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DOI
10.1016/j.fm.2014.03.006

Publication date
2015

Document Version
Final published version

Published in
Food Microbiology

Food Microbiology 45 (2015) 63–70

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Quantitative analysis of the effect of specific tea compounds on germination and outgrowth of *Bacillus subtilis* spores at single cell resolution

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ARTICLE INFO

Article history:
Available online 16 March 2014

Keywords:
Polyphenols
Catechins
Tea
Bacillus subtilis
Spore germination and outgrowth
Live cell imaging
SporeTracker

ABSTRACT

Tea is one of the most widely consumed beverages in the world and known for its antimicrobial activity against many microorganisms. Preliminary studies have shown that tea polyphenols can inhibit the growth of a wide range of Gram-positive bacteria. However, the effect of these compounds on germination and outgrowth of bacterial spores is unclear. Spore-forming bacteria are an aggravating problem for the food industry due to spore formation and their subsequent returning to vegetative state during food storage, thus posing spoilage and food safety challenges. Here we analysed the effect of tea compounds: gallic acid, gallocatechin gallate, Teavigo (>90% epigallocatechin gallate), and theaflavin 3,3′-digallate on spore germination and outgrowth and subsequent growth of vegetative cells of *Bacillus subtilis*. To quantitatively analyse the effect of these compounds, live cell images were tracked from single phase-bright spores up to microcolony formation and analysed with the automated image analysis tool "SporeTracker". In general, the tested compounds had a significant effect on most stages of germination and outgrowth. However, germination efficiency (ability of spores to become phase-dark) was not affected. Gallic acid most strongly reduced the ability to grow out. Additionally, all compounds, in particular theaflavin 3,3′-digallate, clearly affected the growth of emerging vegetative cells.

1. Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed non-alcoholic beverages in the world. Its complex composition includes carbohydrates, amino acids, proteins, alkaloids (caffeine, theophylline and theobromine), volatile compounds, polyphenols, minerals, and trace elements (Bansal et al., 2013). Polyphenols, particularly flavonoids, are the bioactive compounds associated with tea that are considered to provide several health benefits, such as reduction of cholesterol and obesity, as well as protection against cardiovascular disease and cancer (Dulf et al., 2012; Khan and Mukhtar, 2007). The flavan-3-ols are the common flavonoids in tea. On the basis of their degree of polymerization flavan-3-ols are divided into three subclasses, i.e. monomers, dimers, and oligomers. The monomers include catechin compounds such as catechin, gallocatechin, gallocatechin gallate, epicatechin, and epigallocatechin-3-gallate. The dimers comprise of theaflavins such as theaflavin, theaflavin-3-gallate, and theaflavin-3,3′-digallate, whereas the oligomers includes tannin derivatives of unknown structure (Bansal et al., 2013).

A major class of bioactive compounds of green tea is formed by the catechins. They act as powerful antioxidants and are well known to be safe for human consumption (Friedman, 2007). Hence this class of compounds has been part of people's diet for long time and is expected to have additional applications in the food industry. Studies have been published on the beneficial effects of green tea on human health (Nakayama et al., 2011; Yen and Chen, 1995). Usually, green tea contains about 30–50 mg/L of catechins, i.e.

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http://dx.doi.org/10.1016/j.fm.2014.03.006
0740-0020/

about 10% of the tea dry weight (Shigemune et al., 2012). Green tea extracts are also sold as nutraceuticals, i.e. health-promoting dietary supplements (Lai and Roy, 2004). An example is commercially available Teavigo, which is composed of epigallocatechin-3-gallate (>90%), other catechins (5%) and caffeine (<0.1%). Catechins are also known for their antimicrobial activity against many microorganisms. Study has shown that the catechins have stronger antibacterial effects on Gram-positive bacteria than Gram-negative bacteria (Nakayama et al., 2008). The vegetative state of spore forming bacteria is also affected by catechins (Friedman et al., 2006; Nakayama et al., 2008). Hara-Kudo et al. showed that tea polyphenols have antibacterial effects on *Clostridium* spores, however no effect was observed in *Bacillus cereus* spores (Hara-Kudo et al., 2005). A detailed analysis of the effects of these compounds on the time-to-start of germination, germination time itself, and time to first division (outgrowth of bacterial spores) is not available.

Bacillus subtilis is an aggravating problem for the food industry as they form dormant and stress resistant spores. If such spores survive preservation treatments their germination and outgrowth allows them to return to the vegetative state during food storage. Such events may thus lead to food spoilage (Ghosh and Setlow, 2009; Hornstra et al., 2009; Moir, 2003). To get to a better understanding of the effect of antimicrobial compounds on germination and outgrowth inhibition we have recently reported on a novel tool to analyse the heterogeneity in spore germination and outgrowth inhibition at the single spore level (Pandey et al., 2013). Here our studies were aimed at a quantitative analysis of the effect of four different characteristic tea compounds on germination and outgrowth of *B. subtilis* 1A700 spores at single cell resolution using live cell imaging. The tea compounds were first screened by assessing their antimicrobial effect at the population level in microtiter plates using optical density measurements. We tested the catechins (flavan-3-ol monomer): gallocatechin gallate, and Teavigo (>90% epigallocatechin-3-gallate) and one type of theaflavin (flavan-3-ol dimer): theaflavin 3,3′-digallate. In addition we tested the effect of gallic acid, which is a phenolic weak acid (pK_a of 4.5) that is found in tea leaves both as a free compound and as a component (gallate) of many flavan-3-ols.

