GERMAN FEDERAL INSTITUTE FOR RISK ASSESSMENT

BfR
Bundesinstitut für Risikobewertung

Preparation of quantitative *Campylobacter* reference material for use in proficiency tests and for performance testing of culture media

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The need for reference material

- traceability of results to a recognised reference
- to ensure that results are reliable, accurate and comparable
- even if obtained in different laboratories

- **method validation**
- **determination of measurement uncertainty**
- **calibration**

- **quality control**
  e. g. performance testing of culture media according to ISO 11133:2014

  - applies to microbiological laboratories producing culture media for their own use
  - initial culture with known quantity is needed: e. g. ~ 10^2 cfu / plate (quantitative use, solid media)
  - "ready to use" culture is convenient

- **proficiency tests**
  - verification of performance in quantitative detection

Commercial availability of *Campylobacter* reference material

- Only 2 providers of quantitative *Campylobacter* reference material:

  1) National Food Agency (Sweden): lyophilisates

  → not suitable for all matrices (e. g. raw milk, caecal content…)

  2) Biosisto (previously CHEK; The Netherlands): cryo cultures

  → stated concentration could not be verified by us (2017)

- Resulting consequence for our proficiency test in 2017 (caecal content, chicken):
  - preparation of own quantitative reference material (cryo cultures)
  - mail goal: stress-resistant cells
  - strains used: *C. jejuni* WDCM 00005, *C. coli* WDCM 00004, *C. lari* DSM 11375, (*Arcobacter butzleri* DSM 8739)

Protocol

storage culture (-80°C) → Columbia blood agar
24-48 h; 37°C; in microaerobic incubator (5% O_2, 10% CO_2 and 85% N_2)

Subculture → Columbia blood agar
for 18 ± 2 h under same conditions

- inoculation of Brain Heart Infusion with low OD
- incubation in a shaking incubator (37°C)
- growth until early stationary phase
- adding requested concentration to ice-cold cryo medium
- filling the vials under constant gentle stirring of the cryo culture
- shock freezing in liquid nitrogen

Protocol II

Critical points:

- regrowth of storage culture from -80°C stock should be feasible during 24 h
- cultivation medium for *C. lari*: Bolton broth instead of BHI
- prewarming of cultivation medium, anaerobic jar and shaking incubator
- growth at 37°C
- composition of the media for cultivation and freezing
- avoiding oxygen stress and temperature changes
- cultures should stay on ice for at least 2 h before freezing
- a reduction of ~ 1 log CFU / ml due to freezing should be considered

Protocol III

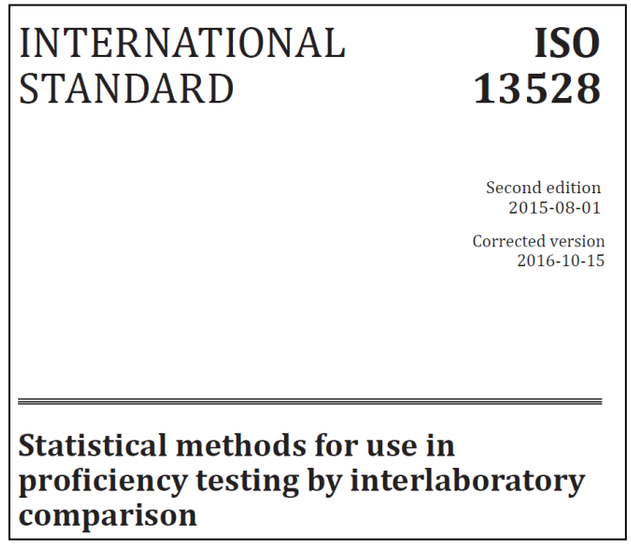
In order not to impair cell growth we estimated the time of incubation:

	C. jejuni	*C. coli*	*C. lari*
no. of doublings	3.47 ± 0,24	4.48 ± 0.36	3.20 ± 0,07
generation time	1.9 ± 0.27 hours	1.3 ± 0.06 hours	2.2 hours ± 0.16
time of incubation	6.6 ± 0.54 hours	6.1 ± 0.16 hours	7.0 ± 0.60 hours

