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Growth of Shiga-Toxin producing *Escherichia coli* (STEC) and bovine feces background microflora in various enrichment protocols

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Running headline: STEC AND FECES FLORA IN ENRICHMENT

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Abstract

20 Cattle are an important reservoir for STEC and eating food contaminated with faecal material is a frequent source of human STEC infection. It is thus essential to reliably determine the prevalence of STEC contamination in cattle. Currently, different enrichment protocols are used before the detection of Shiga-Toxin producing *Escherichia coli* (STEC) in faecal samples. However, there have not been any studies performed that have compared the effectiveness of these various enrichment protocols for the growth of non-O157 STEC in 25 faecal samples. The objective of this present study was to characterize the effects of different enrichment factors on the simultaneous growth of the feces background microflora (BM) and two non-O157 STEC strains. The different factors studied were the basal medium (TSB and EC), the effect of novobiocin in the broth (N+ or N-) and the incubation temperature (37°C or 30 40°C). The BM and STEC growth data were simultaneously fitted by using a competitive growth model. The STEC final levels obtained after 24h were higher for the protocols with novobiocin and/or EC compared to the others. However, novobiocin inhibited the growth of one STEC strain. We observed that the addition of novobiocin into broths is not advisable for optimal growth conditions. Moreover, given high BM and low STEC levels often observed in 35 feces, predictions made with the growth model highlighted that false negative results could more likely appear with protocols using TSB without novobiocin than with protocols using EC. In conclusion, the use of EC broth in enrichment protocols seems to be more appropriate for detecting non-O157 STEC from bovine faecal samples. This can help avoid false negative results that cause an underestimation of the STEC prevalence in cattle.

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Keywords: *Escherichia coli*; STEC; bovine feces background microflora; enrichment protocol; competitive growth model.

1. Introduction

Shiga-Toxin producing *Escherichia coli* (STEC) are a significant public health threat
45 in many industrialized countries (World Health Organization, 1998). Cattle are the primary
reservoir for STEC, and human diseases are most often associated with eating food or
drinking water contaminated by cattle manure (Bopp et al., 2003).

Several studies were carried out on bovine faecal samples to evaluate STEC prevalence in
order to monitor STEC emergence and its implication in faeces-contaminated food and
50 drinking water (Cobbold and Desmarchelier, 2000). Different methods of screening for STEC
are currently available (e.g. PCR, IMS, ELISA), and most of them require an enrichment step
in order to increase their ability to detect low levels of STEC (Cui et al., 2003).

Several types of enrichment protocols characterized by different factors are currently used for
the growth of STEC. The main enrichment protocol factors are the enrichment broth (basal
55 medium), the addition of selective ingredients, the temperature and the duration of the
incubation period. Vimont et al. (2006) reported that the Trypticase Soy Broth (TSB) and the
E. coli broth (EC) are the most frequently used enrichment broths. They are often
supplemented with novobiocin and incubated between 16 to 24 hours at 35-37°C for STEC
growth. Nevertheless, some authors have also reported an optimal growth temperature of non-
60 O157 strains around 41°C (Gonthier et al., 2001).

However, no study has compared the effectiveness of these various enrichment protocols for
the growth of non-O157 STEC in fecal samples (Vimont et al., 2006). Consequently, it
appears difficult to compare the prevalence of STEC in cattle published in different studies.

Furthermore, the enrichment step may be difficult to control because of the presence of the
65 background microflora, which may influence the growth of STEC. So far there has been little
information concerning the interaction between STEC and feces background microflora. It

must be emphasized that with a goal of optimizing the enrichment conditions, it is essential to understand the interaction between the two floras during simultaneous growth.

The aim of the present study was to investigate the effect of different enrichment factors on the simultaneous growth of the feces background microflora and two non-O157 (STEC) transformed strains. The different factors studied were the basal medium (TSB and EC), the addition of novobiocin in broth (N⁺ or N⁻) and the incubation temperature (37°C and 40°C). In order to visualize the potential effects of these factors and thus to optimize the enrichment step, a follow up and a modeling of the simultaneous growth of the background microflora and STEC strains were performed.