2. Materials and methods

2.1. Strain and spore preparation

Spores of the well characterised *B. subtilis* 168 laboratory wild-type strain 1A700 (*trpC2*) were used throughout the study. Spores were prepared in a defined minimal medium buffered to 7.4 with 3-(*N*-morpholino) propanesulfonic acid (MOPS) and harvested as described before (Abhyankar et al., 2011; Kort et al., 2005). The harvested spore crop contained more than 99.9% of phase-bright spores and was stored in distilled water at 4 °C.

2.2. Tea compounds and germination conditions

Gallic acid monohydrate, (−)-gallocatechin gallate, Teavigo (>90% (−)-epigallocatechin gallate), and theaflavin 3,3′-digallate were obtained from Sigma–Aldrich. The spores used for germination and outgrowth experiments were first heat-activated in distilled water for 30 min at 70 °C. Germination and outgrowth of heat-activated spores was triggered in defined minimal (MOPS-buffered) medium (pH 7.4) supplemented with 10 mM L-asparagine, 10 mM glucose, 1 mM fructose, and 1 mM potassium chloride (AGFK).

To test the inhibitory effect of different tea compounds, heat-activated spores were transferred to a microtiter plate reader (MultiSkan FC, Thermo Fisher Scientific) containing defined minimal medium (pH 7.4), supplemented with AGFK and different concentrations of tea compounds. Microtiter plates were incubated at 37 °C and the optical density at 600 nm (OD_{600}) was measured every 5 min for 16 h under rigorous shaking. The following concentrations were tested: 15, 30, 60, 120 μM gallic acid and 2.5, 5, 10, 20, 40 μM gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate. Per condition four technical replicates (wells in one microtiter plate) were used and two different plate experiments were performed with one and the same spore crop.

2.3. Microscope-slide preparation and time-lapse microscopy

A closed air-containing chamber developed by us recently was used for phase-contrast image acquisition (Pandey et al., 2013). In brief, a cast was prepared by attaching a Gene Frame® to a standard microscope slide and cover slip. A thin, semisolid matrix pad (160 μm) of 1% agarose-medium was made on a cover slip. Defined minimal (MOPS-buffered) medium (pH 7.4), supplemented with AGFK and containing no or different concentrations of tea compounds was used. The following compounds were tested: 120 μM gallic acid, 40 μM gallocatechin gallate, 40 μM Teavigo, and 40 μM theaflavin 3,3′-digallate. The pad was loaded with 1 μl heat-activated (70 °C for 30 min) spores of OD_{600} 7.9 and the cover slip (containing the pad) was placed in upside down position onto the Gene Frame®. The resulting chamber was used for time-lapse microscopy.

Time-lapse images were acquired by using a temperature-controlled boxed incubation system set at 37 °C. The specimens were observed with 100X/1.3 plane Apochromatic objective (Axiovert-200 Zeiss, Jena, Germany) and images were taken by a CoolSnap HQ CCD camera (Roper Scientific) using Metamorph software 6.1 (Molecular Devices). Phase-contrast time-lapse series were recorded at a sample frequency of 1 frame per 1 min for 8–10 h. Maximally 9 different fields of view were recorded in parallel per experiment and in each field of view, on average 10 spores were identified and followed in time. This resulted in the analysis of approximately 90 spores from the start of each imaging experiment. Three different microscopy experiments for each stress condition and five for the control condition, with maximally nine technical replicates (recorded fields of view on one slide), were performed with one and the same spore crop.

2.4. Image analysis with SporeTracker

The phase-contrast microscope recorded the complete sequence of spore germination, outgrowth and cell divisions of bacteria emerging from spores. In their dormant state *B. subtilis* spores appeared as bright spots. As the spores germinated, their microscopic appearance became phase-dark. To follow the germination and outgrowth process, and subsequent cell division in time, the decrease in pixel intensity and increase in surface area were analysed, respectively. To measure these parameters the image analysis tool "SporeTracker", <http://simon.bio.uva.nl/objectj/examples/sporetracker/SporeTracker.htm>, was developed (Pandey et al., 2013). This macro runs in combination with ObjectJ, <http://simon.bio.uva.nl/objectj>, which is a plugin for ImageJ <http://rsb.info.nih.gov/ij>. SporeTracker is configured to measure the time-to-start of germination, germination time (duration of phase-bright to phase-dark transition), the outgrowth time (duration from phase-dark to first division), as well as the generation time of vegetative cells emerging from the spores in any desired time frame. During outgrowth the emerging cell bursts out of the germinated spore and sheds its spore coat. Additionally, the "burst or shedding time" was introduced in SporeTracker and is defined as the duration from the phase-dark stage of the spores until the time

of burst or shedding of the spore coat. The program generates the corresponding plots and numerical output from any number of movies. Our recent article describes SporeTracker in detail (Pandey et al., 2013).

All separate stages of development from dormant spores to dividing vegetative cells of control spores were compared with those of spores germinated in the presence of tea compounds and fitted according to the most appropriate distributions, which were log normal for the time-to-start of germination and normal for the other stages (data not shown). All data of the stress experiments were compared with the control conditions and differences in variance were tested with *F*-tests. Depending on the results of the *F*-tests (significant difference in variance when $P < 0.01$) the appropriate *t*-tests (equal or unequal variance) were performed to test differences in the mean.

