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Agaric acid reduces *Salmonella* biofilm formation by inhibiting flagellar motility

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ABSTRACT

Salmonella biofilms are a common cause of contaminations in the food or feed industry. In a screening for novel compounds to combat biofilm-associated foodborne outbreaks, we identified agaric acid as a *Salmonella* Typhimurium biofilm inhibitor that does not affect planktonic growth. Importantly, the remaining biofilm cells after preventive treatment with agaric acid were significantly more sensitive to the common disinfectant hydrogen peroxide. Screening of a GFP-promoter fusion library of biofilm related genes revealed that agaric acid downregulates the transcription of genes responsible for flagellar motility. Concurrently, swimming motility was completely abrogated in the presence of agaric acid, indicating that biofilm inhibition occurs via interference with the motility phenotype. Moreover, agaric acid also reduced biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. Agaric acid thus shows potential as an anti-virulence compound that inhibits both motility and biofilm formation.

Introduction

Bacteria typically live in dense communities encapsulated by a self-produced matrix, commonly known as biofilms. These biofilms are highly tolerant to antibiotics, disinfectants and mechanical removal, giving rise to chronic infections or contaminations [1–3]. The highly tolerant and persistent nature of biofilms causes enormous problems in a wide variety of sectors, including medicine, food industry and agriculture [4–6]. The failure of current strategies to completely prevent or remove biofilms invokes a strong need for novel biofilm inhibitors. Preventive strategies that block initial adhesion seem most promising because of the low permeability of already established biofilms [7].

One biofilm forming pathogen that is particularly problematic in the food and feed industry is *Salmonella*. Globally, there are an approximate 94 million cases of *Salmonella* each year, leading to 155 000 deaths [8]. More than 85% of these cases are estimated to be foodborne, making *Salmonella* the most common cause of bacterial foodborne outbreaks. In 2017, the European food safety agency reported over 90 000 cases of illness due to *Salmonella*, resulting in 156 deaths [9].

In an ongoing screening for novel anti-biofilm compounds, we identified agaric acid as a potent *Salmonella* biofilm inhibitor. Agaric acid or 2-hydroxynonadecane-1,2,3-tricarboxylic acid is a fatty acid naturally produced by certain fungi. This compound has previously been reported as an inhibitor of the mitochondrial adenine nucleotide exchange reaction and inducer of mitochondrial permeability [10]. Historically, agaric acid has been used as an anhidrotic to symptomatically treat extreme sweating in tuberculosis patients [11]. Additionally, at high dosages, agaric acid can inhibit the nervous, respiratory, and circulatory systems in lower animals [10]. Therefore, agaric acid has also been utilized as a metabolic inhibitor in animal experiments [12]. However, no antimicrobial properties have been described.

In this work we show that agaric acid – when used in a preventive manner - inhibits *Salmonella* biofilm formation: it significantly reduces both the number of bacteria and the amount of biomass adhering to abiotic surfaces via downregulation of flagellar rotation genes and inhibition of swimming motility. Importantly, the reduced biofilm formation leads to more effective treatment with hydrogen peroxide, a common disinfectant in the food industry.

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Results & discussion

Agaric acid reduces Salmonella biofilm formation

A crystal violet based screening assay using the Calgary biofilm device revealed agaric acid as a potent inhibitor of *Salmonella* Typhimurium biofilm formation. Agaric acid significantly prevented biofilm formation at concentrations higher than 100 μM, reaching 99,9% inhibition at 800 μM (Fig. 1A). Crystal violet staining measures the total biomass attached to a surface, thus combining cells and biofilm matrix. In order to study whether agaric acid reduces the number of bacteria attaching to the surface, the number of CFUs in biofilms grown on the bottom of a glass petri dish was determined. This assay revealed that agaric acid also significantly reduces the number of *Salmonella* cells attaching to the surface, although this inhibition was weaker than the biomass inhibition as measured by crystal violet staining (Fig. 1C). Microscopic analysis confirmed that biofilms grown in presence of agaric acid are more sparse compared to the control (Fig. 1D).

This inhibition was not due to a bactericidal effect as planktonic growth was not inhibited (Fig. 1B). Moreover, planktonic growth was enhanced at the highest concentration, indicating that agaric acid prevents attachment and results in more bacteria remaining in the planktonic phase. The minimal inhibitory concentration (MIC) of agaric acid under the same conditions as the biofilm assay was measured as 8 mM, further confirming that no bactericidal effects occurred at concentrations relevant for biofilm inhibition.

Agaric acid inhibits flagellar motility

To unravel the mechanism by which agaric acid inhibits biofilm formation, an in house developed reporter GFP-promoter fusion library was screened. This library contains reporters for 130 *Salmonella* genes related to biofilm formation, including genes regulating matrix production, fimbriae and flagella synthesis, quorum sensing and c-di-GMP regulation [13]. A time-lapse of the first 24h of biofilm formation in microtiter plates was performed to identify genes that are differentially transcribed in the presence of 100 μM agaric acid. As these reporter fusions express the stable GFPmut3 variant as a fluorophore, the measured fluorescence values are the accumulation of fluorescence over time [13] (Fig. 2, Figure S1).

