Combination of mild heat and plant essential oil constituents to inactivate resistant variants of *Escherichia coli* in buffer and in coconut water

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Abstract

The growing demand for minimally processed foods with clean labels has stimulated research into mild processing methods and natural antimicrobials to replace intensive heating and conventional preservatives, respectively. However, we have previously demonstrated that repetitive exposure of some bacteria to mild heat or subinhibitory concentrations of essential oil constituents (EOCs) may induce the emergence of mutants with increased resistance to these treatments. Since the combination of mild heat with some EOCs has a synergistic effect on microbial inactivation, we evaluated the potential of such combinations against our resistant *E. coli* mutants. While citral, carvacrol and *t*-cinnamaldehyde synergistically increased heat inactivation (53.0ºC, 10 min) of the wild-type MG1655 suspended in buffer, only the combination with carvacrol (200 µl/l) was able to mitigate the increased resistance of all the mutants. Moreover, the combination of heat and carvacrol acted synergistically inactivating heat-resistant variants of *E. coli* O157:H7 (ATCC 43888). This combined treatment could synergistically achieve more than 5 $\log_{10}$ reductions of the most resistant mutants in coconut water, although the temperature had to be raised to 57.0°C. Therefore, the combination of mild heat with carvacrol appears to hold promise for mild processing, and it is expected to counteract the development of heat resistance.

Keywords: mild heat; carvacrol; citral; *t*-cinnamaldehyde; hurdle technology; *E. coli* O157:H7; resistance development; coconut water

1. Introduction

For well over a century, thermal processing remains the most widely used technology to attain microbial safety and stability of foods. However, the demand for minimally processed foods has stimulated the food industry to reduce the intensity of thermal treatments and to look for alternative mild treatments that better preserve nutritional and sensorial properties of the fresh product, while maintaining high levels of microbial reduction. In order to guarantee sufficient control of foodborne pathogens, mild heating methods generally need to be combined with other food preservation techniques in a hurdle-type approach, especially in low-acid and/or low-moisture foods, in which microorganisms display their highest heat tolerance (Cava-Roda *et al.*, 2012; Kim and Kang, 2017a). In this context, hurdle approaches that improve microbial inactivation during heat treatment are preferable over hurdle approaches that aim for improved growth inhibition throughout the food shelf-life, since these may allow the pathogens to become more tolerant to subsequent stresses (Fong and Wang, 2016; Gayán *et al.*, 2016a) and to become more virulent (Slanec and Schmidt, 2011; Dawoud *et al.*, 2017). In addition, when hurdle technology is used to reduce the heat load of thermal processes, it is important that the new process is still capable of inactivating the most heat resistant variants of the pathogens of concern. Several studies have documented the existence of natural variants with elevated resistance to heat or other stresses used in food preservation (Abee *et al.*, 2016; Li and Gänzle, 2016a). In previous work, we have demonstrated that process-resistant variants can be also selected in the laboratory. Indeed, recurrent exposure of the notorious *Escherichia coli* O157:H7 to progressively intensifying heat treatment with intermittent enrichment rapidly selected for mutants with increased heat resistance that also displayed cross-resistance to high hydrostatic pressure (HHP) (Gayán *et al.*, 2016b).

One of the most efficient and attractive hurdles to combine with heat treatment are natural antimicrobials, in particular plant essential oils (EOs) and their constituents (EOCs). Many EOs and EOCs are multifunctional, having besides antimicrobial also antioxidant activity and alleged health promoting benefits (Calo *et al.*, 2015; Cui *et al.*, 2019). In addition, many EOCs have received the Generally Recognized As Safe (GRAS) status from the U.S. Food and Drug Administration (U.S. FDA, 2011). However, there are some weak points as well, which have hampered the commercial use of EOs and EOCs as food preservatives. First and foremost, the concentrations required to obtain the desired antimicrobial effect in foods often cause undesirable off-flavours. In addition, prolonged exposure of pathogenic bacteria to subinhibitory concentrations can induce the emergence of mutants with elevated resistance to both bacteriostatic and bactericidal concentrations of EOCs, and these mutants show cross-resistance to some other compounds and to heat (Chueca *et al.*, 2016; Berdejo *et al.*, 2019a). Of interest, the combination of EOCs with mild heat or other processes may hold promise to reduce or overcome the off-flavour problem, since certain EOCs can synergistically improve inactivation of mild processing technologies at sensorially acceptable concentrations (Espina *et al.*, 2014a; Berdejo *et al.*, 2019b). In particular, mild heat treatment in combination with carvacrol and citrus components such as citral and (+)-limonene has a synergistic lethal effect against a wide range of bacteria (Ait-Ouazzou *et al.*, 2011; Pagán *et al.*, 2018; Arioli *et al.*, 2019). Also *t*-cinnamaldehyde strongly enhances the efficacy of heat treatment (Juneja and Friedman, 2008; Amalaradjou *et al.*, 2010). With regard to the issue of resistance, however, it remains to be elucidated whether the synergistic combinations of heat and EOCs are also effective against heat or EOC resistant strains.