3. Results

3.1. Effect of different tea compounds on germination, outgrowth, and subsequent vegetative growth of B. subtilis spores at the population level

Different concentrations of four tea compounds (gallic acid, gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate) were screened in a microtiter plate reader by measuring the OD_{600} of germinating and outgrowing spores in time. Fig. 1 displays different stages of germination, outgrowth of spores, and subsequent growth and death of vegetative cells (confirmed by microscopy and counts of colony forming units (data not shown)) in the absence and presence of various concentrations of the four different tea compounds. These morphological stages of germination and (out) growth have been analysed microscopically ((Keijser et al., 2007) and coincides with our live imaging data (see further on)). Spore germination starts with the interaction of germinants with germinant receptors such as GerA, GerB and GerC (Setlow, 2003). During this process partial rehydration and subsequent swelling of the spore core occur. Due to rehydration of the spore core the refractive index of the germinating spore (OD_{600}) drops. This initial small drop in refractive index of the spore suspension with time can be seen within the first 1 h of the experiment. However, due to the low starting OD_{600} of 0.04 (which corresponds to an OD_{600} of 0.2 in a 1-cm-path-length spectrophotometer) that we used, the drop is not that pronounced in the graphs of Fig. 1. After germination, the bursting of the spore coat and the subsequent emergence of the vegetative cell takes place (outgrowth). In the control condition this can be seen as an approximate horizontal line at the lowered OD_{600} within the time frame of 1–3 h. Finally, the OD_{600} becomes proportional to the number of vegetative cells, which can be observed within the time frame of 3–8 h, depending on the conditions tested. The decline/death phase appears due to nutrient limitation and can be seen in the time frame of 10–16 h for the control condition.

Incubating *B. subtilis* spores in the presence of different concentrations of the four tested tea compounds did not reveal any apparent differences in germination behaviour when compared to the control condition (Fig. 1). This suggested that the selected tea compounds have no clear effect on spore germination. However, gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate did clearly cause more extended outgrowth phases with higher concentrations (Fig. 1B–D). Additionally, the maximum growth rate of vegetative cells emerging from spores was increasingly affected (decreased) with higher concentrations of gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate. Gallic acid concentrations up to 60 μM did not have a clear effect on germination, outgrowth, and