→ determination of the cell concentration at OD_{600} = 0.2 (1 cm cuvettes):

log 8.75 ± 0.31 CFU / ml

Homogeneity test: procedure


INTERNATIONAL STANDARD

ISO 13528

Second edition 2015-08-01

Corrected version 2016-10-15

Statistical methods for use in proficiency testing by interlaboratory comparison

- Performed according to ISO 13528:2015: Annex B

- *Select a number g of proficiency test items […], where g ≥ 10*

  - the vials were numbered according to the order of filling

  - the 10 vials were chosen along the whole filling line to assure quality during the entire procedure

- *Prepare m ≥ 2 test portions from each proficiency test item*

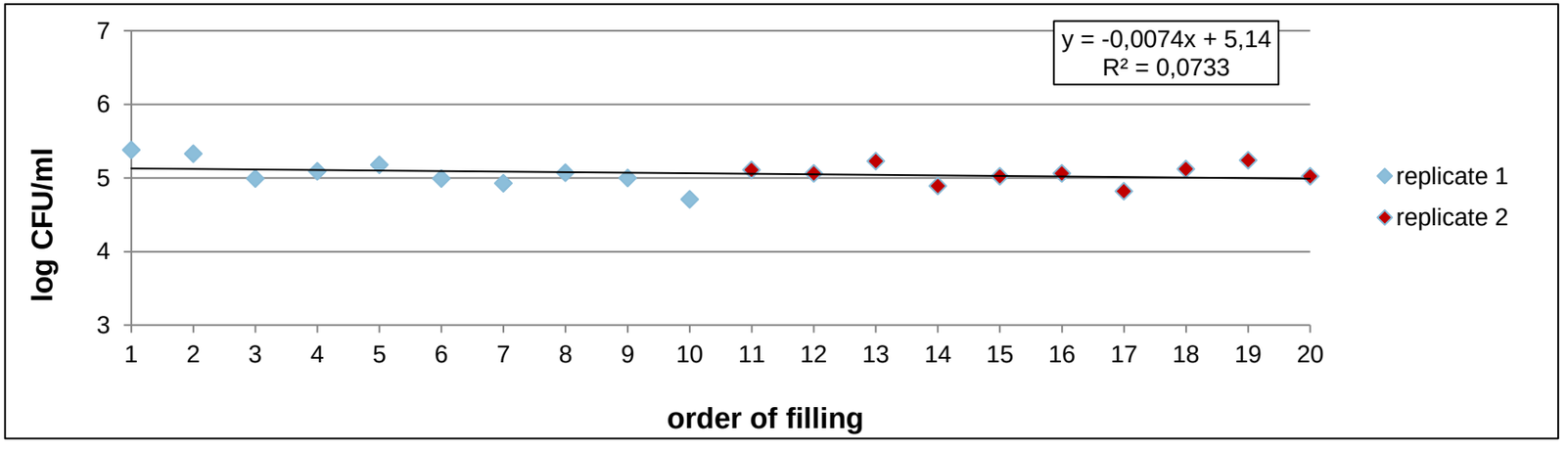
  - performing the procedure for the first replica ; discarding the dilution series

  - same procedure for the second replica (new dilution series)

