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Quantification of live bacteria by quantitative EMA-PCR

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Introduction: Quantitative PCR is a powerful tool for studying population dynamics of bacteria in complex samples, e.g. the population dynamics of starter cultures in cheese ripening. Unfortunately DNA of killed bacteria is stable for weeks in biological samples, compromizing the quality of the qPCR based data. A method for distinguishing live and dead Gram negative bacteria using ethidium monoazide (EMA) has previously been published (Rudi et al 2004). In the present work we demonstrate the use of this method for the Gram positive bacterium *Lactobacillus curvatus*

Discussion:
EMA treatment of the gram positive bacterium *L. curvatus* differentially inactivating DNA from dead bacteria as compared to live bacteria. The signal qPCR signal from dead bacteria is reduced by approximately 8 Ct or 99% more than the signal from live bacteria. EMA treatment of samples will dramatically improve the quality of qPCR derived data in studying population dynamics of complex biological samples.

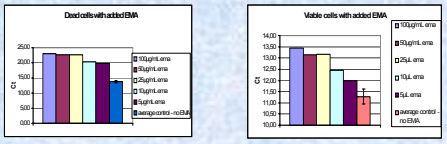

Figure1: The effect of EMA concentration on blocking of DNA from EMA treated samples of live and dead bacteria respectively Maximum effect of EMA treatment is achieved with 25µM of EMA, which was chosen as standard condition. At this condition the signal from live bacteria was inhibited 1,9 Ct or approximately 70%, whereas the signal from dead bacteria was inhibited 9,5 Ct or approximately 99,8%

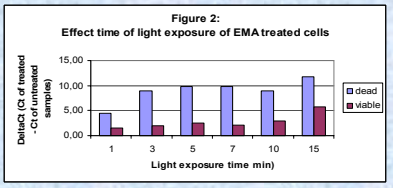

Figure 2: The effect of time of light exposure of EMA treated samples.
Maximum effect of light treatment is achieved after 5 minutes of light treatment, which rthen was choosed as standard condition

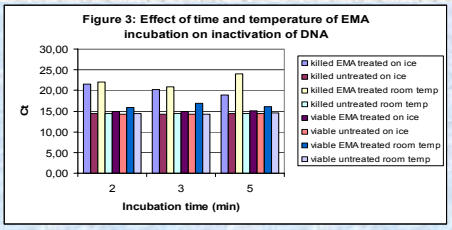

Figure 3: The effect of time of incubation time and incubation temperature during EMA treatment of samples.
Maximum effect of light treatment is achieved after 5 minutes of EMA incubation at room temperature, which then was choosed as standard condition.

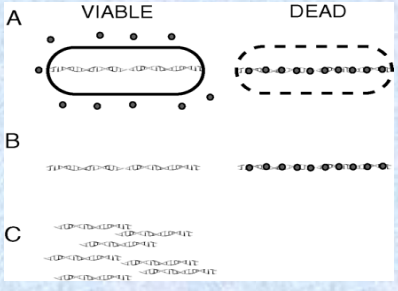

Figure5. Schematic representation of EMA-PCR. (A) EMA is added to the test sample containing both viable and dead cells. EMA penetrates the dead cells and binds to the DNA. Light exposure for 5 min leads to covalent binding and inactivation of free EMA. EMA does not enter viable cells. (B) There are two populations of DNA after purification. The DNA population from viable cells is unstained, while the DNA from the dead cells is covalently bound to EMA. (C). The unstained DNA from viable cells is PCR amplified, while the DNA from dead cells with bound EMA cannot be amplified. (Rudi et al. 1994)

Materials & Methods

Preparation of EMA-stock solution.
5mg EMA was dissolved in 1 mL sterilized H_2O. The solution was kept in freezer at –18ºC.

EMA treatment of samples:
Exponentially growing *L. curvatus* or ethanol killed *L. curvatus* was added ethidium monoacide as indicated. After incubation on ice or at room temperature, samples were exposed to a 150 W halogen bulb light source. The light exposure were performed on ice to avoid excessive heating of samples

Sample preparation
Frozen ON-culture (kept in glycerol) was used. To ensure that the bacteria growth was in the log phase, 10mL MRS bouillon was inoculated with the above culture, and incubated anaerobically at 30ºC for two hours. The culture was mixed by vortexing and aliquoted.

Killing of bacterial cells: Was performed by adding of ethanol to a final concentration of 70% for ten minutes

Primerdesign: Was performed using primer 3. Primerkoncentration was optimised by titration.
Forward primer: Lb F LBF 100pmol/µL 5'-ATG GAA GAA CAC CAG TGG CG
Revers primer: Lb F LBF 100pmol/µL 5'-ATG GAA GAA CAC CAG TGG CG

PCR running conditions:
The PCR was performed on an Applied Biosystems SDS 7500 system, using Power SYBR Green (Applied Biosystems). The cycling conditions was a two-step PCR protocol with 40 cycles of annealing/elongation at 60° C and denturation at 95° C.

References
1) Use of Ethidium Monoazide and PCR in Combination for Quantification of Viable and Dead Cells in Complex Samples. Knut Rudi, Birgitte Moen, Signe Marie Drømtorp, and Askild L. Holck.
Applied and Environmental Microbiology, Vol. 71, No. 2, 2005, p.1018-1024