EURL-*Campylobacter*

Study ‘Detection of *Campylobacter* in raw milk’

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EURL-*Campylobacter* Workshop 2021

Background

- Pasteurization - effective way to improve milk safety.
- But there is an increased demand for raw milk (unpasteurized) from cows, and also an increase of suppliers (sold on the internet, hand-to-hand at farms/local markets or through milk-filling stations).
- In-line milk filters, the bulk tank milk (BTM) or milk from milk-filling stations are all examples of samples analysed for presence of *Campylobacter* in raw milk.
- *Campylobacter* is difficult to detect in raw milk due to rapid decline of colony-forming units (CFU). Standard procedure for detection of *Campylobacter* (ISO 10272-1:2017):
  - ✓ Milk + selective enrichment broth 1:10 (Preston or Bolton)
  - ✓ Plating mCCDA (+ second selective isolation medium)

Viable but non-culturable (VBNC) state

- *Campylobacter* can outlast unfavorable environmental conditions in a metabolic inactive state - the viable but non-culturable (VBNC) state.
- Wulsten et al. 2020[1] showed that *Campylobacter* go into VBNC state in raw milk. Used a viability PCR (PMA[2]-qPCR) and compared results with results from enumeration by cultivation according to ISO 10272-2:2017.
  - CFU significantly decreased over time in a strain-dependent manner.
  - All three strains went into VBNC state.
  - A proportion of bacteria died over time, but bacteria from all three strains could be reactivated to grow on mCCDA at 37°C in an atmosphere with extremely low oxygen partial pressure (1% O_2 in combination with 3.5% H_2) balanced by N_2

[1]Wulsten, I., Galeev, A., and Stingl, K. Underestimated Survival of *Campylobacter* in Raw Milk Highlighted by Viability Real-Time PCR and Growth Recovery. Frontiers in Microbiology, 2020.
[2]Propodium monoacide

Milk and pH

- The pH in fresh raw milk is 6.5-6.7.

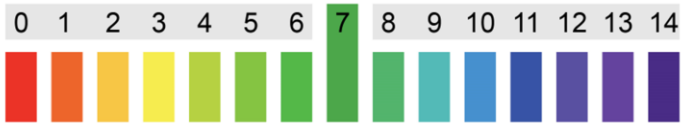
- The pH of milk decreaces over time (the milk goes sour). This occurs as bacteria in milk convert the sugar lactose into lactic acid.

- A lower pH:
  - ✓ Increases the activity of Lactoperoxidase, naturally present in milk.
  - ✓ Lactoperoxidase oxidases thiocyanate (often naturally present in milk – 65% of samples[3]) in presence of hydrogen peroxide (produced by lactic acid bacteria) - bactericidal effect. Used to preserve milk.
  - ✓ Limited effect in raw milk due to low levels of thiocyanate and hydrogen peroxide.

- One study[4] showed that an increase of pH (to 7.5) was beneficial for detection of *Campylobacter* in naturally contaminated raw milk (suggested due to inhibition of lactoperoxidase system).

[3]Yong et al. Investigation of concentration of thiocyanate ion in raw cow's milk from China, New Zealand and the Netherlands. Food Chem, 2017. [4]Beumer et al. The occurrence of *C. jejuni* in raw cow's milk. J App Bact, 1988.

Milk and pH



- According to international standards for microbiology of food pH should be adjusted at sampling or before analysis.
  - ✓ ISO 6887-5 [5] : to pH 7.0
  - ✓ Bacteriological Analytical Manual (BAM) chapter on *Campylobacter*: to pH 7.6

- EURL organised a survey for NRLs 2018: None of the 13 NRLs that analysed milk for *Campylobacter* adjusted the pH.
  - ✓ Also, a variation in choice of enrichment broth (Bolton or Preston broth)

[5]ISO 6887-5 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products).

