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ORIGINAL ARTICLE

Synthetic brominated furanone F202 prevents biofilm formation by potentially human pathogenic *Escherichia coli* O103:H2 and *Salmonella* ser. Agona on abiotic surfaces

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Keywords
biofilms, *E. coli* (all potentially pathogenic types), food safety, *Salmonella*.

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Abstract

Aims: Investigate the use of a synthetic brominated furanone (F202) against the establishment of biofilm by *Salmonella* ser. Agona and *E. coli* O103:H2 under temperature conditions relevant for the food and feed industry as well as under temperature conditions optimum for growth.
Methods and Results: Effect of F202 on biofilm formation by *Salmonella* ser. Agona and *E. coli* O103:H2 was evaluated using a microtiter plate assay and confocal microscopy. Effect of F202 on bacterial motility was investigated using swimming and swarming assays. Influence on flagellar synthesis by F202 was examined by flagellar staining. Results showed that F202 inhibited biofilm formation without being bactericidal. F202 was found to affect both swimming and swarming motility without, however, affecting the expression of flagella.
Conclusions: F202 showed its potential as a biofilm inhibitor of *Salmonella* ser. Agona and *E. coli* O103:H2 under temperature conditions relevant for the feed and food industry as well as temperatures optimum for growth. One potential mode of action of F202 was found to be by targeting flagellar function.
Significance and Impact of the Study: The present study gives valuable new knowledge to the potential use of furanones as a tool in biofilm management in the food and feed industry.

Introduction

Preparation and processing of food is considered as an important route for cross-contamination with pathogenic bacteria. This may subsequently cause human illness through contaminated food products (Reij and Den Aantrekker 2004; Moretro and Langsrud 2011). Many *Escherichia coli* pathotypes and *Salmonella enterica* subspecies *enterica* serovars are known as potential foodborne pathogens (Krogvold *et al.* 2011; Campioni *et al.* 2012; King *et al.* 2012; Raguenaud *et al.* 2012).

Escherichia coli is a complex group of bacteria that are abundant inhabitants of the intestinal tract of all warm-blooded animals including humans (Nataro 2006). Several *E. coli*, including Enteropathogenic *E. coli* (EPEC), are associated with foodborne infection with common symptoms like diarrhoea, vomiting and low-grade fever (Nataro and Kaper 1998). EPEC are defined as *E. coli* isolates positive for the intimine gene (*eae*) but lacking verotoxin genes and are divided into two subgroups: typical EPEC and atypical EPEC (aEPEC). Typical EPEC have only been isolated from humans as aEPEC have been isolated from both humans and animals (Trabulsi *et al.* 2002; Carneiro *et al.* 2006). aEPEC isolated from animals have been shown to be potentially pathogenic to humans as they possess virulence genes

necessary to cause illness (Monaghan *et al.* 2013). *Salmonella enterica* subspecies *enterica* consists of more than 1500 serovars and includes most of the serovars responsible for disease in mammals (Popoff and Le Minor 2005; Threlfall 2005). Even though less studied than *Salmonella* ser. Typhimurium, *Salmonella* ser. Agona is known to be capable of causing food poisoning disease and has in 2011 e.g. been linked to a large multistate outbreak of salmonellosis in the US caused by the ingestion of contaminated papaya (http://www.cdc.gov/salmonella/agona-papayas/ Accessed on 17.10.2013). Symptoms of salmonellosis is often acute gastroenteritis with self-limiting symptoms like diarrhoea, headache, stomach-ache and fever (www.cdc.gov, 12.06.2012; www.fhi.no, 12.06.2012).

Salmonella are frequently found in the feed industry and are known to be a costly problem for this industry (Veldman *et al.* 1995; Alvarez *et al.* 2003; Lunestad *et al.* 2007). In Norway, *Salmonella* ser. Agona is one of the most important serovars for this industry as specific clones have been shown to persist in some feed and fish meal factories for several years (Nesse *et al.* 2003).

Persistence and survival of pathogens in food and feed processing environments has been linked to biofilm formation (Lunden *et al.* 2000; Borucki *et al.* 2003; Møretrø and Langsrud 2004; Vestby *et al.* 2009b). Biofilms are defined as matrix-enclosed bacterial populations that are attached to a surface, an interface and/or to each other (Costerton *et al.* 1995, 1999). It is well known that biofilm enhances the tolerance of bacteria, including pathogenic *E. coli* and *Salmonella*, to antibacterial solutions and antibiotics (Costerton 1999; Scher *et al.* 2005; Moretro *et al.* 2009; Wang *et al.* 2012). The increased resistance to antibacterial treatments of bacteria in biofilms is attributed to several factors including reduced diffusion through the matrix, physiological changes, reduced growth rates and production of enzyme degrading antimicrobial agents (Kumar and Anand 1998).