2. Materials and methods

2.1. Inoculum preparation

The two STEC strains used in the present study (referred to as Strain 1 and Strain 2), were both isolated from dairy cattle feces during the course of a previous study. These strains did not belong to the serogroup O26, O157, O111 or O55 and their exact serotypes were not determined. However, they were characterized for the presence of genes coding virulence factors, *stx1* and *stx2* coding the Shiga-Toxins 1 and 2 respectively, as well as *eae* and *ehx* coding intimin and EHEC-hemolysin, respectively. Strain 1 and Strain 2 were *stx1*⁻, *stx2*⁺, *eae*⁻, *ehx*⁻ and *stx1*⁺, *stx2*⁺, *eae*⁻, *ehx*⁺ respectively. These strains were electroporated in the presence of a plasmid vector pGFPuv (ClonTech) carrying ampicillin resistance and the green fluorescent protein (GFP), according to the protocol described by Delazari et al. (1998). More precisely, the transformation was carried out by setting the electroporation apparatus at 2.5 kV and 25 µF with the pulse controller adjusted to 200Ω. An electrical pulse of 4.7 msec was applied. Point twenty-five µg of GFP-ampicillin resistance plasmid (pGFP) was added to the microcentrifuge tubes containing the bacterial cells. The plasmid and cells were mixed by pipetting, and then transferred into an Invitrogen 0.2cm cuvette. With the cuvette in the

sample chamber, a pulse was applied using the indicated apparatus settings. Aliquot of 0.1 ml of the transformant suspension was spread on Brain Heart Infusion (BHI, 51009, bioMérieux) agar (M3012, Kalys) (BA) plates containing 150 µg of ampicillin per ml (Ampicillin Sodium salt, eu0400, Euromedex) (BA-A). The resulting ampicillin resistant transformed colonies showed bright green fluorescent color when viewed with a handheld UV light (Herolab). This transformation was done in order to make the numeration of the two STEC strains among the abundant bovine feces background microflora during the growth follow up experiments easier. Moreover, it must be emphasized that no difference in behaviour was noted between the transformed non-O157 strains and their parental strains. Indeed in a previous study carried out in our laboratory (Fremaux et al., 2006), we had observed the same decline kinetics of the transformed and parental strains in manure microcosms experiments by using the MPN PCR *stx* method. Moreover, these last authors also showed the stability of the GFP plasmid under selective and non-selective conditions. Prior to the challenge testing, two green fluorescent colonies corresponding to each STEC transformed strain were individually grown in BHI at 37°C in order to reach late exponential growth. After 24 hours, the two cultures had yielded a density around 10^8 CFU.ml⁻¹ and these precultures were used for the inoculation of bovine faecal samples.

2.2. Microbiological culture media

In different stages of the study, various microbiological culture media were used. Tryptone Medium (Fluka-Biochemika, Switzerland) was used for the preparation of serial dilutions throughout the experiment. Tryptone Soya Broth (TSB, bioMérieux, Marcy-l'Etoile, France) and *E. coli* Broth (EC, Fluka-Biochemika, Switzerland) were tested with the addition of 20 mg.l⁻¹ novobiocin (TSB.N+, EC.N+) (Sigma, Steinheim, Germany) or without novobiocin (TSB.N-, EC.N-), for the study of the enrichment protocols. PCA (Plate Count Agar) was used as a non-selective medium for the enumeration of the feces background

microflora (BM). BHI agar with ampicillin (BA-A) ($150 \mu\text{g}\cdot\text{ml}^{-1}$) was used as a selective medium for the enumeration of STEC transformed strains visualized by the appearance under a UV light.

120 2.3. *Feces sample inoculation*

Bovine faecal samples were collected from a dairy farm located in the southeast of France. These samples were mixed together and 25 gram portions of feces were aseptically weighed and deposited in 16 flasks and stored at 4°C for less than 24h before the experiments. The precultures of the two STEC transformed strains were serially diluted to reach a final
125 concentration of $3 \log_{10}$ (CFU ml^{-1}) in fresh Tryptone Medium. One milliliter of each dilution was then individually inoculated into a series of eight different flasks containing the 25 g portions of feces. After manual homogenization, the 16 flasks (8 for each strain) were stored overnight at 4°C . The content of these 16 flasks was transferred into a stomacher bag in which 225 ml of TSB.N-, or TSB.N+, or EC.N-, or EC.N+ were added according to the growth
130 enrichment protocol. The temperature of each broth was around 25°C . Hence, a final concentration of roughly 4 CFU of STEC per milliliter was obtained for each stomacher bag.