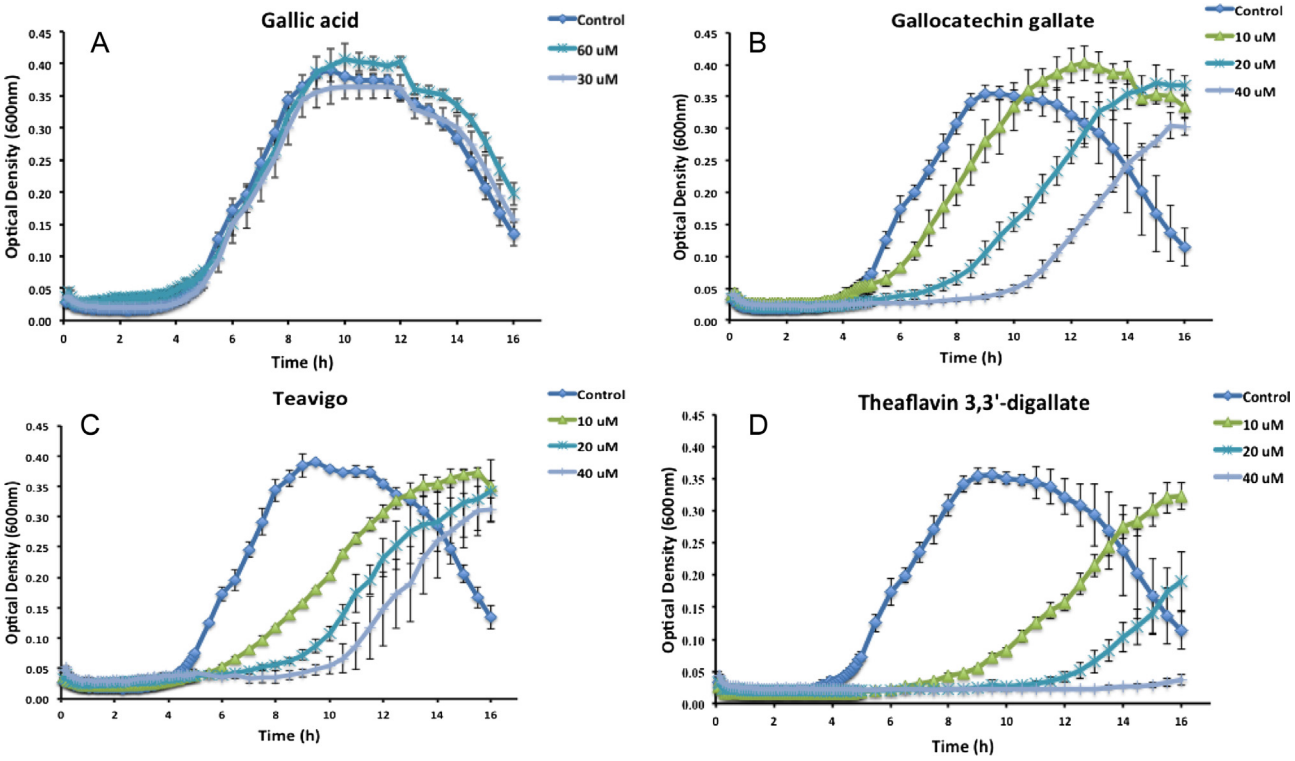

Fig. 1. Inhibitory effect of different tea compounds on the germination, outgrowth, and subsequent vegetative growth of *B. subtilis* 1A700 spores as measured in microtiter plates. Heat-activated spores (70 °C for 30 min) were cultivated in defined minimal (MOPS-buffered) medium (pH 7.4) including AGFK and supplemented with various concentrations of gallic acid, gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate (panels A–D). The OD_{600} was measured every 5 min for 16 h. Error bars indicate the standard deviation of four technical replicates. Measurements of once every 30 min were shown after 5 h for clarity reasons.

subsequent vegetative growth of *B. subtilis* spores (Fig. 1A). Although a higher end-concentration (120 μM) of gallic acid was tested, the high absorbance of the chemical itself (yellow colour in solution) disturbed the OD_{600} measurements severely (data not shown). However, visual inspection of the microtiter plates after 16 h of incubation did reveal (out)growth inhibition of the spores caused by 120 μM gallic acid when compared to the control. To confirm if the inhibitory effect was due to an antimicrobial effect of the tea compounds and not merely because of a change in pH by adding the tea compounds, the pH of the cultures was measured. No changes in pH were observed when spores were incubated in defined minimal medium (buffered to pH 7.4 with 80 mM MOPS) and in the presence of the selected tea compounds (data not shown). The results presented here are in line with previous observations, which showed that polyphenols, and especially catechins, inhibit the growth of vegetative cells and not germination of spores of *Bacillus* species (Friedman et al., 2006; Hara-Kudo et al., 2005; Shigemune et al., 2012).

OD_{600} readings from cultures reflect the result of the sum of the individuals in the whole population. In such studies it cannot be easily observed whether specific phases of germination and outgrowth are affected, and more importantly, how homo-/heterogeneously the outgrowing spores respond to a given stress. Therefore, we aimed at studying spore germination and outgrowth in more detail at the single spore level for those inhibitory concentrations that showed a clear effect of the tea compound on either of these processes (discussed below).

3.2. Effect of different tea compounds on germination, outgrowth, and subsequent vegetative growth at single cell resolution

Using live cell imaging we assessed the effect of the selected tea compounds on germination, outgrowth, and subsequent vegetative growth of *B. subtilis* at the single spore level. The aim was to choose a concentration of each tea compound, which showed a clear effect on the life cycle of *B. subtilis* from germination to (out)growth (Fig. 1). Time-lapse microscopy was performed for 8 h for control conditions and 10 h for stress conditions (see Fig. 2 and Movies S1–S5 of the Supplementary Data). Spores were able to germinate and grow out in presence of the tea compounds. Heterogeneity in spore germination and outgrowth is clearly observed in all, including control, conditions (Fig. 2). Not all spores germinate or grow out within the timeframe of the experiment. Vegetative growth (~number of cells per microcolony) seems to be affected by at least gallocatechin gallate and theaflavin 3,3′-digallate (Fig. 2). When all individual data are taken together, the germination efficiencies of stressed spores with tea compounds (ranging from 94.1% to 96.7%) was comparable to that of unstressed spores (95.1%) (Fig. 3). The ability of spores to grow out was affected when spores were stressed with tea compounds. Fig. 3 shows that, compared to unstressed spores (82.1% outgrowth efficiency), 120 μM gallic acid (56.5%) had the largest effect whereas 40 μM Teavigo (80.9%) had the least effect on the ability to grow out.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.03.006.

The different phases of germination, outgrowth, and subsequent vegetative growth of stressed and unstressed spores were analysed in detail with SporeTracker. The obtained results from all individual data can be found in Table S1 of the Supplementary Data. Interestingly, the results showed that 120 μM gallic acid caused a significant effect ($P < 0.01$) on the mean time and the variance of the time-to-start of germination (Table 1, and Fig. 4). Gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate stress caused no effect on the mean time, but showed a significant effect on the variance of time-to-start of germination ($P < 0.01$). The germination time itself was hardly affected by the four compounds (see Table 1 and Fig. S1 of the Supplementary Data). The variance of the germination time was only affected by Teavigo. Compared to the control (4.4 ± 1.0 min), gallic acid, Teavigo and theaflavin 3,3′-digallate did cause a small, but significant reduction in the mean germination time (4.0 ± 1.0, 4.0 ± 0.8 and 4.0 ± 0.9 min, respectively). At this point we have no explanation why these compounds would enhance the rate of germination. Both the burst/shedding time and the time of outgrowth were clearly affected by all four tea compounds (see Table 1, Fig. 5 and Fig. S2 of the Supplementary Data). All compounds significantly extended the mean time needed for the spores to grow out ($P < 0.01$). The spores stressed with gallic acid, gallocatechin gallate, Teavigo, theaflavin 3,3-digallate needed on average 87.0 min, 49.2 min, 28.8 min, and 68.0 min, respectively more time to grow out (Table 1). The variance of the burst/shedding time and time of outgrowth were also significantly increased by gallic acid, gallocatechin gallate, and Teavigo, however not by theaflavin 3,3′-digallate. Finally, significant differences were found for the mean doubling time and variance after development to vegetative cells between cells exposed to the tea compounds and control incubations (see Table 1 and Fig. 6).