Therefore, this study aimed to examine the potential of combined treatments based on mild heat and carvacrol, citral, (+)-limonene oxide or t-cinnamaldehyde to mitigate the heat and EOC resistant mutants of *E. coli* that we have previously isolated (Hauben *et al.*, 1997; Vanlint *et al.*, 2011; Chueca *et al.*, 2016; Gayán *et al.*, 2016b). Once the most effective combination was selected, the combined treatment was validated in coconut water as a low acidic food model using O157:H7 heat resistant variants. Coconut water, extracted from young coconut liquid endosperm, is gaining popularity as a natural carbohydrate-electrolyte rich beverage, but it needs to be mildly processed to maintain its qualities and to reduce food poisoning risk (Awua *et al.*, 2012; Gabriel and Arellano, 2014). The combination of mild heat and EOCs may be a promising strategy to reduce negative impact of thermal treatment on coconut water quality.

2. Material and Methods

2.1. Bacterial strains and cultures

E. coli MG1655 and ATCC 43888 (serotype O157:H7) and their heat and EOC resistant derivatives shown in Table 1 were used throughout this study. Strains were first precultured in test tubes containing 5 ml of Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK), which were inoculated with three single colonies and then incubated aerobically on an orbital shaker (140 rpm; Heidolph Vibramax 100, Schwaback, Germany) for 12 h at 37°C. Subsequently, the precultures were diluted 1/500 in a flask containing 50 ml of TSB and incubated for 24 h at 37ºC to obtain stationary phase cultures containing about 2×10^9 CFU/ml.

2.2. Treatment media and EOC reagents

As treatment medium, 0.1 M MES (2-(N-morpholino) ethanesulfonic acid; PanReac AppliChem, Darmstadt, Germany) buffer adjusted to pH 5.3 ± 0.1 with 1 M NaOH was used as a model system for coconut water. The buffer was filter-sterilized and stored in the dark at 4°C for up to one week. Coconut water (Vita Coco, London, UK), thermally sterilized by the manufacturer and with a pH of 5.3 ± 0.1, was purchased in a local market in Belgium. Aliquots of 50 ml of coconut water from the same batch were stored frozen and thawed 30 min before use.

Carvacrol (≥ 98%), citral (≥ 96%) and (+)-limonene oxide (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while *t*-cinnamaldehyde (99%) was purchased from Acros Organics (Fairlawn, NJ, USA).

2.3. Heat and EOC (combined) treatment

Cells from a stationary phase culture were first harvested by centrifugation (6000 × g, 5 min) and resuspended in an equal volume of 0.1 M MES buffer or coconut water. Heat and EOC treatment conditions (*i.e.*, temperature, time and EOC concentration) were chosen to detect maximum synergistic lethal effects according to preliminary experiments (Ait-Ouazzou *et al.*, 2013; Espina *et al.*, 2013a). For EOC treatment, cells were diluted 1/100 in the treatment medium supplemented with a final concentration of 200 µl/l of each EOC and incubated for 10 min at room temperature. For heat treatment, cells were diluted 1/100 in a closed polypropylene tube (Sharlab, Barcelona, Spain) containing 900 µl of treatment medium prewarmed at 53.0ºC, 55.0ºC or 57.0ºC (± 0.5ºC) and incubated for 10 min in an FX heating block (mod. ZE/FX, Zeulab, Zaragoza, Spain). When EOC and heat treatment were combined, each EOC was directly added to a tempered tube to a final concentration of 200 µl/l prior to cell inoculation. During heat treatments, temperature of the treatment medium was