Halogenated furanones were first discovered to inhibit bacterial colonization of the red algae *Delisea pulcra* (Kjelleberg and Steinberg 2001). Synthetic brominated furanones have since then been shown to be promising in the use against biofilm formation by different bacterial species in nonbactericidal concentrations (Ren *et al.* 2001; Hentzer *et al.* 2002; Rice *et al.* 2005; de Nys *et al.* 2006; Lonn-Stensrud *et al.* 2007; Han *et al.* 2008; Lianhua *et al.* 2013) and to potentiate the effect of antibiotics and disinfectants against bacteria in biofilm (Janssens *et al.* 2008; Vestby *et al.* 2010). The mode of action for furanones is not yet fully understood, but has e.g. been suggested to be by interference with the AI-2 quorum sensing system in oral streptococci and *Staphylococcus epidermidis* (Lonn-Stensrud *et al.* 2007, 2009; Benneche *et al.* 2008) and interference with flagellar synthesis in *Salmonella* ser. Typhimurium (Janssens *et al.* 2008). Functional flagella have been shown to be important in initial attachment and in biofilm development for both *E. coli* and *Salmonella* (Janssens *et al.* 2008; Mika and Hengge 2013; Vikram *et al.* 2013).

The aim of this study was to investigate the use of a synthetic brominated furanone against the establishment of biofilm by aEPEC and non-aEPEC strains of *E. coli* O103:H2 along with *Salmonella* ser. Agona under temperature conditions relevant for the food and feed industry as well as temperature optimum for growth. Basic mode of action studies were performed.

Materials and methods

Bacterial strains and culture conditions

A total of 15 strains were used in this assay. This includes eight strains of *E. coli* O103:H2, and seven strains of *Salmonella enterica* subspecies *enterica* serovar Agona (Table 1). All isolations were carried out at different private or official laboratories and verified at the National Reference Laboratory for nonhuman isolates of *Salmonella* and Shiga-toxin producing *E. coli* at the Norwegian Veterinary Institute.

The strains were stored at −80°C in Brain Heart Infusion broth (BHI; Difco, BD, Franklin Lakes, NJ, USA) supplemented with 15% glycerine (Merck KGaA, Darmstadt, Germany) and recovered on blood agar at 37 ± 1°C overnight. The bacterial cultures were then transferred into Luria Bertani broth (LB; Merck) and incubated statically overnight (18–24 h) at 37 ± 1°C to obtain an overnight working culture.

Furanone preparation

The brominated furanone tested, F202 (Fig. 1) was synthesized at the University of Oslo, Department of Chemistry, as previously described (Benneche *et al.* 2006). The furanone was dissolved in absolute ethanol (Kemetyl Norge AS, Vestby, Norway) to a concentration of 1 mol l^{-1} and further diluted to working solution 10 000 μmol l^{-1} in DEPC water (Invitrogen, Carlsbad, CA, USA). The furanone working solutions were stored at −20°C and thawed immediately before use.

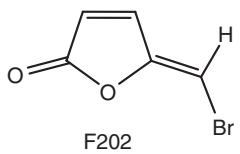
Bactericidal determination of furanone F202 on pure cultures

Determination of furanone F202 bactericidal action on pure cultures was determined for *Salmonella* ser. Agona and *E. coli* O103:H2 according to a method described by Janssens *et al.* (2008) with slight modifications (Janssens

Table 1 Bacterial strains used in this study

Strain ID number	Genus	Characteristics	Origin	Biofilm OD_{595} values			Reference
				12°C	20°C	37°C	
2000-01-2168-10 (2168-10)	*Salmonella* ser. Agona		Feed factory	1·530	1·510	0·049	Vestby *et al.* (2010)
2002-01-71-3 (71-3)	*Salmonella* ser. Agona		Feed factory	0·856	0·986	0·025	Moretro *et al.* (2009); Vestby *et al.* (2009b)
2002-01-2147-7 (2147-7)	*Salmonella* ser. Agona		Feed factory	1·479	1·576	0·061	Vestby *et al.* (2009b)
2006-01-2021-3 (2021-3)	*Salmonella* ser. Agona		Feed factory	0·967	1·283	n.d.	Vestby *et al.* (2009b)
2000-01-2168-4 (2168-4)	*Salmonella* ser. Agona		Human (clinical)	1·205	1·497	0·029	Vestby *et al.* (2009a)
2000-01-2168-3 (2168-3)	*Salmonella* ser. Agona		Human (clinical)	0·993	1·802	0·024	Vestby *et al.* (2009a)
2000-01-2168-7 (2168-7)	*Salmonella* ser. Agona		Human (clinical)	1·347	1·779	0·031	Vestby *et al.* (2009a)
2006-22-1153 (1153)	*E. coli* O103:H2	*eae*$^+$, lacking *stx*	Sheep survey	1·330	1·726	0·009	Sekse *et al.* (2013)
2007-60-10651 (10651)	*E. coli* O103:H2	*eae*$^+$, lacking *stx*	Sheep survey	1·179	2·130	0·677	Sekse *et al.* (2013)
2007-60-10709 (10709)	*E. coli* O103:H2	*eae*$^+$, lacking *stx*	Sheep survey	1·362	1·843	0·071	Sekse *et al.* (2013)
2006-22-1242 (1242)	*E. coli* O103:H2	Lacking *stx* and *eae*	Sheep survey	2·175	1·863	1·038	Sekse *et al.* (2013)
2006-22-1246 (1246)	*E. coli* O103:H2	Lacking *stx* and *eae*	Sheep survey	1·159	1·634	0·816	Sekse *et al.* (2013)
2007-60-10568 (10568)	*E. coli* O103:H2	*eae*$^+$, lacking *stx*	Sheep survey	1·047	1·434	0·811	Sekse *et al.* (2013)
2006-22-1271 (1271)	*E. coli* O103:H2	Lacking *stx* and *eae*	Sheep survey	1·724	1·930	2·084	Sekse *et al.* (2013)
2007-60-10705 (10705)	*E. coli* O103:H2	Lacking *stx* and *eae*	Sheep survey	1·394	1·848	0·204	Sekse *et al.* (2013)

n.d., Not done.