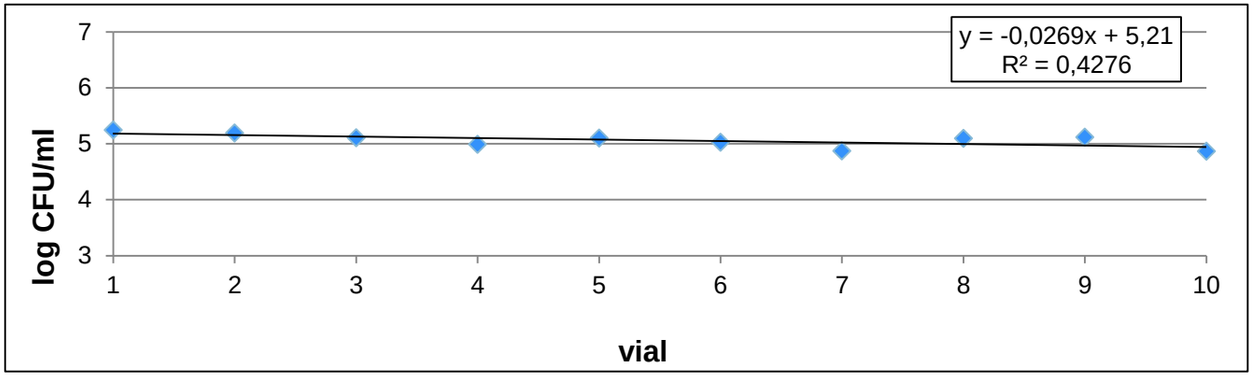
Homogeneity test: assessment criteria

→ 3 checks should be used to assure that the homogeneity test data are valid for analysis

1) Examine the results for each test portion in order of measurement to look for a trend



2) Examine the results for proficiency test item averages by production order for a trend



3) Compare the differences between replicates and […] test for statistically significant difference between replicates

Homogeneity test: analysis

- compare the between-sample standard deviation s_s with the standard deviation for proficiency assessment σ_{pt}
  - if σ_{pt} is not known in advance one option is to check for statistically significant differences using a one-way analysis of variance (ANOVA)

- we considered the batch as homogeneous when following assessment criteria are met:
  - F-value < critical F-value (the result is significant at 0.05 significance level)
  - p-value > 0.05 (the differences of the means are not statistically significant)

Anova: single factor variance analysis (cfu/ml)