149 continuously monitored with a thermocouple (Almemo 2450, Ahlborn, Holzkirchen,
150 Germany), and temperature fluctuations remained within ±0.5ºC. After treatment,
151 samples were aseptically retrieved and survivors were recovered as indicated below.
152
153 **2.4. Determination of viability and synergy calculation**
154 Samples were serially diluted in 0.1% (w/v) peptone water (Oxoid), and a 100-µl
155 sample of each dilution was spread-plated onto Tryptone Soy Agar (TSA; Oxoid)
156 plates. After 24 h of incubation at 37°C, plates containing between 20 and 200 colonies
157 were counted, so that the quantification limit was 200 CFU/ml (equivalent to about 5
158 $\log_{10}$ reductions). The logarithmic reduction was calculated as $\log_{10}(N_0/N)$, in which N_0
159 and N represent the count in CFU/ml prior and after treatment, respectively. The lethal
160 interaction between heat and each EOC was estimated by subtracting the reduction
161 values obtained by application of each individual hurdle from the reduction reached by
162 the combined treatment, as previously described (Feyaerts *et al.*, 2015). A combined
163 treatment was defined as synergistic, antagonistic or additive when the sum of the
164 reductions for the individual hurdles was significantly lower, higher or equal,
165 respectively, than the reduction obtained by the combined treatment.
166
167 **2.5. Statistical analysis**
168 Statistical analyses (ANOVA and *t* test) were carried out using the software GraphPad
169 PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA), and differences were
170 regarded as significant when P was ≤ 0.05. All microbial inactivation data shown in
171 figures correspond to averages and standard deviations calculated from at least three
172 replicates performed in different working days.
173

3. Results and Discussion

3.1. Evaluation of synergistic lethal effect between heat and EOCs on *E. coli* MG1655

The occurrence of synergistic lethal effect by the combination of mild heat with carvacrol, citral, (+)-limonene oxide or *t*-cinnamaldehyde was first explored in the wild-type (WT) MG1655 strain. These experiments were carried out in MES buffer (pH 5.3) since food components might provide heat and EOC protection (Espina *et al.*, 2014a; Maté *et al.*, 2017). Fig. 1 shows the individual inactivation of the WT strain by heat (53.0ºC, 10 min) and each EOC (200 µl/l, 10 min) compared to the inactivation by the combined treatments (53.0ºC, 200 µl/l, 10 min). While heat treatment alone reached 1.4 $\log_{10}$ reductions and each EOC barely changed viability, the combined treatment with carvacrol, citral or *t*-cinnamaldehyde synergistically decreased survival to below the quantification limit (> 5 $\log_{10}$ reductions). The synergistic effect between heat and carvacrol or citral on *E. coli* inactivation has been reported in a wide range of buffer systems and foods (Ait-Ouazzou *et al.*, 2013; Kim and Kang, 2017b; Pagán *et al.*, 2018). However, the synergy of *t*-cinnamaldehyde with mild heat has been barely investigated despite the fact that this compound has shown a strong synergistic lethal effect with other physical food preservation methods such as HHP and pulsed electric fields (Pina-Pérez *et al.*, 2012; Feyaerts *et al.*, 2015; Li and Gänzle, 2016b). As such, the addition of cinnamon essential oil (100 µl/l) to apple cider reduced the thermal resistance of *E. coli* O157:H7 at mild temperatures (48ºC–54ºC) (Knight and McKellar, 2007), and the addition of *t*-cinnamaldehyde (0.15%–1.00% (v/w)) to ground beef synergistically improved *E. coli* O157:H7 inactivation during cooking (55.0ºC–62.5ºC) (Juneja and Friedman, 2008; Amalaradjou *et al.*, 2010).

In contrast to carvacrol, citral and *t*-cinnamaldehyde, the combination of (+)-limonene oxide with heat did not enhance ($P > 0.05$) inactivation (Fig. 1). We previously reported that (+)-limonene combined with mild heat acted synergistically for *E. coli* O157:H7 inactivation (Espina *et al.*, 2013b; Espina *et al.*, 2014a), and that both (+)-limonene oxide and limonene are effective for growth inhibition and inactivation of MG1655 (Chueca *et al.*, 2016). In this work, we tested for the first time the potential synergistic lethal effect between (+)-limonene oxide and heat on *E. coli*. Compared to (+)-limonene, the lack of synergy between (+)-limonene oxide and heat could be attributed to differences in their chemical structure and/or in the treatment medium used in previous studies, since pH and composition can markedly influence the antimicrobial activity of (+)-limonene (Espina *et al*., 2013b; Espina *et al*., 2014a).

3.2. Lethal effect of the synergistic combinations of heat and EOC on resistant variants of *E. coli* MG1655

Subsequently, the synergistic combinations of heat (53.0ºC, 10 min) and EOC (*i.e.*, carvacrol, citral or *t*-cinnamaldehyde; 200 µl/l) was evaluated in heat and EOC resistant derivatives of MG1655 that we previously obtained by directed evolution (Fig. 2; Table 1). These included three heat resistant mutants: DVL10, which emerged by exposing MG1655 to successive cycles of progressively intensifying heat shock and resuscitation (Vanlint *et al.*, 2011), and DVL1 and LMM1020 mutants, which were selected for increased HHP resistance but also displayed a marked level of cross-resistance to heat (Hauben *et al.*, 1997; Vanlint *et al.*, 2011). On the other hand, mutants resistant to carvacrol, citral and (+)-limonene oxide (in this work designated as MTCAR, MTCIT and MTLOX, respectively) were selected for growth resistance to increased concentrations of these compounds, which coincided with enhanced tolerance to lethal

concentrations of all the EOCs and to heat (Chueca *et al.*, 2016). Unfortunately, a MG1655 mutant with increased *t*-cinnamaldehyde tolerance was not yet available. Data on heat and EOC resistance of *E. coli* mutants compared to their corresponding WT strain reported in our previous studies are compiled in Table S1.