Remarkably, the transcription of central biofilm regulatory genes such as *csgD* and *rpoS* was not downregulated in the presence of agaric acid. Additionally, the transcription of downstream genes such as *csgB* and *adrA*, respectively responsible for the production of curli fimbrae and cellulose, was not influenced by agaric acid [14,15]. However, from 12h onwards transcription of the flagellar sigma factor *fliA* that induces the expression of class III flagellar genes, was significantly inhibited by agaric acid. This downregulation was not caused by decreased transcription of *flhDC*, the master regulator of motility in *Salmonella* [16], as the transcription of *flhDC* was increased compared to the control between 9h and 15h. Additionally, transcription of the anti-sigma factor FlgM was reduced. FlgM directly binds FliA and inhibits the expression of class III genes. The FlgM protein is secreted by the flagellum-specific export apparatus after completion of its construction, effectively coupling flagellar assembly with transcriptional regulation. The expression of *flgM* is induced by FliA in a negative feedback loop [17]. The combined repression by agaric acid of both sigma factor and anti-sigma factor appeared to have a complex effect on the transcription of downstream class III flagellar genes: the transcription of *flgK* and *motA* was significantly reduced, whereas transcription of *tdcA, flgB, fljB* and *fliC* was not decreased at consecutive points (Figure S1). The differential response of class III flagellar genes could possibly be explained by a different affinity of FliA for the promoter regions of these genes. In support of this hypothesis, previous work in *E. coli* showed that different class III genes are induced at different time points, with *flgM* and *motA* being expressed after the other genes [18]. This indicates that FliA has a lower affinity for the promoter regions of these two genes, possibly rendering them more sensitive to FliA repression.

The downregulated *flgK* gene codes for a hook-associated protein that stabilizes the hook-filament junction together with FlgL [19], whereas motor protein MotA is essential for driving the rotation of the flagella [20]. The reporter fusion data therefore suggest that agaric acid inhibits flagellar motility. Motility and biofilm formation are inversely regulated in *Salmonella* via the secondary signal molecule c-di-GMP. However,

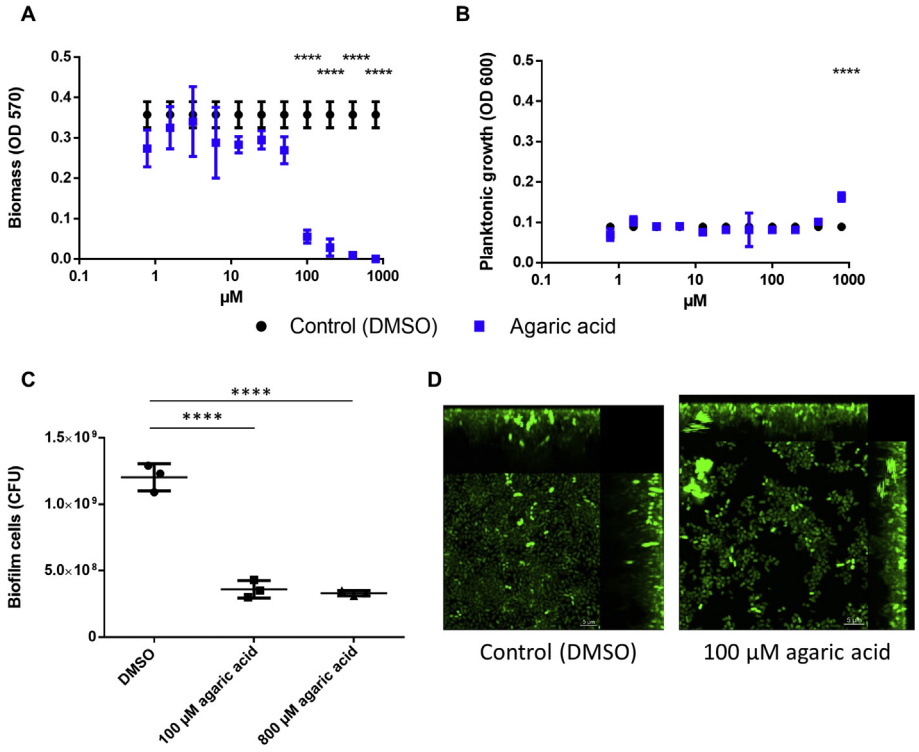

Fig. 1. Agaric acid has no bactericidal effect but strongly inhibits *Salmonella* biofilm formation.
A) Agaric acid inhibits biofilm formation of *Salmonella* Typhimurium ATCC 14028 in a concentration dependent manner as measured via crystal violet staining in the Calgary biofilm device. The mean and standard deviation of three biological repeats are shown. Significant differences were determined via a one-way ANOVA with Bonferroni multiple comparisons corrections. B) Agaric acid does not inhibit planktonic growth at concentrations relevant for biofilm inhibition. Planktonic growth was measured as the OD_{600} of the liquid culture after 48h incubation in the Calgary biofilm device. The mean and standard deviation of three biological repeats are shown. Significant differences were determined via a one-way ANOVA with Bonferroni multiple comparisons corrections. C) Agaric acid also reduces the number of cells that attach to the bottom of a glass petri dish. Significant differences were determined via a one-way ANOVA with Bonferroni multiple comparisons corrections. D) Maximum intensity projection top and side view of fluorescently labelled *Salmonella* biofilms showed that the presence of agaric acid results in less dense and scattered biofilms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