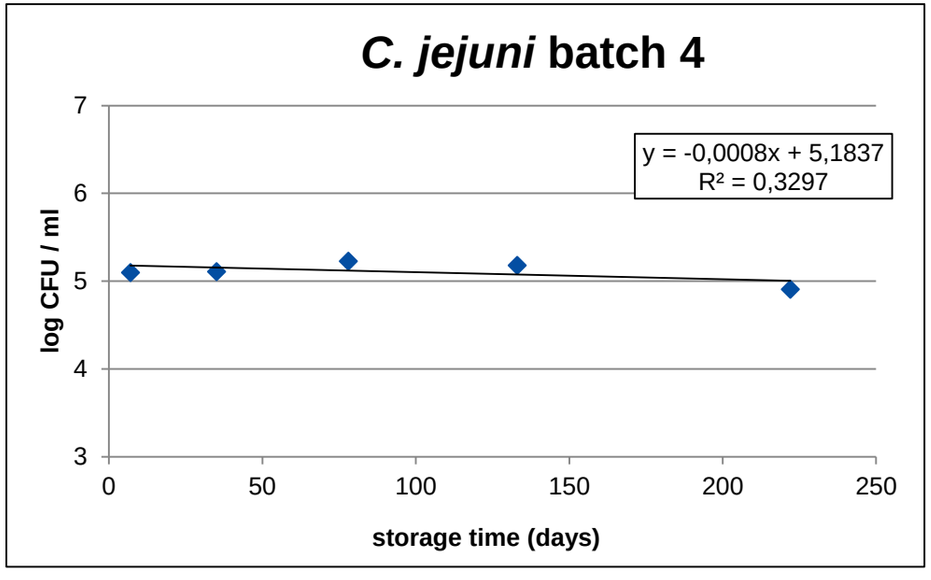
Summary

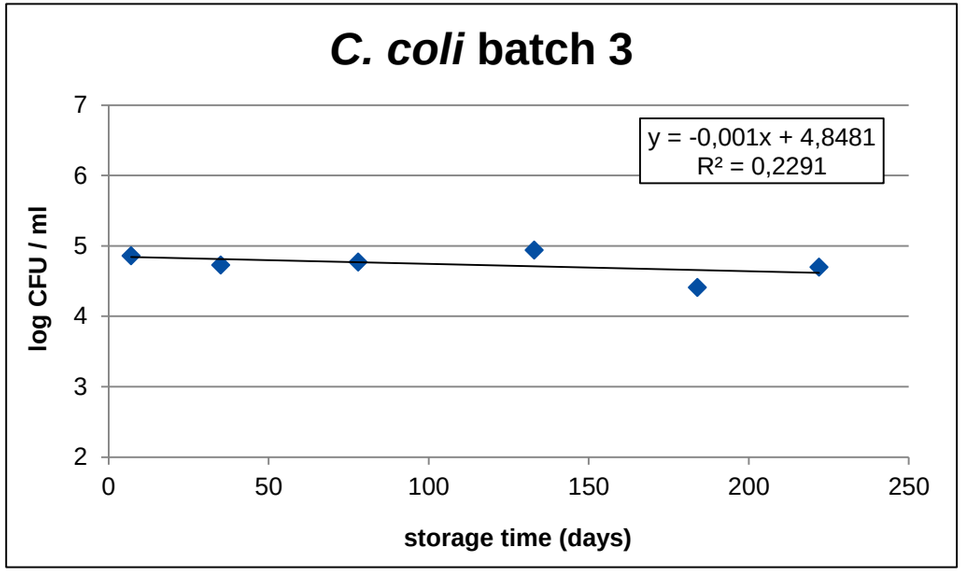
Groups	*Count*	*Sum*	*Average*	*Variance*
Cryo 3	2	9,95	4,975	0,14045
Cryo 4	2	9,87	4,935	0,02645
Cryo 27	2	9,87	4,935	5E-05
Cryo 28	2	9,97	4,985	0,00845
Cryo 52	2	10,05	5,025	0,00125
Cryo 53	2	10,23	5,115	0,01125
Cryo 77	2	9,9	4,95	0,045
Cryo 78	2	9,91	4,955	0,10125