The combination of heat and carvacrol exhibited a synergistic lethal effect of more than 3 $\log_{10}$ cycles on all the EOC and heat (cross)-resistant MG1655 mutants tested, resulting in an inactivation higher than 5 $\log_{10}$ cycles in all the strains (Fig. 2). In contrast, synergy between citral and heat appeared in all the strains except in DVL1, whose heat inactivation was only increased ($P \leq 0.05$) by about 0.7 $\log_{10}$ cycles. *t*-Cinnamaldehyde, finally, synergistically increased heat inactivation of MTCAR and MTCIT, while in the other mutants the combination only exerted an additive (*i.e.*, DVL10 and MTLOX) or even an antagonistic lethal effect (*i.e.*, DVL1 and LMM1020). Thus, although the combination of heat with carvacrol, citral or *t*-cinnamaldehyde could synergistically improve inactivation of the MG1655 parent, the synergy between heat and citral or *t*-cinnamaldehyde was lost against some of its heat and EOC (cross)-resistant mutants, and only the combination of heat with carvacrol retained its synergistic interaction against all the derivative strains.

3.3. Synergistic inactivation of resistant *E. coli* MG1655 and ATCC 43888 variants by the combination of heat and carvacrol in coconut water

In view of the large synergy between heat and carvacrol on MG1655 and its heat and EOC resistant derivatives, this particular combination was then tested in coconut water (pH 5.3). Please note that all the strains showed significantly ($P \leq 0.05$) higher heat resistance (53.0ºC, 10 min) in coconut water than in MES buffer (compare white bars in Figs. 1, 2 and 3), probably due to the presence of coconut water components, of

currently unknown nature, that protect cells against heat lethal effects. However, the addition of carvacrol (200 µl/l) synergistically enhanced inactivation by more than 4 $\log_{10}$ reductions in all the strains, with the exception of DVL1, which only exhibited 2.5 $\log_{10}$ cycles of synergistic lethal effect (Fig. 3). In fact, the degree of synergy displayed by this mutant in coconut water was much lower ($P \leq 0.05$) than the effect observed in MES buffer (> 4.3 $\log_{10}$ cycles; Fig. 2A).

Since consumption of unpasteurized fruit juice has been involved in several foodborne disease outbreaks (Vojdani *et al.*, 2008), the U.S. FDA compels juice manufacturers to develop a Hazard Analysis Critical Control Point (HACCP) system and recommends the application of a decontamination process that reaches at least 5 $\log_{10}$ reductions of the pathogen of concern (U.S. FDA, 2001). This processing is also critical to ensure coconut water safety because it is prone to microbial contamination during extraction, and its nutrient richness and low acidity may support growth of pathogenic contaminants (Awua *et al*., 2012; Gabriel and Arellano, 2014). Gabriel and Arellano (2014) reported that a cocktail of *E. coli* O157:H7 strains displayed higher heat resistance than a *Salmonella enterica* and *Listeria monocytogenes* cocktail in coconut water, suggesting that the former should be regarded as the target pathogen to accomplish the U.S. FDA performance criterion. However, the U.S. FDA (2007) also identifies *Clostridium botulinum* as a critical hazard in low-acid pasteurized juice, and therefore design of minimal processing should take into account appropriate control of this pathogen.

Therefore, we also tested the carvacrol and heat combined treatment (53.0ºC, 200 µl/l, 10 min) in *E. coli* ATCC 43888 (serovar O157:H7) and its heat resistant variants obtained by directed evolution (Gayán *et al.*, 2016b). These variants were previously obtained after reiterative exposure of ATCC 43888 to progressively intensifying heat