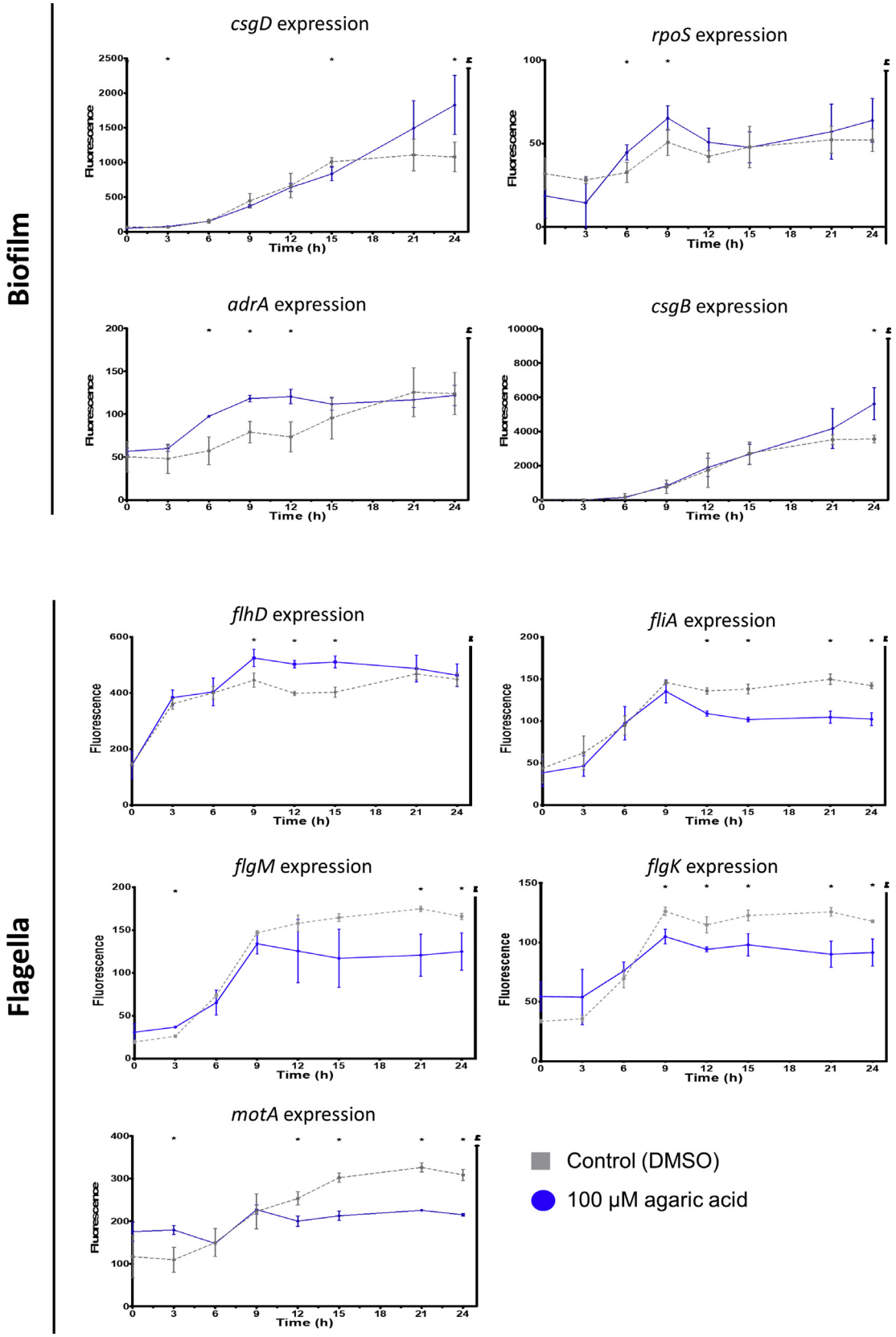

Fig. 2. Agaric acid downregulates transcription of flagella genes. The fluorescence as a measure of gene transcription at different time points is shown for *Salmonella* Typhimurium ATCC 14028 grown in DMSO (grey) or 100 µM agaric acid (blue). The mean and standard deviation of three biological repeats are depicted. Asterisks indicate significant differences as determined by a two-tailed Student t-test ($P < 0.05$). Unexpectedly, genes important for biofilm formation were upregulated by agaric acid. Agaric acid did reduce the transcription of Class II and III flagella genes. Transcription profiles for all genes of the reporter fusion library are shown in Figure S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

although the expression of genes necessary for flagellar motility is downregulated during biofilm maturation [14], the initial adhesion on plastic or glass surfaces requires both flagella and active motility [21,22]. Inhibition of flagellar motility thus potentially explains the biofilm inhibitory effect of agaric acid. To confirm whether these changes in gene transcription also lead to phenotypic changes in motility, flagella of *Salmonella* grown in presence and absence of agaric acid were visualized by staining. However, no differences in flagellar appearance could be observed between the two conditions (Fig. 3A). A soft agar swimming assay was then performed to validate whether the downregulation of flagella genes in presence of agaric acid leads to reduced motility. In the absence of agaric acid, *Salmonella* formed a halo with an average diameter of 195 mm indicating migration from the start position via flagellar motility (Fig. 3B). Agaric acid completely abrogated this migration as no halo was formed, indicating a clear effect on the motility phenotype. Concurrently, a *motA* deletion mutant was also found to not show any migration, confirming that *motA* expression is necessary for flagellar motility under the conditions tested. These results indicate that agaric acid can completely inhibit motility, most likely not via inhibition of flagella synthesis but due to a downregulation of the flagellar motor protein MotA. In line with this hypothesis, deletion of *motA* inhibited biofilm formation to a similar extent as agaric acid. Importantly, addition of agaric did not further reduce biofilm formation in this deletion mutant, providing support for a mechanism where agaric acid reduces biofilm formation via inhibition of flagellar motility (Fig. 3C).