2.4. *Enrichment protocols*

Growth experiments were carried out with the two STEC strains in the presence of BM for different enrichment protocols, which were the result of the combination of two
135 temperatures (37°C or 40°C), two basal media (TSB, EC) and the presence or absence of novobiocin (N+, N-). For this purpose, the 16 samples obtained at the end of the bovine faecal sample inoculation were stomached for 1 min and left at room temperature for a 40 min regeneration step.

2.5. Growth monitoring

140 At each sampling time (every 2 hours for the 8 enrichment protocols), the number of viable cells of BM and STEC was determined by plating 1.0 ml of the appropriate dilutions of the samples onto PCA and 0.1ml onto BA-A. After incubation, the various PCA and BA-A plates were counted so as to provide the respective BM and STEC growth data for each enrichment protocol.

145 2.6. Modelling of individual BM and STEC growth kinetics

Two growth models were used to fit the observed growth kinetics of both BM and STEC for the eight protocols tested. The first model is a very simple one in two phases with three parameters (Mckellar and Lu, 2004) (model 1). It only describes the exponential and the stationary phases and does not take into account any lag phase:

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$$y(t) = \begin{cases} y_0 + \frac{\mu_{\max}}{\ln(10)} \times t & \text{if } t < t_{\max} \\ y_0 + \frac{\mu_{\max}}{\ln(10)} \times t_{\max} & \text{if } t \geq t_{\max} \end{cases} \quad (1)$$

where $y(t)$ is the bacterial density (\log_{10} (CFU ml⁻¹)) at time t (hours), y_0 is the initial bacterial density (\log_{10} (CFU ml⁻¹)), μ_{\max} is the maximum specific growth rate (hours⁻¹), and t_{\max} is the time at which the stationary phase begins, i.e. the time at which the maximum

155 bacterial density is reached.

The second model that has four parameters, incorporates a third phase, a lag phase of duration λ (hours) (model 2). It has been described with different names and by various authors (McKellar and Lu, 2004). Curves described by this model show an abrupt transition (breakpoint) between the lag and the exponential phases:

$$160 \quad y(t) = \begin{cases} y_0 & \text{if } t < \lambda \\ y_0 + \left(\frac{\mu_{\max}}{\ln_{10}} \right) \times (t - \lambda) & \text{if } \lambda \leq t < t_{\max} \\ y_0 + \left(\frac{\mu_{\max}}{\ln_{10}} \right) \times (t_{\max} - \lambda) & \text{if } t \geq t_{\max} \end{cases} \quad (2)$$

2.7. Global modeling of simultaneous growth of BM and STEC

A global model (model 3) for the simultaneous growth of BM and STEC is simply defined as using model 1 (without lag phase) for STEC flora along with model 2 (with lag phase) for the background flora. Model 3 is defined by seven parameters ($y_{0\text{STEC}}$, $\mu_{\max\text{STEC}}$, $t_{\max\text{STEC}}$, $y_{0\text{BM}}$, λ_{BM} , $\mu_{\max\text{BM}}$, $t_{\max\text{BM}}$) which may be estimated by two individual fittings of model 1 and model 2 on STEC and BM growth kinetics, or by a global fitting of model 3. A partial model nested in model 3 is also defined in the same way, but assumes a common value of t_{\max} for the two floras (model 4). This final model has only six parameters

170 ($y_{0\text{STEC}}$, $\mu_{\max\text{STEC}}$, $y_{0\text{BM}}$, λ_{BM} , $\mu_{\max\text{BM}}$, t_{\max}) and is based on the hypothesis according to which a potential competition exists between the two floras studied. More precisely, in model 4, the growth of each microflora is assumed to stop as soon as one microflora has reached its maximum density in the enrichment basal medium. This phenomenon has already been described and is commonly referred to as the Jameson effect (Gimenez and Dalgaard, 2004).