shock with intermittent resuscitation up to the emergence of increased heat resistant mutants (Gayán *et al.*, 2016b). In the present study, we used the three most thermo-tolerant isolates, MT3, MT6 and MT9, which showed more than 10^5-fold higher heat survival (58°C, 15 min) than their parent (Table S1; Gayán *et al.*, 2016b). More specifically, heat resistance of MT6 was 10-fold higher than that of MT3 and MT9 and this mutant incurred 10^3-fold lower sublethal injury in the cell envelopes (Gayán *et al.*, 2016b). As illustrated in Fig. 3, the inactivation by the combined treatment was also synergistic on these strains (ranging from 2.2 to > 4.7 $\log_{10}$ cycles) and reached more than 5 $\log_{10}$ reductions of ATCC 43888 (WT) and its heat resistant mutants MT3 and MT9, although MT6 was only reduced by 2.3 $\log_{10}$ cycles. Subsequently, we examined whether increasing heat treatment intensity could boost the synergy between heat and carvacrol on the most heat resistant mutants. When the treatment temperature was increased to 55.0ºC, the magnitude of the synergy increased by about 1 $\log_{10}$ cycle on DVL1 and MT6, reaching 4.1 and 3.4 $\log_{10}$ reductions, respectively (Fig. 4A). Further temperature increase to 57.0ºC enabled to achieve the target of 5 $\log_{10}$ reductions in both strains, whereas heat inactivation alone only increased about 0.6 $\log_{10}$ cycles (Fig. 4B).

To the best of our knowledge, the detailed mechanism of synergistic *E. coli* inactivation by heat and EOC at molecular and cellular level has not been yet elucidated. It has been proposed that the synergy between heat and EOCs might stem from heat-induced damages in the cell envelopes that facilitate the action of hydrophobic EOCs while impairing resuscitation of injured cells (Ait-Ouazzou *et al*., 2011; Espina *et al.*, 2013b; Arioli *et al.*, 2019). In addition, several authors have shown that the extent of heat and EOC synergy is temperature dependent (Knight and McKellar, 2007; Arioli *et al.*, 2019). Therefore, the higher temperature needed to reach 5 $\log_{10}$ reductions in DVL1

and MT6 could be explained by their increased thermotolerance (Fig. 3 and 4) that likely coincides with increased resistance to thermal sublethal injury. Whole genome sequence analysis of these mutants might shed light on the causes of heat resistance and/or of their more performant cell repair system and in turn on the main cellular targets of the synergistic inactivation of heat and carvacrol combined treatment.

4. Conclusions

Although synergistic combinations of mild heat with EOC have been suggested as a promising approach in minimal food processing to reduce adverse thermal effects on food quality, the present work demonstrates that some compounds, such as citral and *t*-cinnamaldehyde, do not exhibit synergy against EOC and heat resistant variants of *E. coli* that can also display increased resistance to the combined treatment. Carvacrol was the only compound that retained synergy in combination with heat against all the resistant mutants, and therefore the compound of choice to combine with mild heat treatment in order to reduce the heat load and improve quality retention. More specifically, the addition of 200 µl/l of carvacrol increased the thermal reduction of the most thermotolerant derivatives of O157:H7 in coconut water at 57.0°C from less than 2 $\log_{10}$ units to more than 5 $\log_{10}$ units. Although the carvacrol concentration used in this study might fall above the sensory threshold (Espina *et al.*, 2014b), the combination of several EOCs that together synergistically enhance heat inactivation could help to reduce EOC concentrations and/or heating intensity for reaching the 5-$\log_{10}$ reductions goal of the most heat resistant *E. coli* mutants without decreasing sensorial acceptability of coconut water.

Acknowledgements

This work was supported by a postdoctoral fellowship from the Research Foundation of Flanders (FWO-Vlaanderen; to EG), a doctoral fellowship from the Spanish Ministry of Education, Culture and Sports (FPU15/02703 to DB), and grants from the KU Leuven Research Fund (IDO/10/012; METH/14/03), the Spanish Ministry of Science, Innovation and Universities (PGC2018-093789-B-I00), the European Social Fund and the Aragonese Office of Science, Technology and University Research.

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466 **Table 1**. *E. coli* strains used in this study.

Strain	Description	Source
MG1655	Wild-type strain	Blattner *et al.* (1997)
DVL10	Heat resistant derivative of MG1655	Vanlint *et al.* (2011)
DVL1	HHP and heat (cross-)resistant derivative of MG1655	Vanlint *et al.* (2011)
LMM1020	HHP and heat (cross-)resistant derivative of MG1655	Hauben *et al.* (1997)
MTCAR	Carvacrol and heat (cross-)resistant derivative of MG1655. Originally designated as CAR.	Chueca *et al.* (2016)
MTCIT	Citral and heat (cross-)resistant derivative of MG1655. Originally designated as CIT.	Chueca *et al.* (2016)
MTLOX	(+)-limonene oxide and heat (cross-)resistant derivative of MG1655. Originally designated as LIM.	Chueca *et al.* (2016)
ATCC 43888	Wild-type (WT) strain	Uhlich *et al.* (2017)
MT3	Heat resistant derivative of ATCC 43888	Gayán *et al*. (2016b)
MT6	Heat resistant derivative of ATCC 43888	Gayán *et al.* (2016b)
MT9	Heat resistant derivative of ATCC 43888	Gayán *et al.* (2016b)