F202

Figure 1 Chemical structure of furanone F202: (*Z*)-5bromomethylene-2(5*H*)-furanone.

et al. 2008). Briefly, colonies from a blood agar plate that were incubated overnight at 37 ± 1°C were suspended into 5 ml sterile saline until the density of 0·5 McFarland standard was obtained. A total of 10 μl was subsequently transferred to 10 ml IsoSensitest broth (Oxoid Ltd., Cambridge, UK), mixed well using vortex and 45 μl was transferred to a microtiter plate well containing 5 μl furanone solution of seven different concentrations (0·01, 0·1, 1, 10, 25, 50 and 100 μmol l^{-1}). The plate was incubated at 37 ± 1°C for 18–20 h and growth recorded by visual inspection and optical density measurements (Multiscan MS, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 595 nm (OD_{595}). The results for biofilm in microtiter plates are given as: (OD_{595} values with furanone)/(OD_{595} values without furanone) * 100.

Studies on anti-biofilm potential by furanone F202

Microtiter plate assay

The effect of the furanone on biofilm formation by *Salmonella* ser. Agona and *E. coli* O103:H2 was measured as previously described (Vestby *et al.* 2010) with slight modifications. In brief, the furanone was diluted to a final concentration of 12·5, 25 or 50 μmol l^{-1} in polystyrene microtiter plates (NUNC, Nunclon, Roskilde, Denmark) using LB without NaCl (LB ^{wo}/NaCl; bactotryptone 10 g l^{-1}, yeast extract 5 g l^{-1}). In each well, an aliquot of 30 μl overnight bacterial culture was added to 100 μl LB without NaCl. The same volume of sterile distilled water was added to the negative controls. The plates were incubated at 12 ± 1°C for 168 h and at 20 ± 1°C and 37 ± 1°C for 48 h, and biofilm recorded by optical density measurements (OD_{595}). Triplicates of each sample were used and three independent experiments were performed for all strains using freshly prepared solutions. The results are given as amount biofilm formed with furanone (OD_{595} values)/amount biofilm formed without furanone (OD_{595} values) * 100.

Confocal microscopy

Confocal microscopy was used for visualizing the effect of furanone F202 on biofilm formation by *Salmonella* ser. Agona and *E. coli* O103:H2. Overnight cultures were inoculated (10 μl) in sterile centrifuge tubes (Greiner bio-one GmbH, Frickenhausen, Germany) containing 10 ml LB broth ^{wo}/NaCl with 50 μmol l^{-1} furanone or equal volume of sterile distilled water (control). An autoclaved stainless steel coupon (AISI 304, 2B, 7 by 2 by 0·1 cm pieces) was partially submerged into the liquid and incubated for 48 h at 20 ± 1°C. After incubation, the biofilms were stained with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen, Molecular probes Inc, Eugene, OR, USA). The kit reagent was diluted 1 : 1000 in sterile distilled water. Secure seals imagine spacers (Sigma-Aldrich, St Louis, MO,

USA) were used in combination with coverslides (Gerhard Menzel GmbH, Braunschweig, Germany) to protect the biofilm. Stained biofilms were immediately placed in petri dishes containing a piece of paper towel saturated with sterile distilled water to avoid dehydration of the sample. The petri dishes were covered in aluminium foil. Horizontal plane images of biofilms were acquired using an inverted Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss AS, Oslo, Norway). The set excitation/emission wavelengths used were 480/500 nm for Syto9 and 535/617 nm for PI. Images were collected through a 10× Plan-APOCHROMAT objective lens with a *z*-step of 1 μm. The structural quantifications of biofilms (biovolume, roughness and thickness) were estimated using PHILP Matlab program (http://phlip.sourceforge.net/phlip-ml). PHLIP image analysis enables analysis of multi-channel CLSM data. For each CLSM stack, a height projection transformation of the image stack was created where for every point in the *xy* plane, the maximal height *h* of the corresponding foreground pixels in *z* direction was stored. The mean thickness was then calculated as the average of the resulting distribution of pixel height *h* (Xavier *et al.* 2003). The roughness coefficient was obtained by first calculating the standard deviation of the distribution and divided this value by the mean thickness (Heydorn *et al.* 2000).

Studies on effect on bacterial motility by furanone F202 under biofilm conditions

Swimming assay

The method used was adapted from Janssens *et al.* (2008) with modifications. Swimming assay medium was prepared using LB ^{wo}/NaCl with 0·3% agar. The medium was freshly prepared and kept in a waterbath at 55°C until used. The furanone was added to the medium to a final concentration of 50 μmol l^{-1} or the same amount of sterile distilled water in the control. An amount of 3 ml swimming assay medium was pipetted into sterile 6-wells microtiter plates (CORNING® COSTAR® cell culture plates, Corning Inc., Lowell, MA, USA). The medium was subsequently set to cool in room temperature for 1–3 h before 1 μl of overnight cultures were inoculated to the centre of the agar half way down the agar. The microtiter plates were incubated at 12 ± 1°C for 168 h, 20 ± 1°C for 24 h and at 37 ± 1°C for 5 h. The swimming zone diameter was measured by visual inspection using a conventional ruler. At least two independent experiments were performed.