175 2.8. Statistical methods

Fits of models to the BM and STEC data were performed by nonlinear regression (Bates and Watts, 1998) by using the least-squares criterion with the function nls of the R-Software (Ihaka and Gentleman, 1996). Comparisons of nested models (model 2 nested in model 1 and model 4 nested in model 3) were performed using an F test (Bates and Watts,

180 1998).

3. Results

3.1. Individual fits of BM and STEC growth kinetics

Figure 1 demonstrates that Strain 2 did not grow in media in which novobiocin was added (N+). For the other enrichment protocols, the growth of the STEC strains was not inhibited. The STEC growth kinetics seem to be generally characterized by only two phases; an exponential phase followed by a stationary phase. When the two strains grow, model 1 was used to globally fit the data obtained for the two strains. The choice of a null lag phase for STEC data was justified by the fact that no significant difference was found between model 1 (model in two phases) and model 2 (model in three phases) in terms of the residual sum of squares (RSS) for each protocol ($p_{F \text{ test}} > 0.05$).

A similar growth kinetic was obtained with all the enrichment protocols for the background microflora growth. It is characterized by three phases, i.e., a lag phase followed by an exponential and a stationary phase. In all cases, a significantly better fit of the BM growth data was obtained by model 2 than by model 1 ($10^{-5} < p_{F \text{ test}} < 0.03$).

3.2. Global fits of simultaneous growth of BM and STEC

Figure 1 shows that for each of the eight protocols studied, the parameters $t_{\max \text{ STEC}}$ and $t_{\max \text{ BM}}$ seem to be very close. Hence, BM and STEC data were globally fitted by model 4 with a common t_{\max} for the two microfloras. No significant difference was found between model 3 (full model) and model 4 (partial model) in terms of RSS for all the protocols ($p_{F \text{ test}} > 0.05$), except for one protocol (EC.N-.37, $p_{F \text{ test}} = 0.01$). Figure 1 also shows that growth data are well described by model 4 pointing out that the STEC strain growth is stopped as soon as BM reaches its maximal level. Moreover, it is worth noting that this BM maximal level is almost constant from one protocol to another (8.13 to 8.37 $\log_{10}(\text{CFU ml}^{-1})$).

3.3. Impact of enrichment factors on BM and STEC growth

205 Growth inhibition of Strain 2 is observed when novobiocin was added into the enrichment basal media (TSB or EC) which would imply a potential susceptibility of Strain 2 to novobiocin (Fig. 1. and Table 1). For the other protocols, growth of the STEC strains is not inhibited and the STEC initial level (y_{0STEC}) is not significantly influenced by the enrichment protocol tested where the values vary from 0.13 to 0.71 \log_{10} (CFU ml^{-1}). On the contrary, the
210 BM initial level (y_{0BM}) appears to decrease by roughly one \log_{10} for the protocols with EC (from 6.15 to 6.41 \log_{10} (CFU ml^{-1})), compared to those with TSB (from 6.95 to 7.22 \log_{10} (CFU ml^{-1})). Furthermore, the BM lag time (λ_{BM}) values seem to increase when novobiocin is added in the basal medium especially for protocols with TSB (Fig. 1). Finally, t_{\max} values are higher for the protocols in which selective ingredients (novobiocin and bile salts) are used
215 compared to those using no selective ingredients at 37°C or 40°C (Fig. 1 and Table 2).

3.4. Impact of enrichment factors on the maximum STEC level

It is worth recalling that novobiocin inhibited the growth of Strain 2. However for Strain 1, the addition of novobiocin creates a delay in the onset of the Jameson effect (t_{\max} values are higher for protocols with novobiocin than for protocols without novobiocin). This
220 would allow for a prolonged growth of Strain 1 and thus higher $y_{\max STEC}$ values regardless of broth and temperature tested (4.08 to 4.96 \log_{10} (CFU ml^{-1})) (Table 1). In the same way, bile salts (present in EC) also delay the onset of the Jameson effect (with an increase of t_{\max} of more than 2 hours in the absence of novobiocin), which implies $y_{\max STEC}$ values higher for protocols with EC than for protocols with TSB at 37°C or 40°C. Furthermore when
225 novobiocin is added, the temperature has an impact on the $y_{\max STEC}$ values regardless of the basal medium. The $y_{\max STEC}$ values are higher by roughly one \log_{10} for the protocols in which 37°C is used than for those in which 40°C is used.