In line with the microtiter plate results (Fig. 1) and previously published data (Friedman et al., 2006; Hara-Kudo et al., 2005; Shigemune et al., 2012) the analysis of data of individual germinating spores with SporeTracker revealed a strong effect of the tea compounds on the outgrowth of spores and doubling times of vegetative cells (Table 1). Not reported before, the time-to-start of germination and the germination time itself are also significantly affected by the tea compounds, but compared to the effects on vegetative cells, to a relative lesser extent. Gallic acid is here an exception as it delayed the average start of germination with more than a factor 2 (Table 1).

4. Discussion

B. subtilis spores are universally present in foods; they may survive preservation treatments and grow out in end products leading to food spoilage. Hence efforts are being made to eliminate or inactivate these bacterial spores from foods. Germination and outgrowth of spores ensures their return to vegetative cells, thus posing spoilage and food safety challenges for the food industry. Tea compounds may retard both these processes. In order to check the antimicrobial effect of tea components we have studied four different tea compounds, namely gallic acid, gallocatechin gallate, Teavigo, and theaflavin 3,3′-digallate with regard to their effect on germination, outgrowth, and subsequent vegetative growth of *B. subtilis* spores in detail. The performed experiments with populations in microtiter plates clearly revealed an inhibitory effect of the tested tea compounds on the outgrowth of spores and doubling times of emerging vegetative cells (Fig. 1), corroborating previously reported data (Friedman et al., 2006; Hara-Kudo et al., 2005; Shigemune et al., 2012).

Since spore germination and outgrowth progression are often very heterogeneous it is exceedingly challenging to make predictions of microbial stability of food products. Due to this heterogeneity it is difficult to pinpoint which phases of germination and outgrowth are specifically affected by each tea compound. Therefore it is necessary to analyse the behaviour of single spores/cells to quantify the effect and degree of heterogeneity on each life cycle phase. The closed air-containing chamber described in our recent article, has proven to be very useful for the observation of growth of aerobic bacteria and germination and outgrowth of individual spores (Pandey et al., 2013). The newly developed program “SporeTracker”, with its incorporated macros, allowed for comprehensive and efficient data analyses, which include time-to-