Salmonella biofilms are more sensitive to antimicrobial treatment in presence of agaric acid

It is well established that biofilms can be extremely tolerant to

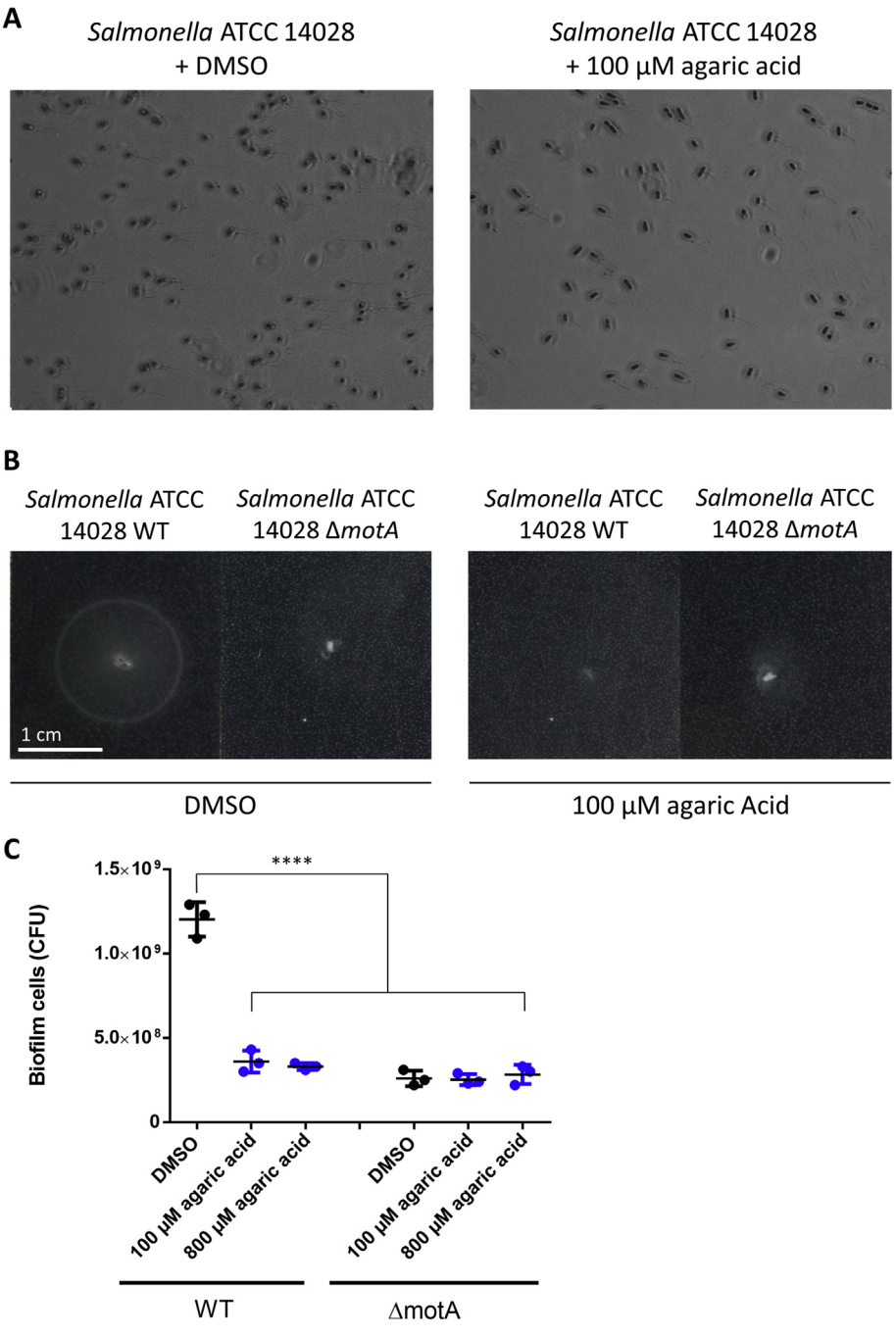

Fig. 3. Agaric acid reduces biofilm formation via inhibition of flagellar motility. A) Phase contrast microscopy showed no difference between flagella of *Salmonella* grown in 100 µM agaric acid or corresponding amount of DMSO as visualized via a crystal violet based flagella staining. One representative repeat of three biological repeats is shown. B) 100 µM agaric acid almost completely inhibited motility in a soft agar swimming, similar to a *motA* deletion mutant. One representative repeat of three biological repeats is shown. C) Deletion of *motA* inhibited biofilm formation to a similar extent as a preventive treatment with agaric acid. Moreover, agaric acid does not further reduce biofilm formation of the *motA* deletion mutant. Three biological repeats are shown. Significant differences were determined via a one-way ANOVA with Bonferroni multiple comparisons corrections. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatment with antimicrobials. To test whether the preventive addition of agaric acid renders the remaining *Salmonella* biofilm cells more susceptible to treatment with common disinfectants or antibiotics, biofilms grown in the presence or absence of agaric acid for 48 h were treated with 0.25% H_2O_2 or 1 μM ciprofloxacin (Fig. 4). Hydrogen peroxide is a commonly used disinfectant in food industry [23,24], whereas ciprofloxacin is a fluoroquinolone antibiotic frequently used to treat *Salmonella* infections [25]. Biofilm formation has been shown to strongly protect *Salmonella* against either compound [26,27].

Biofilms grown in presence of agaric acid were significantly more susceptible to hydrogen peroxide. The increased sensitivity to treatment could be a consequence of the lower number of bacteria present, i.e. the inoculum effect [28]. Moreover, crystal violet staining already showed that agaric acid has a stronger inhibitory effect on the biofilm matrix than on the number of cells, indicating that the remaining attached cells are less protected by the matrix. Agaric acid also further increased the effect of a ciprofloxacin treatment, albeit not significantly.