Swarming assay

Overnight cultures in LB ^{wo}/NaCl were diluted to OD_{600} = 1·0. Swarming motility medium was prepared using bacto-agar 5 g l^{-1}, bacto-tryptone 10 g l^{-1}, yeast extract 5 g l^{-1} and D-(+)-glucose 5 g l^{-1}. Furanone was added to the freshly prepared medium to a final concentration of 50 μmol l^{-1}, or the same amount of sterile distilled water was added in the control. Aliquots of 3 ml swarming medium was pipetted into sterile 6-wells microtiter plates (NUNC, Nunclon, Surface cell culture plates) subsequently let to dry overnight in room temperature. Overnight cultures were spot inoculated (1 μl) on the plates and the plates were incubated at 37 ± 1°C for 10 h. The swarming diameter was measured using a conventional ruler. Each experiment was performed at least twice.

Detection of flagella

Staining of flagella was performed as previously described (Janssens *et al.* 2008) with slight modifications. In short, overnight cultures were diluted 1 : 50 in LB broth ^{wo}/NaCl in the presence of 50 μmol l^{-1} furanone or the same amount of sterile distilled water. The samples were incubated statically at 20 ± 1°C for 17 h before staining. The dye was prepared by mixing 10 parts of mordant solution (2 g tannic acid, 10 ml 5% aqueous phenol and 10 ml saturated aqueous $AlKO_8S_2{\cdot}12H_2O$) with one part stain (12% crystal violet in ethanol). An aliquot of 3 μl sample was applied to a microscope slide (76 × 26 mm, Gerhard Menzel GmbH) and covered with a coverslip (18 × 18 mm, Gerhard Menzel GmbH). The slide was placed horizontally on the bench and 1·5 μl of dye was applied to the edge of the coverslip to stain the sample by capillary action. Samples were observed with 100× magnification using Olympus BX50 light microscope (Olympus Europa Holding GmbH, Hamburg, Germany). Pictures were taken using Samsung Pixion 12, M8910 (Samsung, Ridgefield Park, NJ, USA). The experiment was performed twice.

Statistical analysis

When testing the effect of furanone F202 on bactericidal activity and biofilm formation, corresponding 95% confidence intervals (CI) was calculated using 'CONFIDENCE' function in Excel® 2010 (Microsoft®, Redmond, WA, USA). Means with CI's not including 100% were considered statistical significant. When testing the effect of furanone F202 on the structural quantifications of biofilms (biovolume, roughness and thickness) as found by the confocal microscope experiments, the ANOVA test in Minitab v15.1 (Minitab Inc. State College, PA, USA) was used. The association between the use of the furanone F202 and an effect on bacterial motility in biofilms, as found by the swarming and swimming assays, were analyzed using two-tailed *t*-test using 'T-TEST' function in Excel® 2010. The level of significance was set to $P < 0{\cdot}05$ in all experiments.

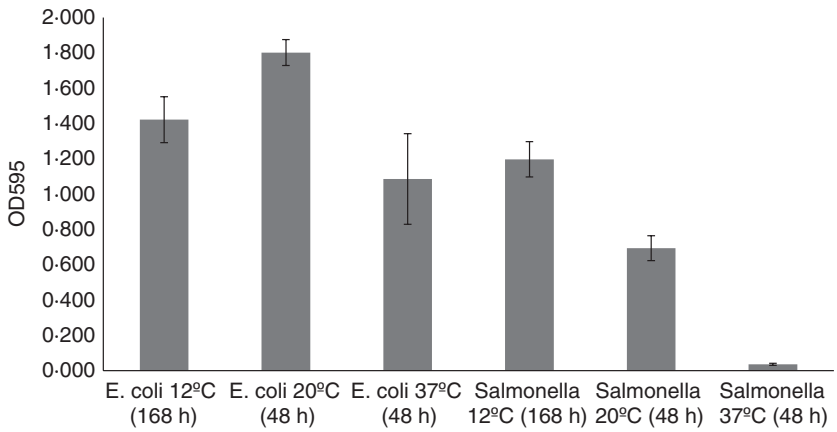


Figure 2 Biofilm on microtiter plates by *E. coli* O103:H2 (all strains) and *Salmonella* ser. Agona (all strains) at 12°C, 20°C and 37°C. Results are given as mean values of OD measurements at 595nm with standard deviations.

Results

Effect of the furanone on bacterial growth and biofilm formation

Bactericidal effect of F202 was determined for two randomly chosen strains for each species, i.e. *Salmonella* 2168-10, *Salmonella* 71-3, *E. coli* 1242 and *E. coli* 1153. Reduced or eliminated bacterial growth as determined by visual inspection was not observed at any of the concentrations tested. A slight inhibitory effect of the furanone was seen as a reduction in the OD_{595} measurements at the highest concentration, i.e. 100 μmol l^{-1} [mean 85 $\pm$ 14% (95% CI)] and 50 μmol l^{-1} was therefore the highest concentration used in further experiments.