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Figure captions

Figure 1. Logarithmic reduction ($\log_{10} N_0/N$) of *E. coli* MG1655 (WT) by mild heat (53.0ºC, 10 min; white bar, H) and each EOC (200 µl/l, 10 min; grey bars: CAR, carvacrol; CIT, citral; LOX, (+)-limonene oxide; *t*-CIN, *t*-cinnamaldehyde) separately and the combination of both hurdles (black bars, H + EOC) in MES buffer (0.1 M, pH 5.3). Dotted line indicates the quantification limit (200 CFU/ml, equivalent to about 5 $\log_{10}$ reductions). Asterisks indicate synergistic combinations based on statistical analysis ($P \leq 0.05$).

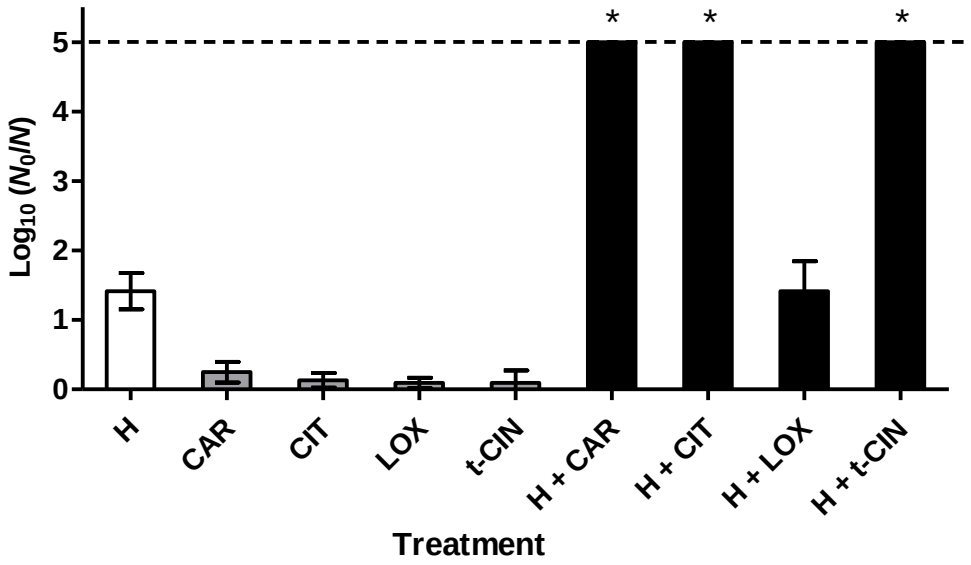
Figure 2. Logarithmic reduction ($\log_{10} N_0/N$) of indicated *E. coli* mutants by mild heat (53.0ºC, 10 min; white bars) and each EOC (200 µl/l, 10 min; grey bars) separately and the combination of both hurdles (black bars) in MES buffer (0.1M, pH 5.3): (A) carvacrol, (B) citral, (C) *t*-cinnamaldehyde. Dotted line indicates the quantification limit (200 CFU/ml, equivalent to about 5 $\log_{10}$ reductions). Asterisks indicate synergistic combinations based on statistical analysis ($P \leq 0.05$).

Figure 3. Logarithmic reduction ($\log_{10} N_0/N$) of *E. coli* MG1655 and ATCC 438888 (WT) and their indicated derivatives by mild heat (53.0ºC, 10 min; white bars) and carvacrol (200 µl/l, 15 min; grey bars) separately and by the combination of both hurdles (black bars) in coconut water (pH 5.3). Dotted line indicates the quantification limit (200 CFU/ml, equivalent to about 5 $\log_{10}$ reductions). Asterisks indicate synergistic combinations based on statistical analysis ($P \leq 0.05$).

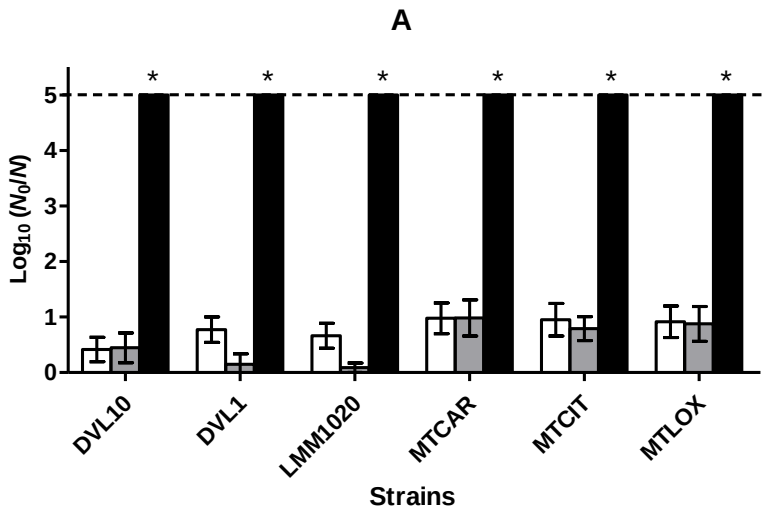
Figure 4. Logarithmic reduction ($\log_{10} N_0/N$) of *E. coli* MG1655 and ATCC 438888 (WT) and their indicated heat resistant derivatives by heat (A) 55.0ºC, 10 min; (B) 57.0ºC, 10 min) in the absence (white bars) or presence of carvacrol (200 µl/l; black bars) in coconut water (pH 5.3). Dotted line indicates the quantification limit (200