Cryo 97	2	9,97	4,985	0,00045
Cryo 98	2	9,86	4,93	0,0338

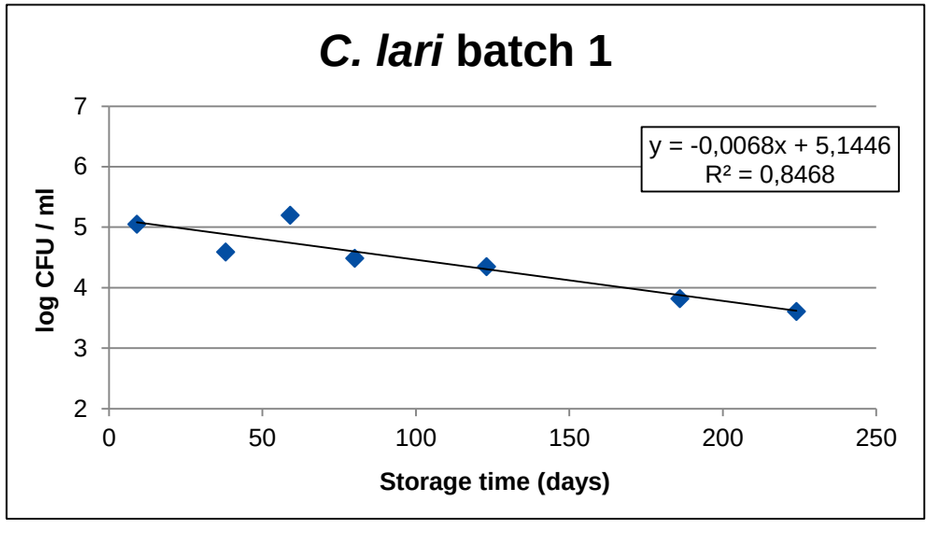
ANOVA

Source of variation	*Square sums (SS)*	*degrees of freedom*	*Mean square sums (MS)*	*F-value*	*p -value*	*F crit*
Between groups	0,05678	9	0,006308889	0,171251056	0,993030424	3,020382947
Within Groups	0,3684	10	0,03684			
Total	0,42518	19				
				F < F crit	p > 0,05	