When all strains were tested in the microtiter plate assay, both *Salmonella* ser. Agona and *E. coli* O103:H2 strains were capable of forming biofilm on polystyrene microtiter plates at 12 and 20°C (Fig. 2). *E. coli* O103:H2 also formed biofilm on polystyrene microtiter plates at 37°C, whereas *Salmonella* ser. Agona formed very little biofilm at this temperature (Fig. 2). For this reason, the effect of furanone on biofilm formation by *Salmonella* ser. Agona was not investigated at 37°C. Likewise, three of the *E. coli* O103:H2 strains which also displayed low OD_{595} at 37°C were not included in the testing of furanone effect at this temperature. The effect of the various concentrations (12·5, 25 and 50 μmol l^{-1}) of furanone F202 on biofilms by *Salmonella* ser. Agona and *E. coli* O103:H2 are shown in Figs 3 and 4, respectively. A reduction of biofilm build-up by *Salmonella* ser. Agona in microtiter plates was found for all three concentrations of the furanone F202 at 20°C ($P < 0{\cdot}05$), but not at 12°C (Fig. 3). For *E. coli* O103:H2, a similar reduction in biofilm build-up for all three concentrations of furanone

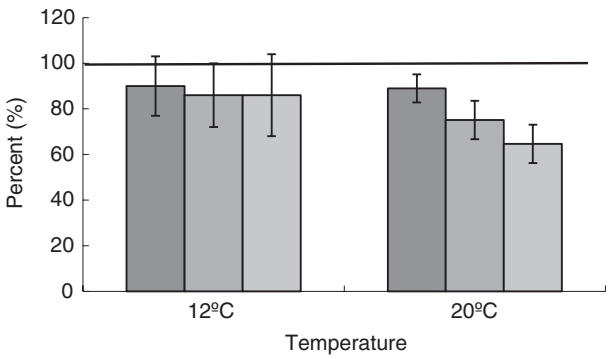


Figure 3 The effect of different concentrations (12·5, 25 and 50 μmol l^{-1}) of furanone F202 on biofilm by *Salmonella* ser. Agona (all strains) on microtiter plates at 12°C and 20°C. Results are given as percentage of biofilm formed in the absence of furanone with 95% confidence intervals. Means with confidence intervals not including 100% are considered statistically significant. (▩) 12·5 μmol l^{-1}-1; (▩) 25 μmol l^{-1}-1 and (▩) 50 μmol l^{-1}-1.

F202 was shown at 37°C ($P < 0{\cdot}05$), and a reduction in biofilm build-up was shown for 25 and 50 μmol l^{-1} of furanone F202 at 20°C ($P < 0{\cdot}05$). At 12°C, a reduction in biofilm build-up by *E. coli* O103:H2 was only shown for 50 μmol l^{-1} of the furanone F202 ($P < 0{\cdot}05$) (Fig. 4).

Effect of the furanone on biofilm formation as visualized by confocal laser scanning microscopy

Salmonella 2168-10 and *E. coli* 1242 were used when visualizing the effect of furanone F202 on biofilm by CLSM. A reduction was shown on biovolume, substrate coverage, mean thickness and roughness of the biofilm grown by *E. coli* 1242 on stainless-steel coupons ($P < 0{\cdot}05$, Table 2, Fig, 5) in the presence of 50 μmol l^{-1} F202. For biofilm by *Salmonella* 2168-10, only the biovolume and the

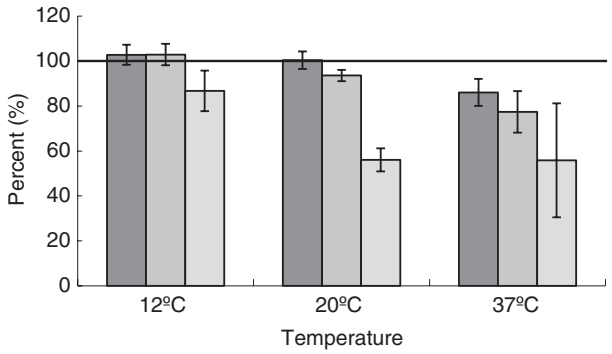


Figure 4 The effect of different concentrations (12·5, 25 and 50 μmol l^{-1}) of furanone F202 on biofilm by *E. coli* O103:H2 on microtiter plates at 12°C and 20°C (all strains), and at 37°C (all strains except three which did not display enough biofilm formation). Results are given as percentage of biofilm formed in the absence of furanone, and 95% confidence intervals are shown. Means with confidence intervals not including 100% are considered statistically significant. (■) 12·5 μmol l^{-1}; (▣) 25 μmol l^{-1} and (□) 50 μmol l^{-1}.

Table 2 Different parameters of biofilm formed by *E. coli* O103:H2 strain 1242 in the presence or absence of 50 μmol l^{-1} of furanone F202 as studied by Confocal Laser Scanning Microscopy

	0 μmol l^{-1} F202	Standard error of mean	50 μmol l^{-1} F202	*P*-value
Biovolume (μm^3)	5·5 × 10^6	611341	3·5 × 10^5	<0·05
Substrate coverage (%)	28·1	3·25	3·5	<0·05
Mean thickness (μm)	75·4	3·12	31·4	<0·05
Roughness	0·4	0·0094	0·2	<0·05

substrate coverage were shown to be reduced ($P < 0·05$) by furanone F202. A slight reduction in mean thickness and roughness was also observed, but this reduction was not significantly associated (Table 3, Fig. 5).