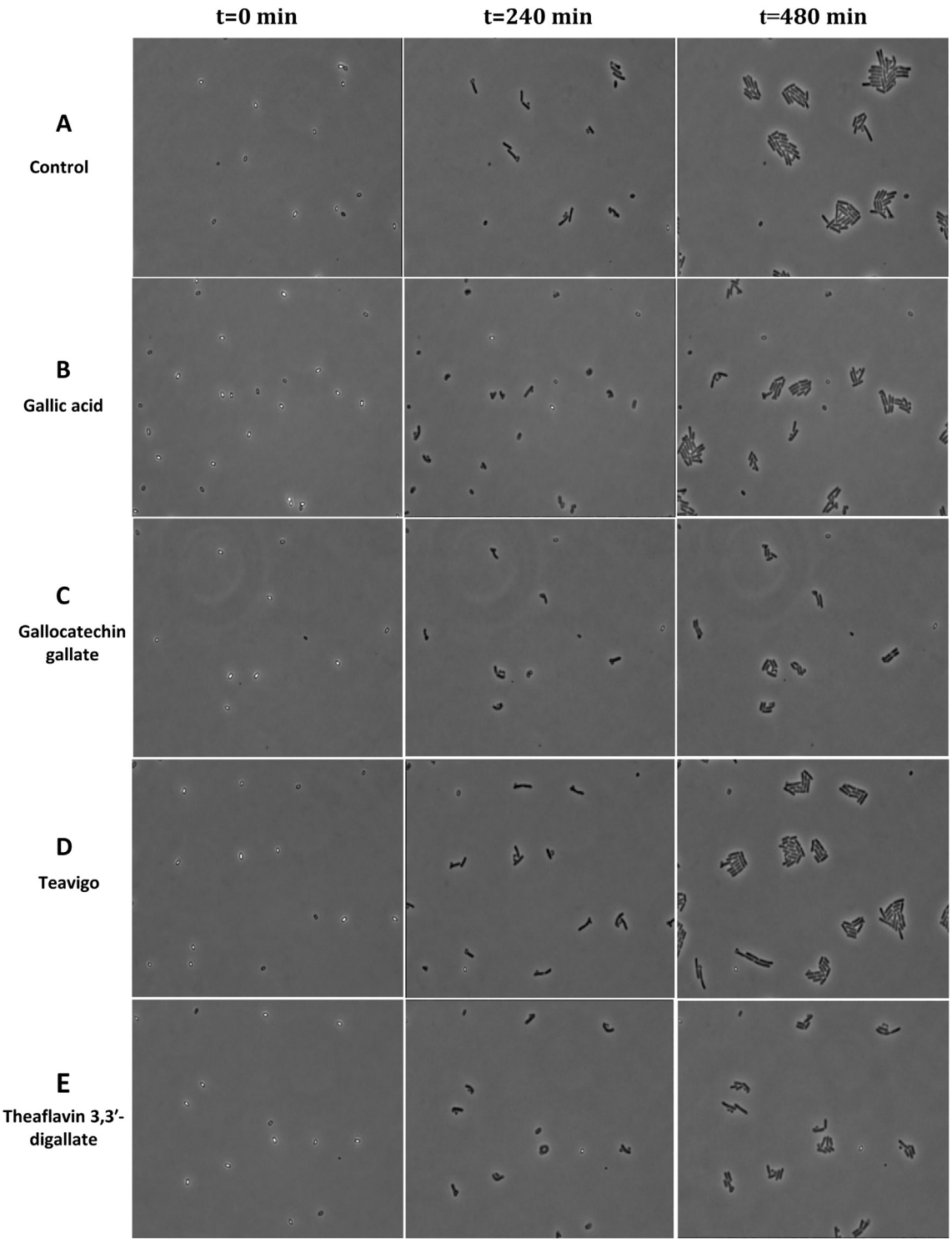

Fig. 2. Time-resolved images showing germination and outgrowth of *B. subtilis* 1A700 spores in the presence of different tea compounds. Heat-activated spores (70 °C for 30 min) were spotted on defined minimal (MOPS-buffered) medium (pH 7.4) including AGFK and supplemented without (control) (A) or with 120 μM gallic acid (B), 40 μM gallocatechin gallate (C), 40 μM Teavigo (D), and 40 μM Theaflavin 3,3′-digallate (E). Germination and outgrowth was followed in time using phase-contrast microscopy for 8–10 h.

start of germination, germination time, burst or shedding time, time of outgrowth and generation time during microcolony formation.

The variability between experiments of the same condition (stress and control) was comparable. For instance, the coefficient of variation for the germination time varied between 0.21 and 0.25 in the control condition (five experiments) and between 0.20 and 0.26 for gallocatechin gallate-stressed spores (three experiments). Consequently, we did not find a significant difference for the germination time of gallocatechin gallate-treated spores when compared to untreated spores (Table 1). The outgrowth and subsequent vegetative growth of cells emerging from tea compound-

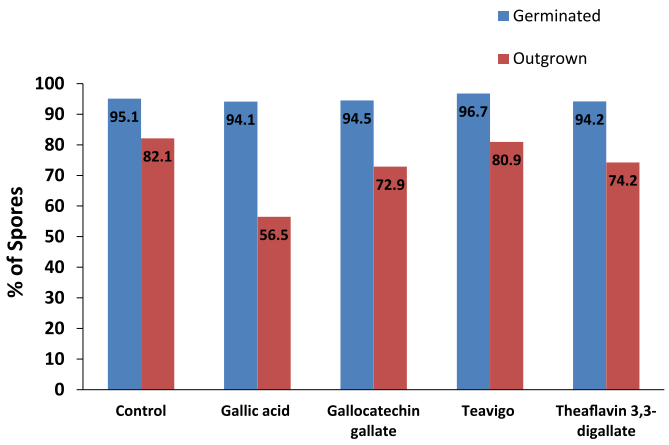

Fig. 3. Effect of different tea compounds on the ability to germinate and grow out. Movies of spores germinated in the presence and absence of tea compounds (see Fig. 2 for details) were analysed with SporeTracker and the spores were scored (by additional manual inspection) for their ability to germinate and grow out. The total number of spores assessed in the control and stress conditions was 486, 264, 277, 426 and 286, respectively.

stressed spores were significantly affected. The coefficient of variation for the generation time of cells growing in the presence of gallocatechin gallate (0.13–0.18) was relatively higher than for the control condition (0.06–0.12). Our results clearly suggest that the tea compounds have effect on vegetative cells (Table 1). The previous studies indicate that catechins in tea may pass through the cell wall, which is mainly composed of peptidoglycan (Vollmer et al., 2008) and bind through hydrophobic interactions to the lipid bilayer (Ajiya et al., 2002; Caturla, 2003; Kajiya et al., 2008). This affinity for the lipid bilayer is characterized by a number of factors such as the number of hydroxyl groups on the B-ring, the presence of a galloyl moiety, which is located on the surface of the lipid bilayers, and the stereochemical structure of the each catechin (Kajiya et al., 2008, 2004; Kumazawa et al., 2004). Finally, a recently published article indicates the mode of action of gallic acid against pathogenic bacteria (Borges et al., 2013). It showed that the compound led to irreversible changes in membrane properties through effects on membrane hydrophobicity, decrease of negative surface charge, and the occurrence of local ruptures as well as pore formation with consequent leakage of essential intracellular constituents (Borges et al., 2013). Theaflavins play an important role as antioxidants, however at high dosage, theaflavins were shown to be

Table 1
Mean values and standard deviation of different stages of germination and outgrowth of individual *B. subtilis* spores germinated in the presence and absence of different tea compounds.[a]