Stability



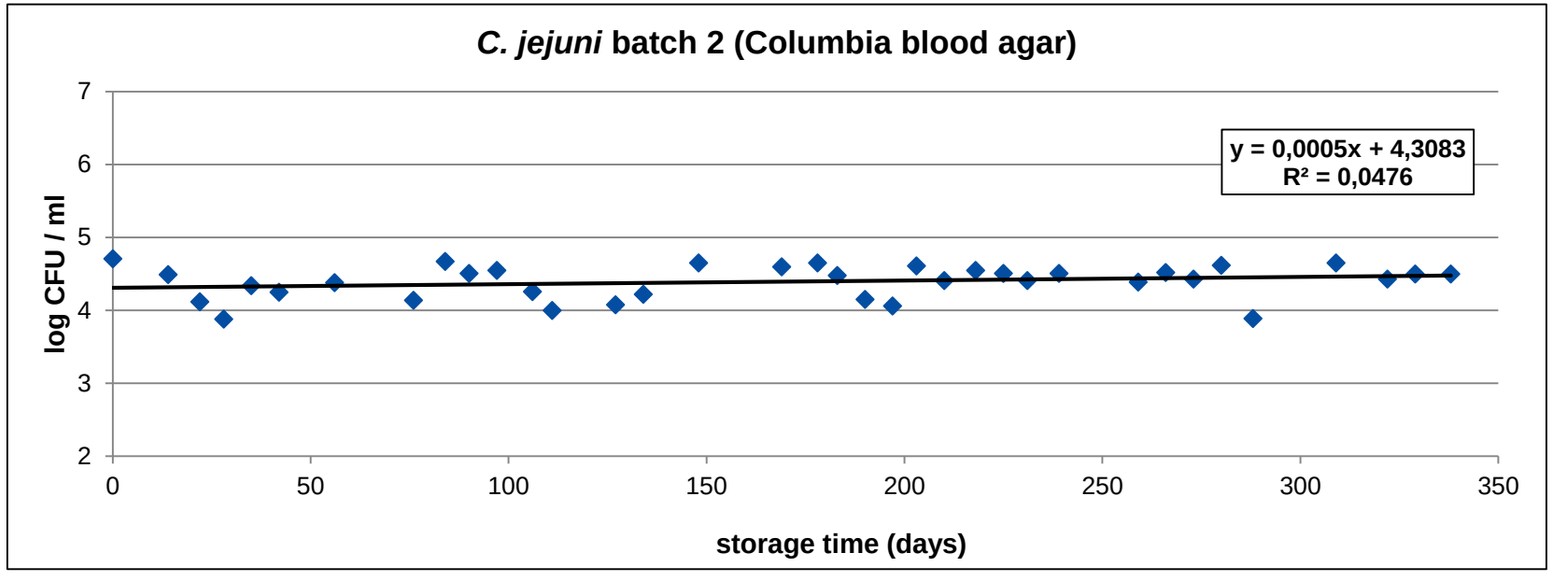




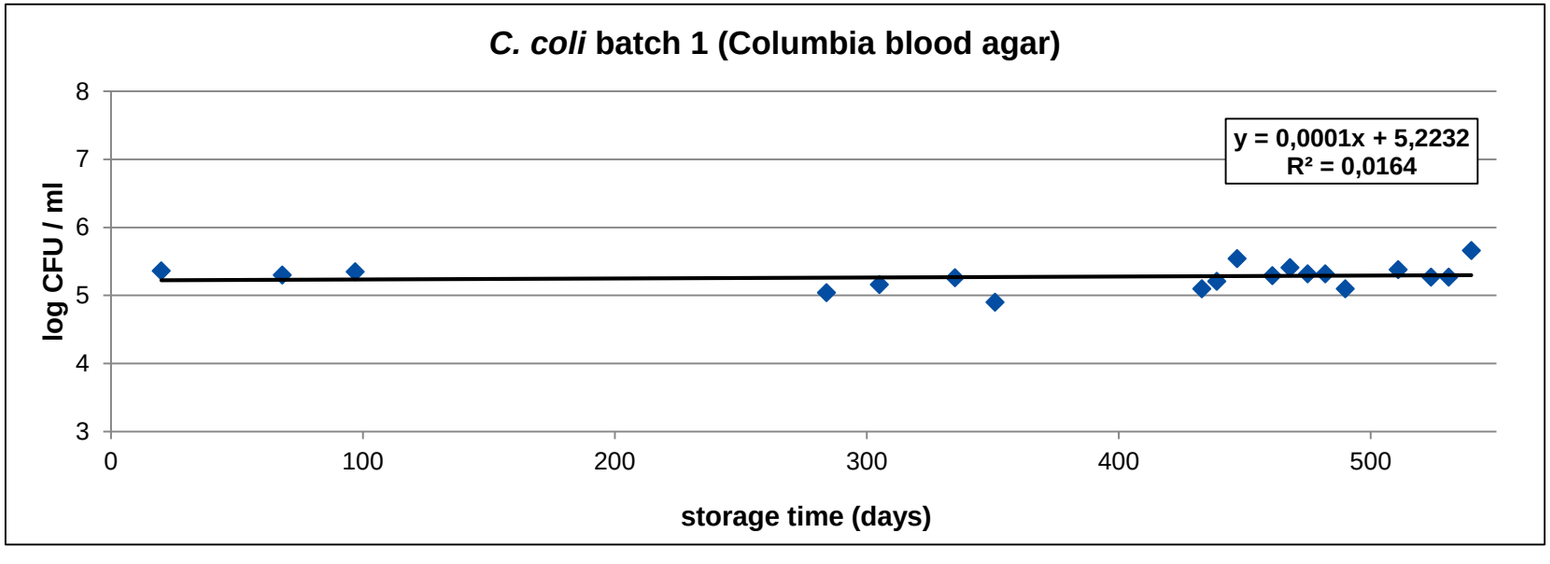
- *Select a number of 2g of the proficiency test items at random, where g ≥ 2*
- all *C. jejuni* and *C. coli* batches appear stable
- *C. lari* batch shows only short-time stability
  → ~ 1.5 log reduction within 224 days