Effect of furanone on motility

Salmonella 2168-10 and *E. coli* 1242 and two additional randomly chosen strains of each species (*Salmonella* 2147-7 and 2168-7, *E. coli* 1153 and 10705) were used in the swimming and swarming assays. Both swimming and swarming motility were affected by furanone F202 as shown in Table 4. The swimming zone diameter was reduced for both *Salmonella* ser. Agona and *E. coli* O103:H2 at 20 and 37°C in the presence of furanone F202 ($P < 0·05$). At 12°C, a reduction in swimming zone diameter was shown for *E. coli* O103:H2 ($P < 0·05$), but the observed reduction on *Salmonella* ser. Agona was not significantly associated ($P = 0·08$). The swarming zone diameter was also reduced for both *Salmonella* ser. Agona and *E. coli* O103:H2 at 37°C in the presence of furanone F202 ($P < 0·05$).

Visual inspection of *Salmonella* 2168-10 and *E. coli* 1242 in the presence and absence of the furanone showed no effect on flagellar production for either *Salmonella* 2168-10 nor *E. coli* 1242 (Fig. 6).

Discussion

In this study, it was shown that a synthetic brominated furanone inhibited the formation of biofilm by *Salmonella* ser. Agona and *E. coli* O103:H2 on two abiotic surfaces (polystyrene and stainless steel), without being bactericidal. The brominated furanone effectively reduced the biofilm build-up by *E. coli* and *Salmonella* on polystyrene at temperatures relevant for food and feed production and a similar effect of the furanone was shown on stainless-steel as visualized by confocal microscopy. Thus this compound may have a potential as a tool against the establishment of biofilm in food and feed production environments.

Due to the increased understanding of the ubiquitousness of biofilms and the protective means a biofilm offers to micro-organisms, various studies on so called 'anti-biofilm' compounds have been reported. These compounds have been shown to selectively block virulence, quorum sensing and/or biofilm formation at concentrations not affecting planktonic growth of the bacteria (Rasmussen and Givskov 2006; Jagusztyn-Krynicka and Wyszynska 2008; Steenackers *et al.* 2010). Halogenated furanones were one of the first groups of such anti-biofilm compounds to be discovered (Kjelleberg and Steinberg 2001; de Nys *et al.* 2006). Our results are in accordance with previous studies showing reduction in biofilm formation at a nonbactericidal concentration of two other furanones on *Salmonella* ser. Typhimurium (Janssens *et al.* 2008) and *E. coli* (Ren *et al.* 2001). On the contrary, a furanone not structurally identical to F202 has been reported to enhance staphylococcal biofilm production at low concentrations (between 1·25 and 20 μmol l^{-1}) (Kuehl *et al.* 2009). However, a similar effect was not observed with F202 in this study.

Bacteria use quorum sensing signalling molecules (autoinducers) to coordinate the gene expression of the community (Miller and Bassler 2001; Schauder *et al.* 2001). This has been found important for the development of biofilms in many different bacterial species (Davies *et al.* 1998; Costerton *et al.* 2007; Li *et al.* 2007; Lonn-Stensrud *et al.* 2007). It has generally been considered that the working action of some furanones is by inhibiting quorum sensing, namely, the AI-2 quorum sensing system (Ren *et al.* 2004; de Nys *et al.* 2006; Lonn-Stensrud *et al.* 2007). This has also been shown for *E. coli* (Ren *et al.* 2004).

