15% NaCl
HMA 1	+	-	+	-
HMA 2	+	-	+	+
HMA 3	+	+	+	+
HMA 4	+	+	+	+
HMA 5	+	-	+	+
HMA 6	+	-	+	-
HMA 7	+	-	+	+
HMA 8	+	+	+	+
HMA 9	+	+	+	+
HMA 10	+	-	+	+
HMA 11	+	+	+	+
HMA 12	+	-	+	-
HMA 13	+	+	+	+
HMA 14	+	+	+	+
HMA 15	+	+	+	-
HMA 16	+	+	+	+
HMA 17	+	-	+	-

The technological properties of the isolated strains, presented in Table 3, showed high variability amongst strains. All *S. equorum* strains had antioxidative enzymes catalase (5.3 – 19.6 AU) and SOD (2.5 – 43.5 % activity). The strain *S. equorum* HMA4 displayed the highest values of catalase and SOD activities while the strains HMA2 and 16 had the lowest levels of catalase and SOD, respectively. About 52% of the isolated strains showed ability to reduce nitrate (Table 3). Only 41.1 and 29.4% of the studied strains had lipolytic and proteolytic activity, respectively. In accordance with the obtained results, Carvalho *et al.,* [30] stated that 42% and 30% of *S. equorum* and *S. xylosus* revealed lipolytic activity and proteolytic ability, respectively, while 65% of the strains reduced nitrate. In the present study, the *S. equorum* strains that showed proteoltic ability displayed greater proteolysis against myofibrillar than sarcoplasmic proteins. This result is in harmony with that reported by Mauriello *et al* [14].

In general, the strain *S. equorum* HMA4 showed good characters of all the studied metabolic properties and grew under all studied conditions. Therefore, this strain was subjected for further work to investigate its ability to inhibit biofilm formation by some foodborne pathogens.

Table 3: Technological properties[a] of *Staphylococcus equorum* strains

Strain	Catalase activity[b] (AU)	SOD activity[c] (%)	Nitrate reduction[d] (mm)	Lipolysis[e] (%)	Proteolysis[f] (mm)
HMA 1	5.3 ± 0.12	14.2± 0.22	7± 0.36	7.05± 0.25	0/0
HMA 2	4.8 ± 0.1	11.3± 0.42	10± 0.43	0	9/0
HMA 3	16.3 ± 0.31	9.5± 0.21	12± 0.51	0	0/0
HMA 4	19.6 ± 0.15	43.5± 1.63	15± 0.37	35.2± 0.12	22/14
HMA 5	6.8 ± 0.17	12.3± 0.19	0	0	0/0
HMA 6	11.1 ± 0.14	15.4± 0.31	7± 0.63	0	2/2
HMA 7	19.1 ± 0.13	22.3± 0.26	10± 0.52	0	0/0
HMA 8	14.2 ± 0.16	14.6± 0.34	0	7.05± 0.17	0/0
HMA 9	13.4 ± 0.22	5.5± 0.12	0	0	12/2
HMA 10	12.0 ± 0.21	13.6± 0.21	8± 0.24	0	0/0
HMA 11	18.2 ± 0.18	5.9± 0.125	0	7.05± 0.19	0/0
HMA 12	5.4 ± 0.12	17.2± 0.42	11± 0.51	0	9/0
HMA 13	7.8 ± 0.15	16.2± 0.22	0	14.1± 0.31	0/0
HMA 14	12.3 ± 0.31	22.0± 0.32	10± 0.35	0	0/0
HMA 15	14.5 ± 0.21	6.8± 0.33	0	7.05± 0.16	0/0
HMA 16	12.1 ± 0.23	2.5± 0.16	0	0	0/0
HMA 17	16.2 ± 0.26	19.2± 0.72	0	7.05± 0.14	0/0

[a] Values are means of three replicates, standard deviations are always lower than 10% of means., [b] Arbitrary units: µmoL of degraded H_2O_2/min/mL of cells with optical density at 600 nm (OD_{600}) =1.0., [c] % of optical density, [d] Diameter of red haloes., [e] % of oleic acid., [f] Diameter of clear zone on myofibrillar/sarcoplasmic proteins.

3.2 Anti-biofilm effect of *S. equorum* HMA4 against foodborne pathogens

S. equorum HMA4 was tested for its ability to inhibit biofilm formation by *S. aureus* and *E coli* O157:H7 at 10 and 15 ℃ in the presence of 10 or 15% NaCl at pH values of 4.5, 5.0 or 5.5. Comparing with the biofilm formation of *S. aureus,* a significant reduction ($p \leq 0.05$) in biofilm formation was detected when *S. equorum* HMA4 and *S. aureus* were synergistically incubated at both tested temperature (10 and 15 ℃) (Fig. 1). In the presence of 10% NaCl, the highest reduction effect (30%) was obtained at 15 ℃ and pH 5.0, whereas the highest reduction percentage in the presence of 15% NaCl was 36% (Fig. 1).