Agaric acid has a broad spectrum activity

It was tested whether agaric acid can also inhibit the biofilm formation of other opportunistic pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* using the Calgary biofilm device (Fig. 5). Agaric acid was found to inhibit biofilm formation of all three species. *E. coli* TG1 was even more sensitive to agaric acid than *Salmonella* as significant inhibition already occurred at 12.5 μM. Similarly to the case of *Salmonella*, planktonic growth was unaffected, except for an increase at the highest concentration. In contrast, agaric acid inhibits both the planktonic growth and biofilm formation of *P. aeruginosa* PA14 and *S. aureus* SH1000. However, *S. aureus* biofilm inhibition occurred already at a lower concentrations than the bactericidal effect, indicating that some biofilm-specific effects take place. Conversely, the main effect on *P. aeruginosa* was bactericidal as planktonic growth was already reduced at lower concentrations than biofilm. However, at high concentrations of agaric acid, biofilm formation is inhibited to a higher extent than planktonic growth.

The flagellar systems of *E. coli* and *Salmonella* show a high degree of similarity on the genetic and functional level [29]. Moreover, *E. coli* also requires normal flagellar function in order to successfully adhere to an abiotic surface [30]. The specific biofilm inhibition of agaric acid on both *Salmonella* and *E. coli* thus further supports our hypothesis that agaric acid prevents biofilm formation via inhibition of flagellar rotation. Contrarily, *S. aureus* does not show flagellar motility, but rather moves via spreading or gliding [31]. Therefore, agaric acid cannot inhibit *S. aureus* biofilm formation via interfering with the expression of genes responsible for flagellar rotation. Additionally, while *Pseudomonas* has flagella that are involved in adhesion and biofilm formation [32], the mainly bactericidal effect of agaric acid indicates that agaric acid has different targets in *Pseudomonas*. The mode of action of agaric acid is thus species dependent.

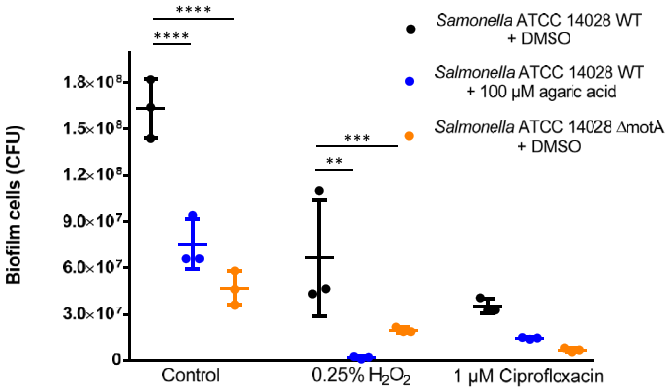

Fig. 4. Agaric increases susceptibility to treatment with H_2O_2. Biofilms, grown in presence of 100 μM agaric or the corresponding amount of DMSO, were treated with 0.25% H_2O_2 or 1 μM ciprofloxacin for 1h. A *motA* deletion mutant was used as a control. Agaric acid significantly reduced the number of biofilm cells that survive treatment with 0.25% H_2O_2 from 6.65 10^7 to 1.71 10^6 cells. Three biological repeats are shown. Significant differences were determined via a two-way ANOVA with Bonferroni multiple comparisons corrections.

Conclusion

Agaric acid was identified as a novel inhibitor of *Salmonella* biofilms that does not reduce planktonic growth. This biofilm specific effect could be a major advantage as it has been hypothesized that there is less selection pressure for resistant mutants if virulence traits such as biofilms are targeted instead of growth [33]. Additionally, it has been suggested that biofilm-specific inhibitors could increase the risk that a contamination spreads as scattering is enhanced [34]. However, this potential drawback is diminished in the case of agaric acid because flagellar motility is abrogated. Moreover, motility in itself is also an important virulence factor, further expanding the possible application fields of agaric acid [35]. Agaric acid thus shows strong potential for industrial and medical use.

Material & methods

Bacterial strains and culture conditions

Overnight cultures (ONCs) of *Salmonella enterica serovar* Typhimurium ATCC 14028, *Escherichia coli* TG1, *Pseudomonas aeruginosa* PA14, and *Staphylococcus aureus* SH1000 were grown at 37 °C, shaken, with aeration, in Luria–Bertani (LB) broth, with 100 μg ml^{-1} of ampicillin if appropriate.

Anti-biofilm assay

The Calgary biofilm device consists of a lid carrying 96 polystyrene pegs that fits into a microtiter plate with a peg hanging into each well. This device was utilized to screen for compounds that prevent bacterial biofilm formation, as described previously [36]. Two-fold serial dilutions of the compounds in 100 μl liquid broth per well were prepared in the microtiter plate. Subsequently, an overnight culture was diluted 1/100 into the respective liquid broth, and 100 μl (~10^6 cells) was added to each well of the microtiter plate, resulting in a total amount of 200 μl medium per well. After placing the lid on the microtiter plate, samples containing *Salmonella*, *Pseudomonas* or *E. coli* were incubated statically in TSB 1/20 for 48 h at 25 °C, whereas *S. aureus* was incubated in undiluted TSB at 37 °C for 48h. After incubation, the lid was removed from the microtiter plate and the liquid culture was transferred to a new microtiter plate prior to determining the planktonic growth in each well via OD_{600} measurements using a Synergy MX multimode reader (Biotek, Winooski, VT). The pegs were washed once in 200 μl PBS and the remaining attached bacteria were stained for 30 min with 200 μl 0.1% crystal violet in an isopropanol-methanol-PBS solution (1:1:18). Excess stain was washed off by placing the pegs in a 96-well plate filled with 200 μl distilled water per well. Afterwards, the pegs were air dried for 30 min and the dye bound to the adherent cells dissolved into 200 μl 30% glacial acetic acid. The OD_{570} of each well was measured using a Synergy MX multimode reader. Data was analysed using the GraphPad Prism 6 software.