CFU/ml, equivalent to about 5 $\log_{10}$ reductions). Asterisks indicate synergistic combinations based on statistical analysis ($P \leq 0.05$).

Figure 1


Log10 (N0/N)
0
1
2
3
4
5
*
*
*
H
CAR
CIT
LOX
t-CIN
H + CAR
H + CIT
H + LOX
H + t-CIN
Treatment

543 **Figure 2**



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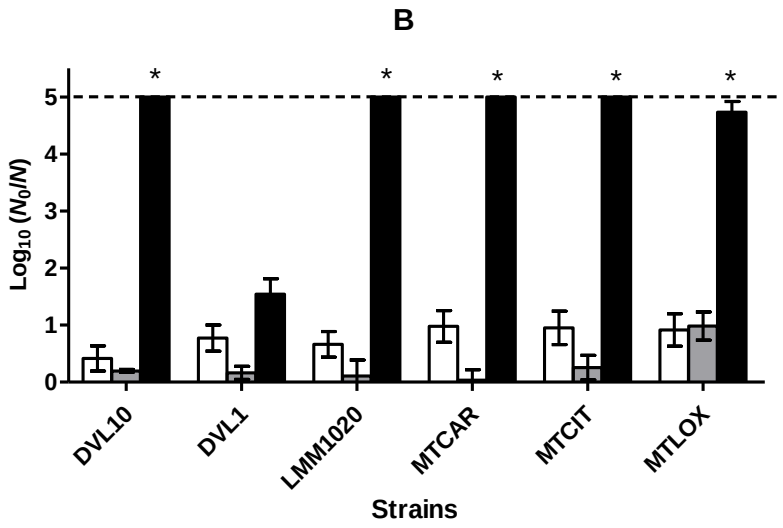

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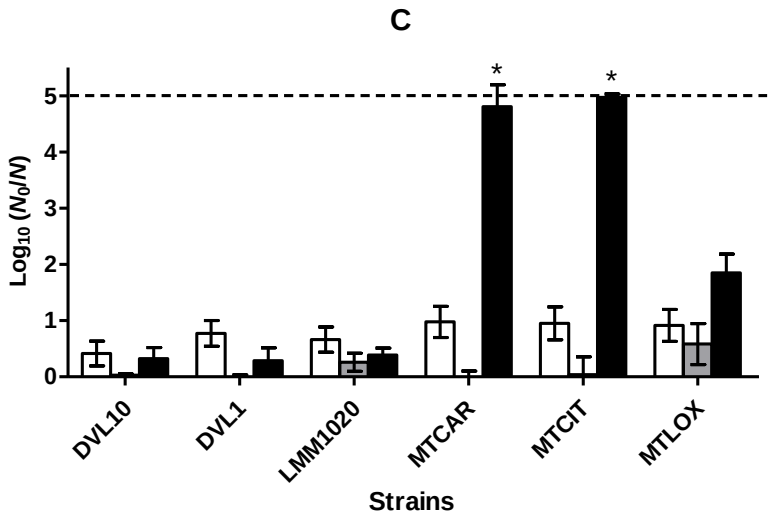