Long-term stability

- data generated during performance testing of culture media



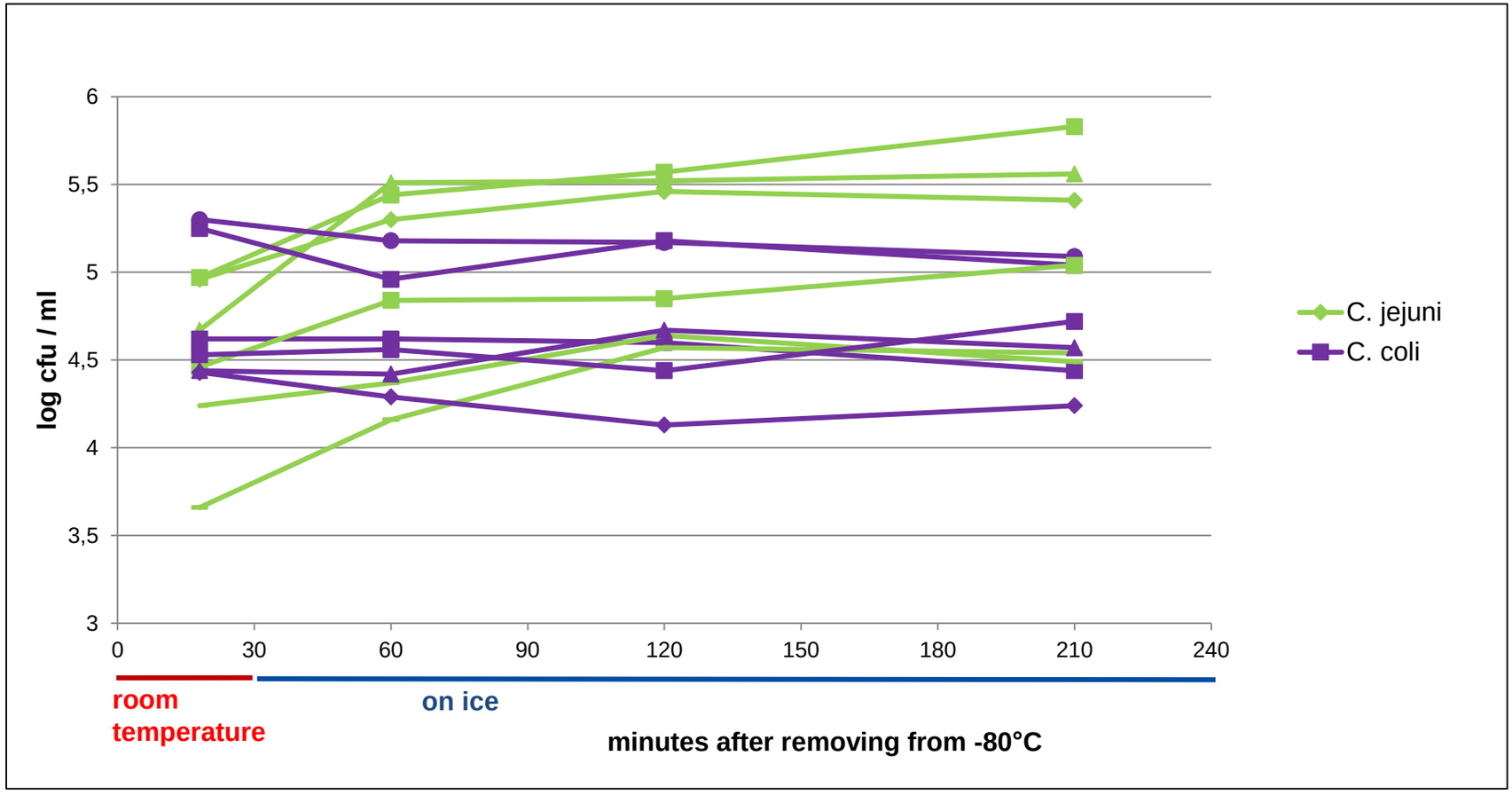
stable for min. 338 days



stable for min. 540 days

Stability of thawed cultures on ice

- determination of stability during storage on ice
- *C. jejuni* batches need longer recovery time than *C. coli* after thawing



procedure before use:

- thawing for 30 min at room temperature
- incubation for another 30 min on ice

Use in proficiency tests

	2017 (caecal content, chicken)				
sample	1	3	4	6	7
species	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*
expected (log CFU / g)	5.23	6.04	7.79	6.04	7.79
median	5.21	6.49	7.52	6.25	7.46
SD_{MAD}	0.47	0.38	0.38	0.43	0.56

	2018 (chicken breast meat)				
sample	1	3	6	7	8
species	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*
expected (log CFU / g)	3.00	2.41	3.84	3.99	3.84
median	2.87	2.05	3.82	3.68	3.51
SD_{MAD}	0.50	0.60	0.41	0.40	0.43

EURL-PT 15 (chicken meat):
lyophilisates
median: 3.13 – 4.62
SD_{MAD}: 0.33 – 0.80

BfR-LVU 2015 (chicken meat):
lyophilisates
Median: 0.70 – 5.17
SD_{MAD}: 0.30 – 0.61

Protocol implementation outside BfR

- one batch of *C. coli* reference material at Wageningen Bioveterinary Research (WBVR) by Conny van Solt and Miriam Koene
  → 2 inoculation levels planned: log 3 and log 5