Minimal inhibitory concentration (MIC) assay

MIC values were determined in a 96-well plate. Two-fold serial dilutions of agaric acid (Sigma) or dimethyl sulfoxide (DMSO) were

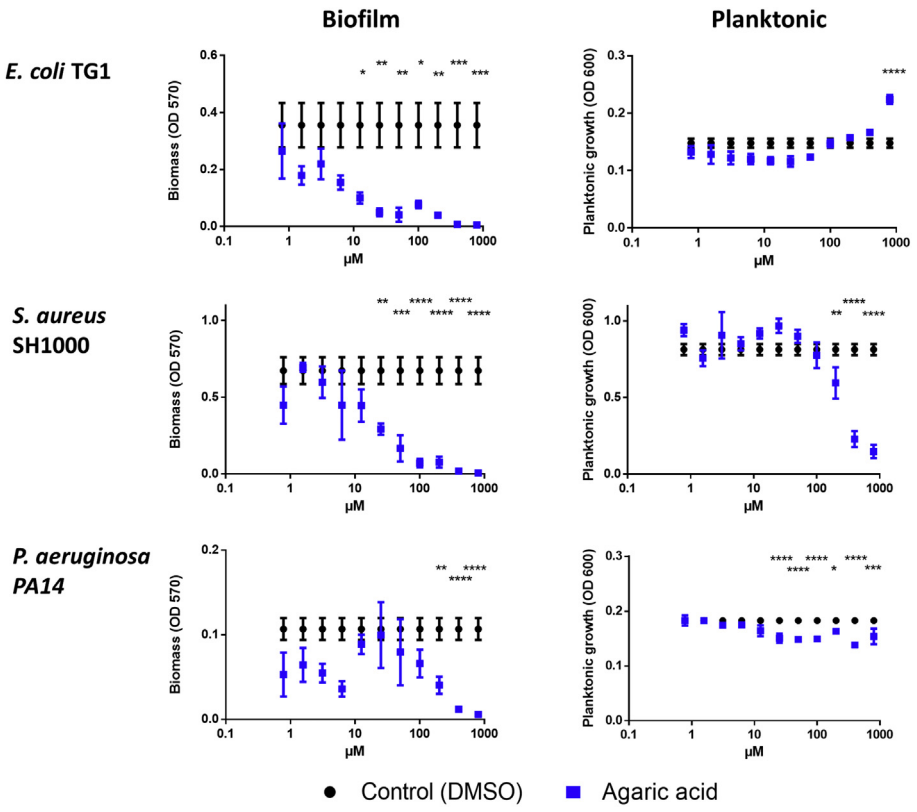

Fig. 5. Agaric acid also reduces biofilm formation of other pathogens. Agaric acid inhibits biofilm formation of *E. coli* TG1, *P. aeruginosa* PA14 and *S. aureus* SH1000 as measured via crystal violet staining in the Calgary biofilm device. The effect on planktonic growth was measured via OD_{600} measurement of the broth in the well. The mean and standard deviation of three biological repeats are shown. Significant differences were determined via a one-way ANOVA with Bonferroni multiple comparisons corrections. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

prepared in 100 μl of TSB 1/20 and 100 μL of the inoculum diluted 1/100 in TSB 1/20 was added. The plate was covered with a breathable sealing membrane and a lid and incubated for 24 h at 25 °C, shaking at 200 rpm. The MIC was defined as the lowest concentration of compound were *Salmonella* growth was lower than the upper bound of the 95% confidence interval of the negative control.

Petri dish biofilm assay

ONCs of *S.* Typhimurium ATCC 14028 were normalized to an OD_{595} of 3.2 and diluted 1/100 in a small (60 mm Ø) glass petri dishes containing 10 ml of 1/20 TSB to which a final concentration of 100 μM agaric or the corresponding amount of DMSO was added. Around $12*10^7$ ml^{-1} cells were inoculated and incubated under static conditions at 25 °C for 48 h. Afterwards, the liquid above the biofilms was poured off and the biofilms were scraped off the bottom of the plate in 1 ml of PBS, passed through a 25 gauge syringe and vortexed to break down the biofilm structure and ensure an homogenous suspension during dilution [37]. The number of colony forming units (CFU) of biofilms was determined by plating.

Microscopic analysis

ONCs of *S.* Typhimurium ATCC 14028 containing the pFPV25.1 plasmid encoding for constitutive GFPmut3 productions were normalized to an OD_{595} of 3.2. 20 μl was added to uncoated glass bottom microwell dishes (35 mm Ø petri dish, 20 mm Ø microwell, Mattek) containing 2 ml 1/20 TSB, 100 μg ml^{-1} of ampicillin and a final concentration of 100 μM agaric acid or the corresponding amount of DMSO. Around $12*10^7$ ml^{-1} cells were inoculated and incubated under static conditions at 25 °C for 48 h. After incubation, the planktonic phase was gently poured off and the biofilm was washed with 1 ml PBS. Biofilms were visualized with a Zeiss LSM880 confocal laser scanning microscope using an 100× oil-immersion objective (α Plan-Apochromat 100x/1.46 Oil DIC M27 Elyra, Zeiss). Images were acquired using the Airyscan detector at 1156 × 1156 resolution size. Images were analysed using Zen Blue (Zeiss).