4. Discussion

Our results demonstrate a simple competitive interaction between non-O157 STEC
230 and the prevailing background microflora during the enrichment step of faecal samples.
Indeed, STEC growth is stopped as soon as BM reaches its maximal level ($\sim 8 \log_{10}$ (CFU
ml⁻¹)), which is fairly consistent regardless of the enrichment protocol used.

Natural compound present in feces might also inhibit the growth of STEC. However we
hypothesize that their effects is certainly negligible due to their low concentration in the
235 enrichment broth (i.e. 25g in 225ml).

This present study also shows that the enrichment protocol factors have an impact on the
simultaneous growth of BM and STEC strains. More precisely, the protocols with novobiocin
and/or EC increased the t_{\max} values, which consequently yielded higher final levels of STEC
obtained after 24h of enrichment (Fig. 1). Indeed, bile salts included in EC broth delay the
240 onset of the Jameson effect by extending λ_{BM} , and thus permitted a longer growth for STEC.
Nevertheless, the action of novobiocin initially directed against background microflora, could
also disadvantage the STEC flora, and could then lead to false negative results. Indeed, our
study displayed no detected growth of Strain 2 for the enrichment protocols in which
novobiocin was added (N+). An additional experiment was performed in order to confirm the
245 potential susceptibility of Strain 2 to novobiocin. The transformed Strain 2 and its parental
strain were cultured on Muller-Hinton broth containing 20 mg.ml⁻¹ of novobiocin and
incubated for 24h at 37°C. No growth of Strain 2 (both the transformed and the non-
transformed strains) was observed. This strongly suggests that the non-O157 Strain 2 is
susceptible to novobiocin (at 20mg.l⁻¹). This result is in concordance with those reported by
250 Uemura et al. (2003) who highlighted Minimum Inhibitory Concentrations (MIC) varying
from 3.13 to >100 mg.l⁻¹ for 57 strains of STEC tested. Moreover, Foster et al. (2003)
demonstrated the superiority of an enrichment protocol without antibiotics for the isolation of

E. coli O157:H7 from bovine feces. The addition of novobiocin in enrichment broths appears to be inadequate for the recovery of STEC.

255 The final level of STEC ($y_{\max STEC}$) obtained at the end of the enrichment step, is obviously expected to be higher than the detection threshold of the screening method used for the STEC detection. It is worth noting that no method is currently validated for detecting non O157 STEC in faecal samples. The immunological methods (ELISA, RIA, immunochromatography, and immunomagnetic separation (IMS)) often combined with
260 detection methods, and the genetic methods (like Polymerase Chain Reaction (PCR)) are the main methods used for the detection of STEC. With no prior enrichment step, a minimum concentration of 3.5 to 4.4 \log_{10} (CFU of STEC g^{-1} of sample) is required to allow the detection of the bacteria after the immunomagnetic separation combined with detection methods (IMS-DM) (Gehring et al., 2004). The PCR detection threshold with no prior
265 enrichment step reported in the literature, is 3 \log_{10} (CFU g^{-1}) (Cui et al., 2003). Figure 1 shows that the protocols with TSB and EC at 37°C or 40°C without novobiocin, allowed STEC levels (after 24h of incubation) to reach the PCR and the IMS detection thresholds. Moreover, it must be emphasized that $y_{\max STEC}$ obviously depends on the initial STEC level in feces ($y_{0 STEC}$). The initial STEC inoculum (0.6 \log_{10} (CFU ml^{-1})) is relatively high in our
270 study because of experimental conditions. Yet, Fukushima et al. (2004) showed that the majority of cattle infected with STEC were low-carriers that contained STEC at a concentration less than 2 \log_{10} (CFU g^{-1}). Hence, for a STEC concentration in feces of 1 \log_{10} (CFU g^{-1}), and by taking into account the 10-fold dilution carried out at the beginning of the enrichment protocol (i.e., 25g of sample transferred into 225 ml of basal media), this
275 corresponds to a $y_{0 STEC}$ value around 1 CFU ml^{-1} . Predictions made by model 4 with this $y_{0 STEC}$ realistic value give final STEC levels from 2.3 to 2.4 \log_{10} (CFU ml^{-1}) for TSB without