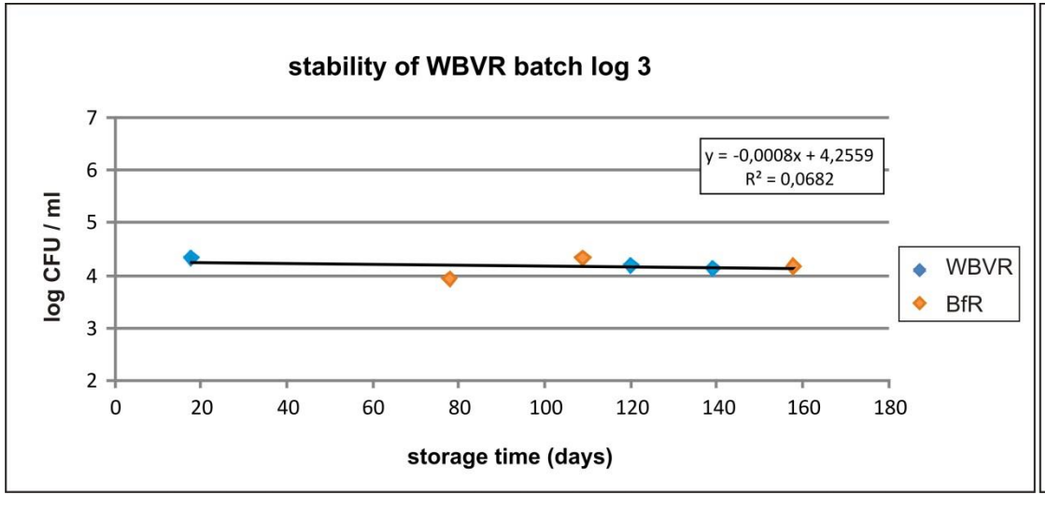
- slight modifications:
  - different composition of atmosphere: 6% O_2, 7.1% CO_2, 7.1% H_2 and 79.8% N_2 (Anoxomat)
  - quantification was performed on HIS agar plates

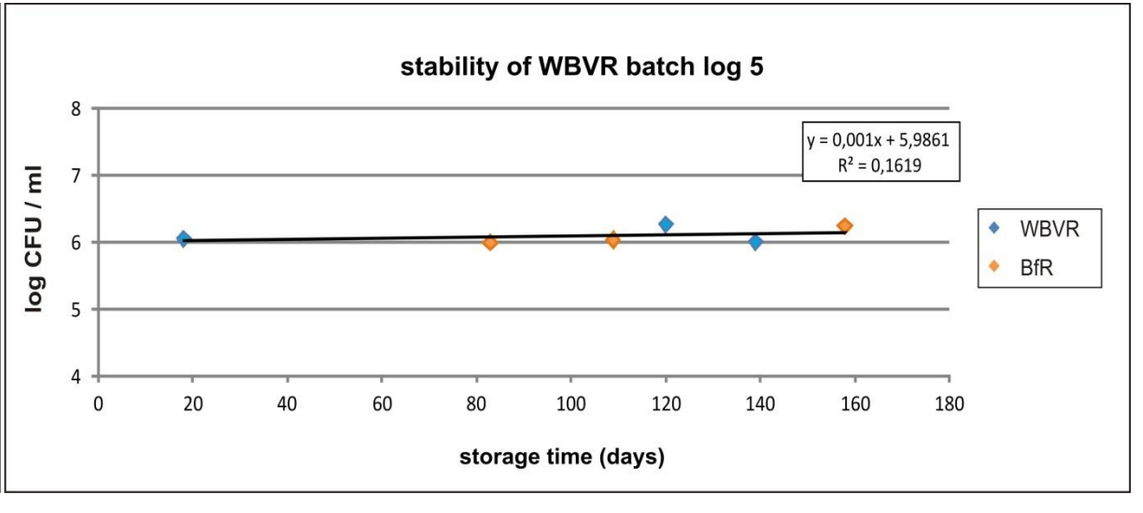
- confirmed level after freezing: log 4 and log 6

- slightly higher loss due to freezing: log 1.14 and log 1.53

- both levels are homogeneous and stable (158 days)

- no differences in results obtained at WBVR and BfR





Summary

- development of a simple and fast protocol for the production of quantitative *Campylobacter* reference material
- the reference material is homogeneous and stable for up to 1.5 years (tested so far)
  → exception: *C. lari*
- after thawing, the vials should be incubated for another 30 min on ice before use
- the reference material is sucessfully used in performance testing of culture media and proficiency tests at BfR
- the protocol works in two different labs with different atmospheres used for cultivation
- publication is in progress, so the protocol will be available for everybody soon

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