Microtiter-plate-based GFP promoter fusion assay

The GFP promoter fusion assay was performed as described previously [13]. Briefly, 1.5 μl of the reporter fusions' ONCs were transferred in three repeats to black polystyrene, clear-bottomed microtiter plates (Greiner bio-one 655096) containing 200 μl of 1/20 TSB with either a final concentration of 100 μM agaric or the corresponding amount of DMSO. Subsequently, the microtiter plates were incubated statically, at 25 °C for 24 h. Every 3 h, the fluorescence (excitation 488 nm, emission 511 nm) and absorbance at 600 nm (OD_{600}) were measured using a Synergy MX multimode reader. For data analysis, blank measurements, using a promoterless pFPV25 vector as control, were subtracted from both the fluorescence and OD_{600}. The ratio between the different OD_{600} values of the strains/conditions was used to normalize any effects on the fluorescence caused by growth differences in the bacteria. Significant differences in the level of fluorescence between treatment and control were determined using a two-tailed Student t-test ($P < 0.05$).

Staining of flagella

An ONC of *Salmonella* Typhimurium ATCC 14028 was normalized to an OD_{600} of 3.2 and diluted 1/100 in 5 ml TSB 1/20, with either a final concentration of 100 μM agaric or the corresponding amount of DMSO as a control. The planktonic cultures were incubated for 24 h at 25 °C, shaking at 200 rpm. The flagella were stained according to Kearns and Losick (2003) [38]. Briefly, 3 μl of sample was applied to a microscopic slide and covered with a 22 mm × 40 mm coverslip. After placing the slide vertically, 10 μl of the stain consisting of ten parts mordant (2 g tannic acid, 10 ml 5% phenol, 10 ml saturated aqueous $AlK(SO_4)_2$) mixed with one part stain (12% crystal violet in ethanol), was applied to the top

edge of the coverslip in order to stain the sample due to capillary forces. Samples were visualized with phase contrast using a Zeiss Axio Imager Z1 microscope with an EC Plan Neofluar (×100 magnification/1.3 numerical aperture) objective.

Soft agar swimming assay

Based on Kim & Surette (2003) [39], swimming plates were made by mixing 30 ml TSB 1/20 with 0.25% agar. These plates contained either a final concentration of 100 μM agaric or the corresponding amount of DMSO. After 2h drying at room temperature, 3 μl of an overnight culture was inoculated by piercing the surface of the agar with the pipette tip. The plates were incubated upright for 24 h at 25 °C, afterwards the size of the halo was measured and visually recorded.

Tolerance assay

To determine the tolerance of mature biofilms, biofilms were grown on microscopy glasses (75 mm × 25 mm) placed vertical in a 50 ml falcon filled with 30 ml TSB 1/20. This set-up allows for easy transfer of mature biofilms as the top of vertical slide sticks out of the medium which allows to grab the slide with a pincer without damaging the biofilm. ONCs of *Salmonella* were normalized to an OD_{600} of 3.2 and diluted 1/100 into the broth containing either a final concentration of 100 μM agaric or the corresponding amount of DMSO. After 48h of static incubation at 25 °C, the glass slide was transferred to a new 50 ml falcon containing either 0.25% H_2O_2, 1 μM ciprofloxacin, or PBS and was incubated for 1 h. Afterwards, biofilms were scraped off the glass slide in 10 ml of PBS, passed through a syringe (25G) and vortexed to break down the biofilm structure and ensure an homogenous suspension during dilution. The number of colony forming units of biofilms was determined by plating.

Data availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Hans Steenackers (hans.steenackers@kuleuven.be).

Declaration of competing interest

The authors declare no competing interests.

CRediT authorship contribution statement

Bram Lories: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Tom E.R. Belpaire:** Methodology, Investigation, Writing - review & editing. **Anna Yssel:** Conceptualization, Writing - review & editing. **Herman Ramon:** Writing - review & editing, Supervision, Funding acquisition. **Hans P. Steenackers:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2020.100022.