novobiocin at 37°C and 40°C after 24h of incubation. It is worth noting that the same levels are predicted after 6 hours (the common enrichment duration in the IMS-DM detection protocol of non-O157 STEC and *E. coli* O157:H7 in food), since the t_{\max} values are lower than 5 hours. When the initial level of contamination by STEC is low, the predictions show that the $y_{\max STEC}$ values obtained after 6 and 24h of enrichment, would be lower than the PCR and IMS-DM detection thresholds. In the same way, predictions made by model 4 with an $y_{0 STEC}$ value around 1 CFU ml⁻¹ for EC without novobiocin at 37°C and 40°C after a 6h enrichment step, give $y_{\max STEC}$ values (from 2.6 to 2.9 log₁₀ (CFU ml⁻¹)) lower than the IMS-DM detection threshold. However given higher t_{\max} values for the protocols with EC, $y_{\max STEC}$ values after 24h of incubation (from 3.2 to 3.4 log₁₀ (CFU ml⁻¹)) would reach the PCR detection threshold and get closer to the IMS-DM detection threshold.

Given the high BM level observed in our fecal bovine samples, the t_{\max} values for protocols with TSB.N- (37 and 40°C) are therefore low (< 5 hours). In this case, it is not useful to increase the time beyond 5-6 hours and predictions showed that false negative results could be obtained when the STEC level of contamination is low. However, the predictions highlight that the protocols with EC.N- seem to be more effective than the protocols with TSB.N- for the enrichment of low STEC contamination levels because the t_{\max} values for EC.N- are higher (> 2.5 hours) than those for TSB.N- (Table 2).

In several studies fecal samples have been used to report prevalence of STEC in cattle. The percentage of STEC in cattle feces ranged from 6% in an American study (Cray et al., 1996) to 71% in a French study (Pradel et al., 2000). The difference of prevalence between countries could be due to different reasons (e.g. different samples sizes, various detection methods...), some of which were discussed in the present study. Indeed, we have demonstrated that the

300 current enrichment protocols are sometimes inadequate. In addition, some use novobiocin that
may inhibit the growth of the target STEC strain, and other protocols may be insufficient to
enable a low initial level of the target strain to reach detection threshold because of the
competition with background microflora. Yet, in winter low levels of STEC are excreted
(Besser et al., 1997) and potential underestimation of the STEC percentage in cattle could
305 certainly be made.

It will be necessary to perform additional experiments with more bovine faecal samples (from
different cattle), various STEC serotypes and STEC stressed cells in order to confirm the
present results and especially the effectiveness of enrichment protocols with EC broth.

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425 **FIGURE CAPTIONS**

Fig. 1. Simultaneous growth kinetics of the two non-O157 STEC transformed strains and background microflora obtained for the eight protocols and theoretical curves obtained by fitting of model 4. ○, Strain 1; Δ, Strain 2. The grey color (dots and curves) represents background flora data sets and the black color represents STEC data sets. The t_{\max} values are represented by the vertical dotted lines.

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Table 1 The maximum concentration of STEC strains 1 and 2 after each enrichment protocol, calculated as the average of the bacterial counts measured after 12h

	Protocols	Strain 1	Strain 2
435	TSB.N-.40	2.97	3.03
	TSB.N-.37	2.90	3.19
	TSB.N+.40	4.08	ND [†]
440	TSB.N+.37	4.96	ND
	EC.N-.40	3 [*]	4.16
	EC.N-.37	3.69	3.39
	EC.N+.40	4.27	ND
	EC.N+.37	4.96	ND

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* value obtained at 24h (not calculated as an average, observations lacking for experimental reasons)

[†] Not Detected (< 4 CFU.ml⁻¹)

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Table 2 t_{\max} obtained for each enrichment protocol

Protocols	t_{\max} (h)	95% confidence intervals of the t_{\max}
TSB.N-.40	4.18	3.86-4.51
TSB.N-.37	4.93	4.46-5.39
TSB.N+.40	8.1	7.68-8.52
TSB.N+.37	8.89	8.31-9.46
EC.N-.40	6.95	6.06-7.84
EC.N-.37	7.34	6.67-8.01
EC.N+.40	8.83	8.11-9.56
EC.N+.37	10.16	9.62-10.69

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