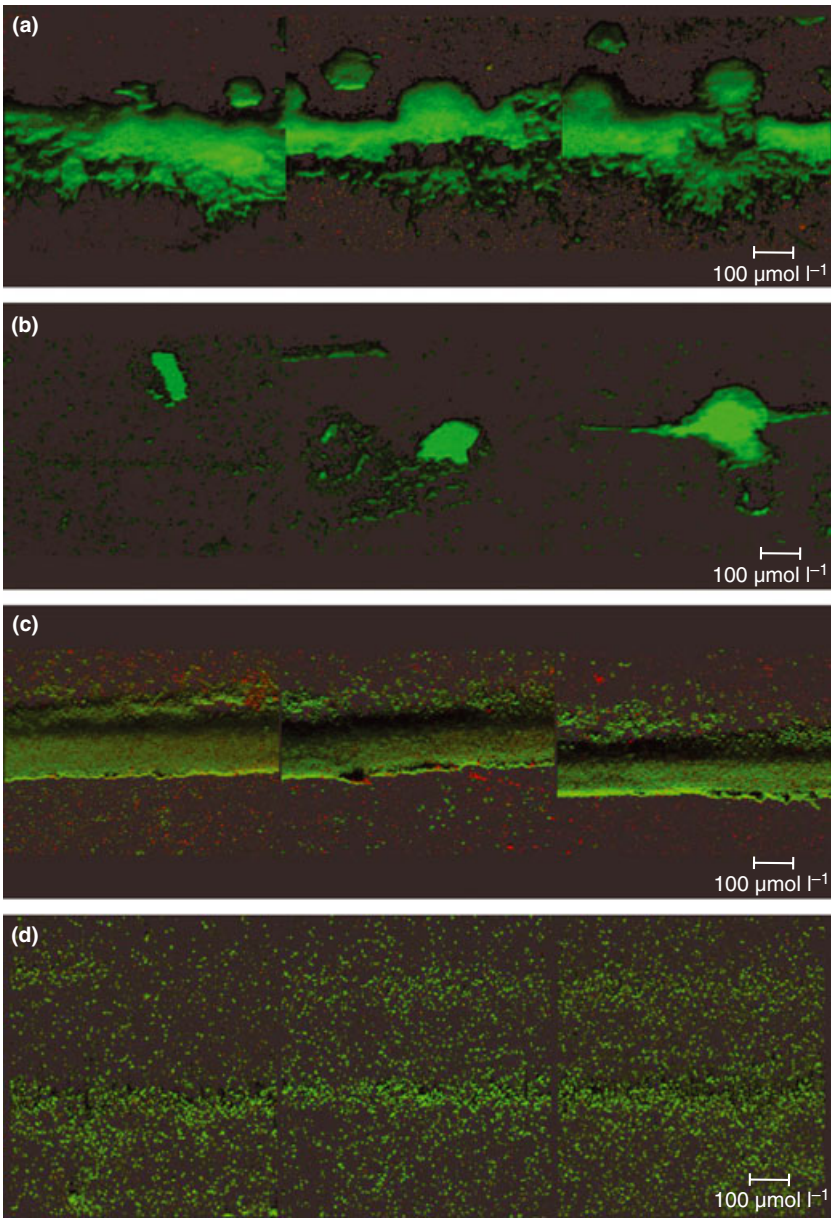


Figure 5 Confocal Laser Scanning Microscopy images of biofilm by *E. coli* strain 1242 and *Salmonella* ser. Agona strain 2168-10 in the presence or absence of 50 μmol l^{-1} furanone. (a) *E. coli* O103:H2 without F202. (b) *E. coli* O103:H2 in the presence of 50 μmol l^{-1} F202. (c) *Salmonella* ser. Agona without F202. (d) *Salmonella* ser. Agona in the presence of 50 μmol l^{-1} F202.

Previous studies have, however, showed that this is probably not the mode of action for *Salmonella* (Janssens *et al.* 2008; Vestby *et al.* 2010). Janssens *et al.* (2008) concluded that the most likely mode of action of furanones on *Salmonella* ser. Typhimurium was by interference with flagellar biosynthesis. In addition, it has been shown that furanones inhibit the expression of flagellar biosynthesis genes in *E. coli* (Ren *et al.* 2004). For *E. coli* it has been reported that surface attachment depends on flagellar action (Pratt and Kolter 1998), and it was suggested that motility was important for both initial interaction with the surface and for movement along the surface. The role of flagella in biofilm development by *Salmonella* is less clear, but flagellar genes have been shown to be upregulated in biofilm of *Salmonella* ser. Typhimurium (Kim and Wei 2009; Crawford *et al.* 2010). For this reason, this study focused on the effect of physical motility properties of *Salmonella* ser. Agona and *E. coli* O103:H2 caused by the furanone. Both swimming and swarming motility were shown to be affected by the furanone F202 (Table 4) without, however, affecting

Table 3 Different parameters of biofilm formed by *Salmonella* ser. Agona strain 2186-10 in the presence or absence of 50 μmol l^{-1} of furanone F202 as studied by Confocal Laser Scanning Microscopy

	0 μmol l^{-1} F202	Standard error of mean	50 μmol l^{-1} F202	*P*-value
Biovolume (μm^3)	$3{\cdot}1 \times 10^6$	188251	$1{\cdot}6 \times 10^6$	$P < 0{\cdot}05$
Substrate coverage (%)	73·9	2·3	54·2	$P < 0{\cdot}05$
Mean thickness (μm)	29·9	4·18	22·7	$P = 0{\cdot}21$
Roughness	0·5	0·0093	0·4	$P = 0{\cdot}20$

the expression of flagella (Fig. 6). This may indicate that the working action of our furanone F202 affected the function of the flagella rather than their synthesis. This seems to be in contrast to the study by Janssens *et al.* (2008) that found that their furanones affected the expression of flagellar genes. However, the furanones used in the studies by Janssens *et. al* and Ren *et. al*. have a different structure than the furanone used in this study and this might explain the differences in the results (Ren *et al.* 2001, 2004; Janssens *et al.* 2008) as differences in structural elements of furanones have previously been found important for *E. coli* biofilm formation and toxicity (Han *et al.* 2008). This may indicate that structurally different furanones may have different modes of action although the biofilm inhibitory potential might be similar.

Swarming motility describes a rapid multicellular bacterial movement across a surface powered by rotating flagella. Swimming motility is also a bacterial movement powered by rotating flagella, but unlike swarming, swimming takes place as individual cells moving in a liquid medium (Kearns 2010). Furanone F202 seemed to affect the function of flagella in both *Salmonella* ser. Agona and *E. coli* O103:H2 as visualized in both swimming and swarming assays in this study although these two modes of motility are controlled by different mechanisms. As only swarming is an example of multicellular behaviour which requires quorum sensing (Kohler *et al.* 2000) and swimming seems to be controlled at the individual cell level, our results indicate that one possible mode of action by the furanone F202 could be by targeting flagella function directly or indirectly rather than by bacterial quorum sensing.