References

[1] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science (New York, N.Y 1999;284:1318–22.
[2] Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol 2005;13:34–40.
[3] Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Cell Microbiol 2009;11:1034–43.
[4] Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F. Biofilms in the food industry: health aspects and control methods. Front Microbiol 2018;9. 898-898.
[5] Velmourougane K, Prasanna R, Saxena AK. Agriculturally important microbial biofilms: present status and future prospects. J Basic Microbiol 2017;57:548–73.
[6] Koo H, Allan RN, Howlin RP, Stoodley P, Hall-Stoodley L. Targeting microbial biofilms: current and prospective therapeutic strategies. Nat Rev Microbiol 2017;15:740–55.
[7] Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. Virulence 2018;9:522–54.
[8] Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM, Studies, f.t.I.C.o.E.D.B.o.I.. The global burden of nontyphoidal Salmonella gastroenteritis. Clin Infect Dis 2010;50:882–9.
[9] EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA Journal 2018 2018;16(12):5500.
[10] García N, Zazueta C, Pavón N, Chávez E. Agaric acid induces mitochondrial permeability transition through its interaction with the adenine nucleotide translocase. Its dependence on membrane fluidity. Mitochondrion 2005;5:272–81.
[11] Bacchi CJ, Ciaccio EI, Koren LE. Effects of some antitumor agents on growth and glycolytic enzymes of the flagellate Crithidia. J Bacteriol 1969;98:23–8.
[12] Freedland RA, Newton RS. Agaric acid [37]. In: Methods in enzymology, vol. 72. Academic Press; 1981. p. 497–506.
[13] Robijns SCA, Roberfroid S, Van Puyvelde S, De Pauw B, Uceda Santamaría E, De Weerdt A, De Coster D, Hermans K, De Keersmaecker SCJ, Vanderleyden J, et al. A GFP promoter fusion library for the study of Salmonella biofilm formation and the mode of action of biofilm inhibitors. Biofouling 2014;30:605–25.
[14] Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ. Salmonella biofilms: an overview on occurrence, structure, regulation and eradication. Food Res Int 2012;45:502–31.
[15] Simm R, Ahmad I, Rhen M, Le Guyon S, Römling U. Regulation of biofilm formation in Salmonella enterica serovar Typhimurium. Future Microbiol 2014;9:1261–82.
[16] Das C, Mokashi C, Mande SS, Saini S. Dynamics and control of flagella assembly in Salmonella typhimurium. Front Cell Infect Microbiol 2018;8. 36-36.
[17] Kutsukake K, Iino T. Role of the FliA-FlgM regulatory system on the transcriptional control of the flagellar regulon and flagellar formation in Salmonella typhimurium. J Bacteriol 1994;176:3598–605.
[18] Kalir S, McClure J, Pabbaraju K, Southward C, Ronen M, Leibler S, Surette MG, Alon U. Ordering genes in a flagella pathway by analysis of expression kinetics from living bacteria. Science (New York, N.Y 2001;292:2080–3.
[19] Ohnishi K, Ohto Y, Aizawa S, Macnab RM, Iino T. FlgD is a scaffolding protein needed for flagellar hook assembly in Salmonella typhimurium. J Bacteriol 1994;176:2272–81.
[20] Muramoto K, Macnab RM. Deletion analysis of MotA and MotB, components of the force-generating unit in the flagellar motor of Salmonella. Mol Microbiol 1998;29:1191–202.
[21] Prouty AM, Gunn JS. Comparative analysis of Salmonella enterica serovar Typhimurium biofilm formation on gallstones and on glass. Infect Immun 2003;71:7154–8.
[22] Mireles JR, Toguchi A, Harshey RM. Salmonella enterica serovar typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. J Bacteriol 2001;183:5848–54.
[23] Meireles A, Giaouris E, Simões M. Alternative disinfection methods to chlorine for use in the fresh-cut industry. Food Res Int 2016;82:71–85.
[24] Ölmez H, Kretzschmar U. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. LWT - Food Sci Technol (Lebensmittel-Wissenschaft -Technol) 2009;42:686–93.
[25] Tabak M, Scher K, Chikindas ML, Yaron S. The synergistic activity of triclosan and ciprofloxacin on biofilms of Salmonella Typhimurium. FEMS Microbiol Lett 2009;301:69–76.
[26] González JF, Alberts H, Lee J, Doolittle L, Gunn JS. biofilm formation protects Salmonella from the antibiotic ciprofloxacin in vitro and in vivo in the mouse model of chronic carriage. Sci Rep 2018;8. 222-222.
[27] Lise Dieltjens KA, Lissens Maries, Lories Bram, Kim Wook, Van der Eycken Erik, Foster Kevin R, et al. Inhibiting bacterial cooperation is an evolutionarily robust anti-biofilm strategy. Nat Commun 2020;11(107).
[28] Brook I. Inoculum effect. Clin Infect Dis 1989;11:361–8.
[29] Albanna A, Sim M, Hoskisson PA, Gillespie C, Rao CV, Aldridge PD. Driving the expression of the Salmonella enterica sv Typhimurium flagellum using flhDC from Escherichia coli results in key regulatory and cellular differences. Sci Rep 2018;8:16705.
[30] Pratt LA, Kolter R. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 1998;30:285–93.
[31] Pollitt EJG, Diggle SP. Defining motility in the staphylococci. Cell Mol Life Sci 2017;74:2943–58.
[32] O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 1998;30:295–304.

[33] Allen RC, Popat R, Diggle SP, Brown SP. Targeting virulence: can we make evolution-proof drugs? Nat Rev Microbiol 2014;12:300.
[34] Fleming D, Rumbaugh KP. Approaches to dispersing medical biofilms. Microorganisms 2017;5:15.
[35] Josenhans C, Suerbaum S. The role of motility as a virulence factor in bacteria. International Journal of Medical Microbiology 2002;291:605–14.
[36] De Keersmaecker SC, Varszegi C, van Boxel N, Habel LW, Metzger K, Daniels R, Marchal K, De Vos D, Vanderleyden J. Chemical synthesis of (S)-4, 5-dihydroxy-2, 3-pentanedione, a bacterial signal molecule precursor, and validation of its activity in Salmonella typhimurium. J Biol Chem 2005;280:19563–8.
[37] Hermans K, Nguyen TLA, Roberfroid S, Schoofs G, Verhoeven T, De Coster D, Vanderleyden J, De Keersmaecker SCJ. Gene expression analysis of monospecies Salmonella Typhimurium biofilms using Differential Fluorescence Induction. J Microbiol Methods 2011;84:467–78.
[38] Kearns DB, Losick R. Swarming motility in undomesticated Bacillus subtilis. Mol Microbiol 2003;49:581–90.
[39] Kim W, Surette MG. Swarming populations of Salmonella represent a unique physiological state coupled to multiple mechanisms of antibiotic resistance. Biol Proced Online 2003;5:189–96.