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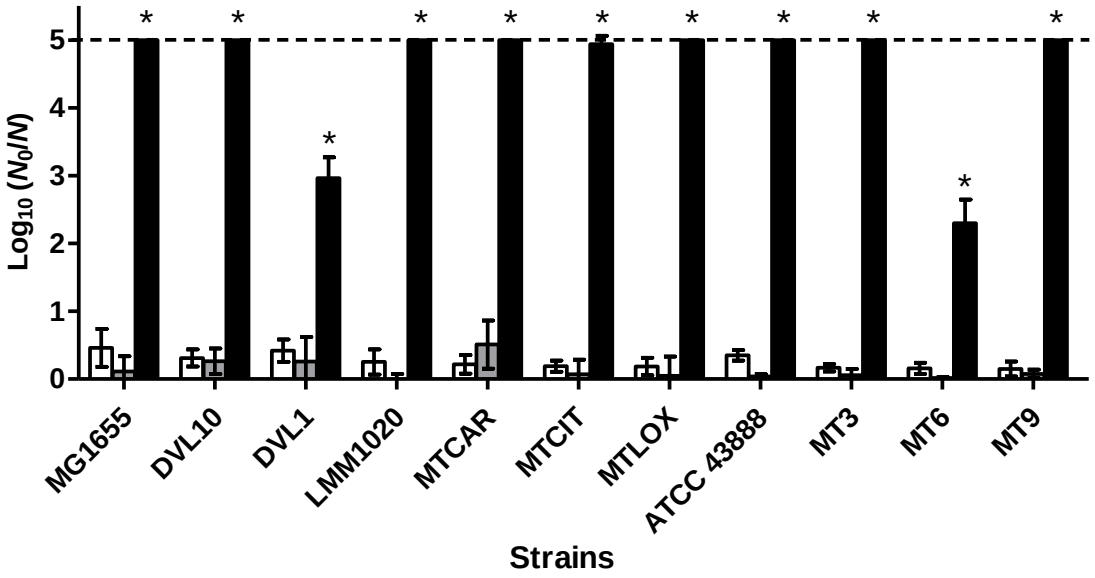
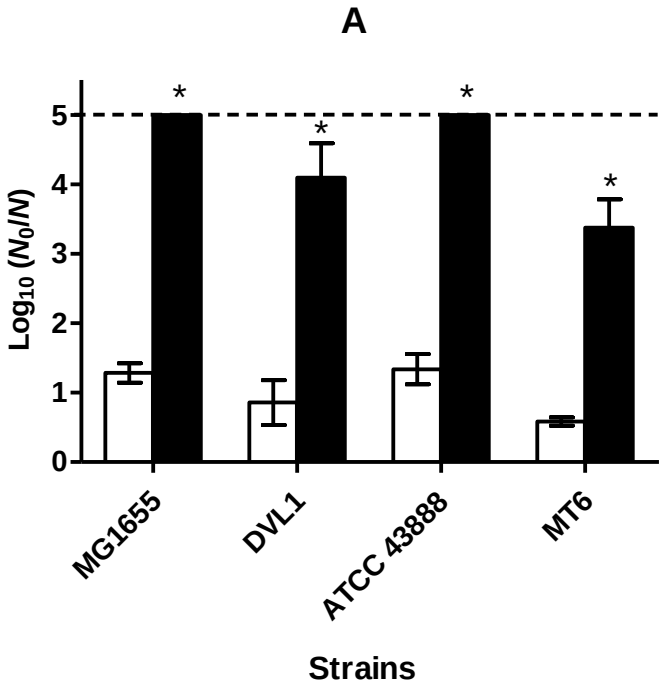
Figure 3


Log10 (N0/N)
0
1
2
3
4
5
*
MG1655
DVL10
DVL1
LMM1020
MTCAR
MTCIT
MTLOX
ATCC 43888
MT3
MT6
MT9
Strains

567 **Figure 4**



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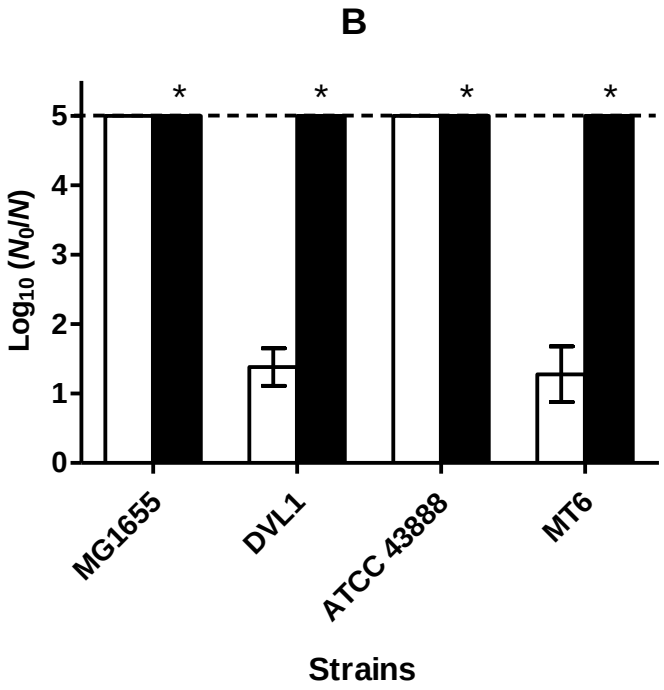

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