Mean ± SD[b]	Treatment				
	None	Gallic acid (120 μM)	Gallocatechin gallate (40 μM)	Teavigo (40 μM)	Theaflavin 3,3′-digallate (40 μM)
Start of germination (min)	33.7 ± 52.8 (n = 275)	75.0 ± 138.6 (n = 96)#*	57.7 ± 110.4 (n = 108)#	37.2 ± 64.1 (n = 187)#	54.2 ± 106.2 (n = 140)#
Germination time (min)	4.4 ± 1.0 (n = 275)	4.0 ± 1.0 (n = 96)*	4.1 ± 1.0 (n = 108)	4.0 ± 0.8 (n = 187)#*	4.0 ± 0.9 (n = 140)*
Burst/Shedding time (min)	114.4 ± 43.2 (n = 226)	179.3 ± 82.3 (n = 50)#*	139.0 ± 59.1 (n = 84)#*	142.6 ± 65.8 (n = 144)#*	137.7 ± 37.6 (n = 113)*
Outgrowth time (min)	236.1 ± 47.3 (n = 197)	323.0 ± 75.2 (n = 43)#*	285.2 ± 77.7 (n = 76)#*	264.9 ± 68.8 (n = 118)#*	304.0 ± 54.4 (n = 105)*
Generation time (min)	78.1 ± 9.3 (n = 274)	99.5 ± 13.5 (n = 98)#*	185.2 ± 30.9 (n = 146)#*	108.5 ± 20.6 (n = 203)#*	418.5 ± 177.3 (n = 140)#*

[a] Spores of *B. subtilis* 1A700 were heat-activated and germinated in defined minimal (MOPS-buffered) medium including AGFK and 120 μM gallic acid, 40 μM gallocatechin gallate, 40 μM Teavigo, and 40 μM theaflavin 3,3′-digallate. Various germination and outgrowth parameters of individual spores were calculated as described in the Materials and methods.

[b] Mean time of different stages is given including the standard deviation. The amount of spores/cells analysed from each stage and gathered from three (compounds) and five (control) microscopy experiments is given in brackets. The hash indicates that the variance of the distributions between the stress and control experiment are significantly different (F-test, $P < 0.01$). The asterisk indicates that the mean of the distributions between the stress and control experiment are significantly different (t-test, $P < 0.01$).

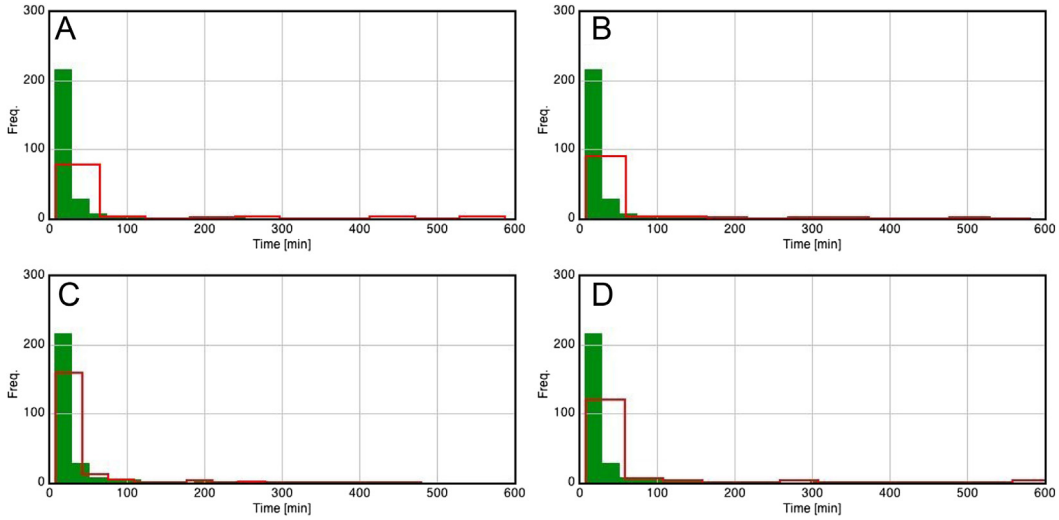

Fig. 4. Analysis of time-to-start of germination of individual spores germinated in the presence of different tea compounds. Movies of heat-activated spores germinated without and with gallic acid (A), gallocatechin gallate (B), Teavigo (C), and theaflavin 3,3′-digallate (D) (see Fig. 2 for details) were analysed with SporeTracker. Frequency distributions of time-to-start of germination of spores in the presence and absence of different tea compounds were calculated and are shown in red (outline) and green (solid), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