The reduction percentage of biofilm formation was ranged from 5 to 24% and from 5 to 36% when 10% and NaCl was added to the incubation medium, respectively. There was significant increase in the reduction percentage of biofilm formation at pH 5.0 and 5.5 comparing with those obtained at pH 4.5 (Fig. 1). This may be due to the negative effect of low pH value (pH 4.5) on the growth of *S. equorum*, as shown in Table 1. Same trend was obtained when *E coli* O157:H7 was used as tested strain (Fig.2). Remarkably, the highest reduction percentage of biofilm formation (34%) was recorded when *S. equorum* and *E coli* O157:H7 were used together in the presence of 15% NaCl, however, there is no significant difference between reduction percentages at pH 5.0 and 5.5 (Fig. 2).
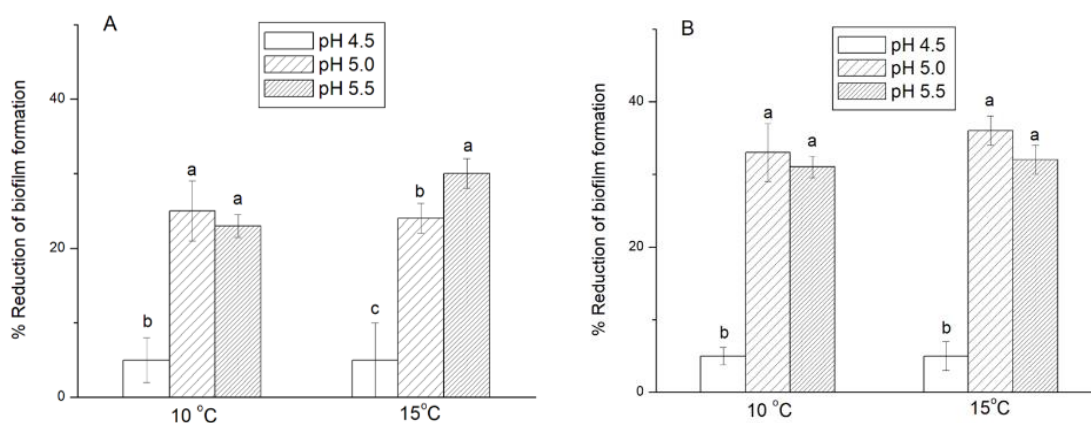

Fig. 1. Inhibitory effect of *Staphylococcus equorum* on biofilm formation by *Staphylococcus aureus* in the presence of 10 %(A) and 15% (B) NaCl at 10 and 15 ℃ at different pH values.

In general, the obtained inhibitory effect on the biofilm formation of both tested pathogens is probably due to the antagonistic behavior of the studied *S. equorum* strain HMA4. To the best of the author knowledge, the present work is the first study that considered the antagonistic effect of *S. equorum* against biofilm formation by foodborne pathogens such as *S. aureus* and *E coli* O157:H7. However, the effect of *L. fermentum* and *L. paracasei* against

biofilm formation by another bacterial pathogen *Pseudomonas aeruginosa* was demonstrated at laboratory scale [27].
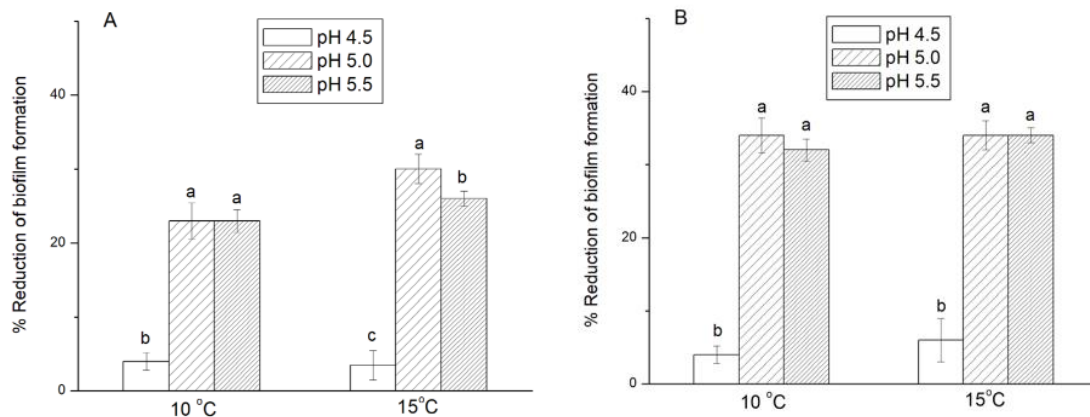

Fig. 2. Inhibitory effect of *Staphylococcus equorum* on biofilm formation by *Escherichia. coli O157:H7* in the presence of 10%(A) and 15% (B) NaCl at 10 and 15 ℃ at different pH values.

In conclusion, besides its role in the developing flavor and enhancing the color of the fermented foods, the obtained results demonstrated that *S. equorum* could be used as a protective starter against biofilm formation by some foodborne pathogens during fermentation of foods.

4. References

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