Temperature serves as a cue to regulate gene expression and biofilm in many different bacteria, and for instance low temperatures have been shown to increase expression of biofilm genes in *E. coli*. (White-Ziegler

Table 4 Motility of *E. coli* O103:H2 (3 strains) and *Salmonella* ser. Agona (three strains) in the presence or absence of 50 μmol l^{-1} furanone F202. Swimming zone diameter (mm) was measured after 168 h of incubation at 12°C, 24 h of incubation at 20°C and 5 h of incubation at 37°C. The swarming zone diameter (mm) was measured after 10 h at 37°C

	Swimming 12°C	Swimming 20°C	Swimming 37°C	Swarming 37°C
Salmonella ser. Agona 0 μmol l^{-1} F202	11·0 ± 2·0	29·0 ± 1·8	16·8 ± 3·7	29·1 ± 1·9
Salmonella ser. Agona 50 μmol l^{-1} F202	7·0 ± 3·0	7·0 ± 0·6	8·9 ± 3·1	16·1 ± 2·1
E. coli O103:H2 0 μmol l^{-1} F202	33·0 ± 2·7	35·0 ± 2·7	12·6 ± 2·8	33·9 ± 0·9
E. coli O103:H2 50 μmol l^{-1} F202	6·0 ± 2·0	7·0 ± 2·0	8·9 ± 1·7	15·6 ± 3·2

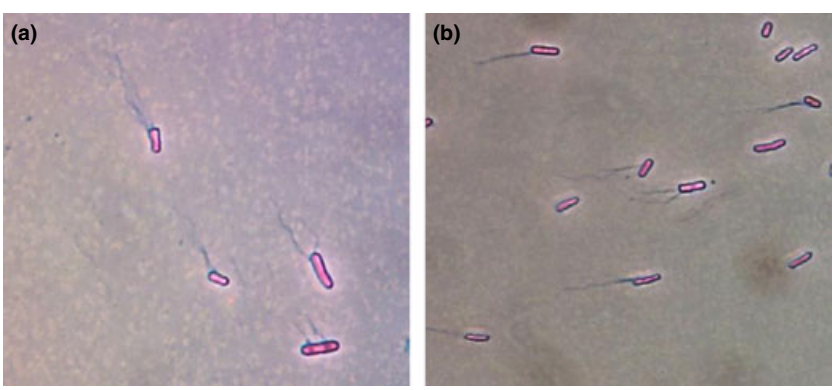


Figure 6 Flagellation of *E. coli* strain 1242. (a) in the absence of furanone. (b) in the presence of 50 μmol l^{-1} F202.

et al. 2008). The isolates of *Salmonella* ser. Agona and *E. coli* O103:H2 used in this study were capable of forming biofilm at a wide range of temperatures. *Salmonella* ser. Agona formed very little biofilm at 37°C and this is in accordance with a study by Schonewille *et al.* (2012) on other *Salmonella* serovars (Schonewille *et al.* 2012). *Salmonella* can grow at a temperature ranging from 7 to 48°C, but are known to be able to proliferate at temperatures as low as below 4°C under special conditions (Popoff and Le Minor 2005; Threlfall 2005). Although, the effect of the furanone on biofilm formation by *Salmonella* ser. Agona in microtiter plates at 37°C was not possible to assess due to little biofilm formed by *Salmonella* ser. Agona at this temperature, the motility tests performed at 37°C, both showed that the furanone also affected *Salmonella* ser. Agona at this temperature. *E. coli* is a mesophilic bacterium that is able to grow well in the temperature range of 21–49°C, although it has an optimum growth temperature at about 37°C. Its growth is gradually impaired at temperatures below 20°C, and the minimum for measurable growth is around 7·5°C (Strocchi *et al.* 2006). This study shows that both *Salmonella* ser. Agona and *E. coli* O103:H2 are able to form biofilm at temperatures which are relevant for the food and feed industry, and they should therefore be considered a potential risk of establishing in these environments. Even at 12°C, the measured OD_{595} in the microtiter assay seems to be due to biofilm rather than just attached cells, since the OD_{595} increased with increasing incubation length (results not shown) and reached similar magnitudes as observed at 20°C. The ability of potential foodborne pathogenic bacteria, like *Salmonella* ser. Agona and *E. coli* O103:H2, to establish and form biofilm at temperatures used in the food industry is of great concern. As bacteria in a biofilm are protected and less sensitive to antibacterial agents (Costerton 1999; Scher *et al.* 2005; Moretro *et al.* 2009), eradication of suchlike bacteria is very difficult. This is a matter of concern that should be taken into considerations when disinfection strategies are selected.

In conclusion, the furanone tested in this study showed potential as inhibitor of biofilm on abiotic surfaces by *Salmonella* ser. Agona and *E. coli* O103:H2. The inhibition was shown at conditions relevant for the food and feed industry, and the compound thereby may have a potential as a tool against the establishment of biofilm in these environments. In addition, the furanone F202 showed potential as a biofilm inhibitor for *E. coli* O103:H2 at optimum growth temperature. Furanones with different structures may have different modes of action and the results indicate that one of the working actions is by targeting the function of the flagella.

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Conflict of interest

No conflict of interest declared.

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