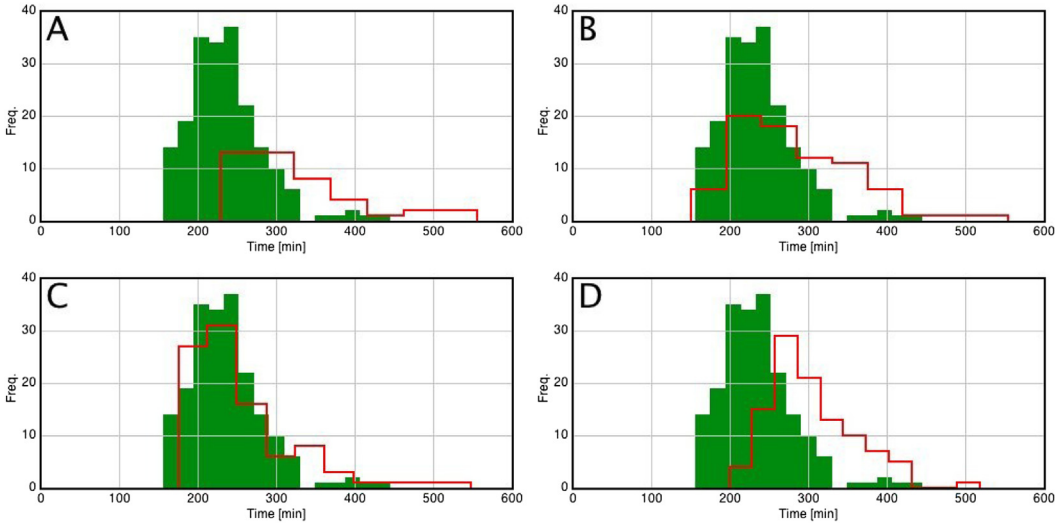

Fig. 5. Analysis of outgrowth times of individual spores germinated in the presence of different tea compounds. Movies of heat-activated spores germinated without and with gallic acid (A), gallocatechin gallate (B), Teavigo (C), and theaflavin 3,3′-digallate (D) (see Fig. 2 for details) were analysed with SporeTracker. Frequency distributions of outgrowth times of spores in the presence and absence of different tea compounds were calculated and are shown in red (outline) and green (solid), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitors of the ATP synthase and Complex I (NADH dehydrogenases) of the respiratory chain of *Escherichia coli* (Li et al., 2012).

Interestingly, we found that the time-to-start of germination was affected by gallic acid (Table 1). Since gallic acid (3,4,5-trihydroxybenzoic acid) is a weak acid (pK_a of 4.5) its mode of action might also involve lowering the intracellular pH and disturb the build-up of a proton gradient in the germinating spore (Ter Beek and Brul, 2010). For instance, Van Melis and co-workers showed that sorbic acid delays the germination and outgrowth of *B. cereus* spores (Van Melis et al., 2011). However, very limited data is available on the antimicrobial activity of gallic acid as a 'classical' weak organic acid. In addition, we speculate that gallic acid might interfere with the interaction of germinant receptors, which are needed for the initiation of germination. Further experiments are needed to evaluate these hypotheses.

In conclusion, the evaluated tea compounds have strong effects on the outgrowth and generation time of vegetative cells after their emergence from the spores. It supports the idea that flavonoids found in tea have an effect on the membrane and membrane constituents. Our results are in line with and corroborate previously reported studies and extend the data to the level of single spore analyses, thus facilitating the assessment of the heterogeneity in the response of *Bacillus* spores to the presence of antimicrobial flavonoids present in tea. Hence, this study can be directed toward possible application of tea compound as food preservatives. A new and interesting observation is that germination, especially the time-to-start of germination by gallic acid, is also somehow affected. For future work linking the single spore analysis data to molecular stress physiology, DNA microarray analysis can be performed on spores germinating in the presence of the antimicrobial compounds studied here. Microarray data can lead to the

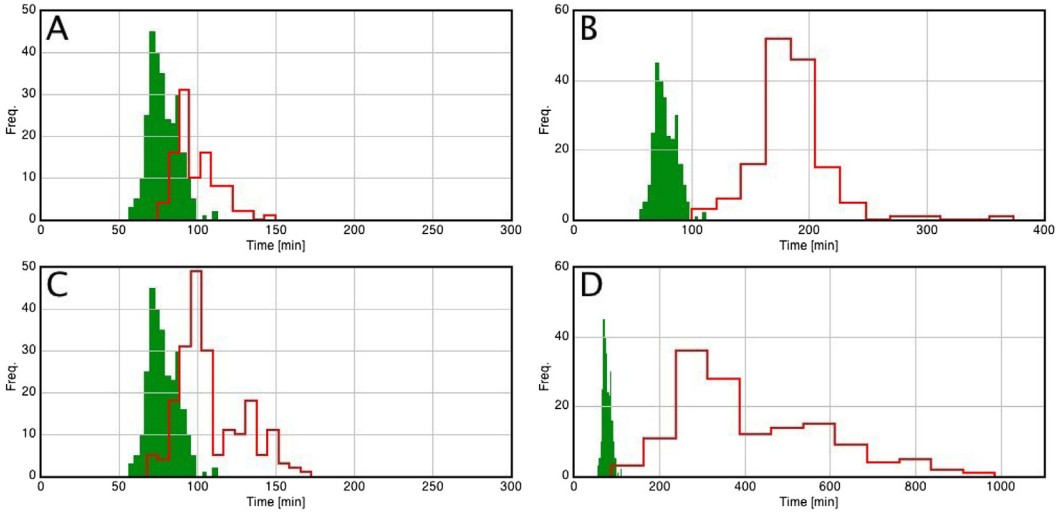

Fig. 6. Analysis of generation times of cells emerging from individual spores germinated in the presence of different tea compounds. Movies of heat-activated spores germinated without and with gallic acid (A), gallocatechin gallate (B), Teavigo (C), and theaflavin 3,3′-digallate (D) (see Fig. 2 for details) were analysed with SporeTracker. Frequency distributions of generation times of cells emerging from individual spores in the presence and absence of different tea compounds were calculated and are shown in red (outline) and green (solid), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identification of suitable stress response related genes that may be used for the generation of fluorescent reporter proteins that can be engineered into *B. subtilis* wild-type strains. Such strains will be instrumental in identifying the fraction and importance of the population switching on the particular stress response (degree of heterogeneity).

Acknowledgements

This work was supported by a grant from the Unilever R&D. A.T.B. is supported by a grant from the Dutch Foundation for Applied Sciences (STW 10431).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.03.006.

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