The organisation of an EURL-NRL collaborative study in 2020

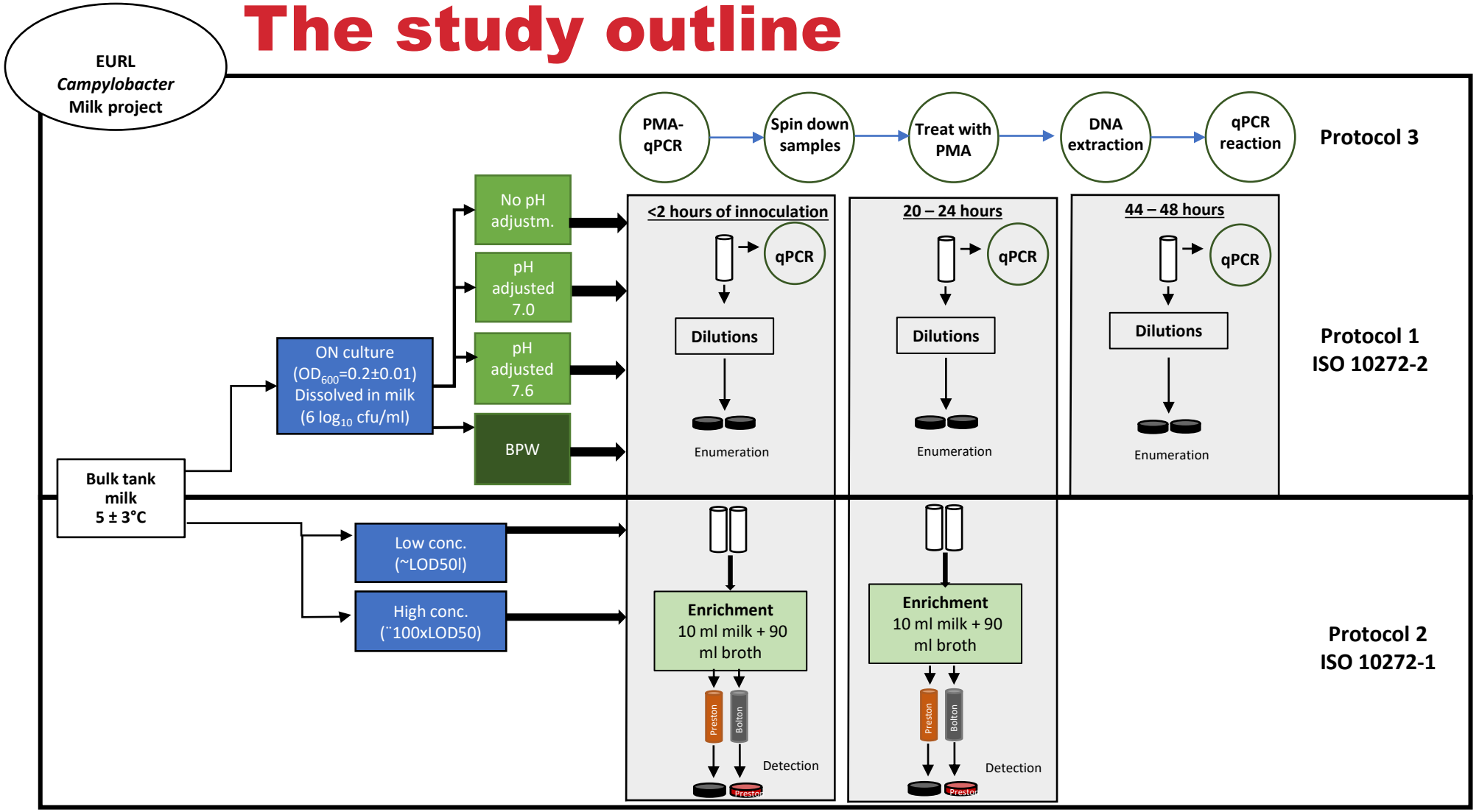
Collaborative study in EURL-NRL network using the same *Campylobacter* strain but participants collected their own milk (close to the laboratory) to answer following questions:

- How does storage of raw milk at refrigerated temperature affect the recovery of CFU of the *Campylobacter* strain over time (up to 48 hours)?
- Does pH adjustment to 7.0 or 7.6 have a positive impact on the detection of the *Campylobacter* strain in raw milk compared to no adjustment of pH?
- Does the choice of enrichment broth, Preston or Bolton, have an impact on the detection of the *Campylobacter* strain?

The organisation of an EURL-NRL collaborative study in 2020

- Invite sent out 20 February 2020 (voluntary participation): 15 registered participants.
- Two sets of vials of low/high conc. with freeze dried strain of *C. jejuni* isolated from milk were sent out with PT packages in March 2020.
- Study protocols were developed together with participants and based on the pilot studies performed by the EURL in the spring 2020. Kerstin Stingl, NRL-DE, provided protocol for PMA-qPCR.
- Participants intended to use local milk collected from bulk milk tank (or vending machine) and start the analysis the same day.
- The study period was June-December 2020.

The study outline

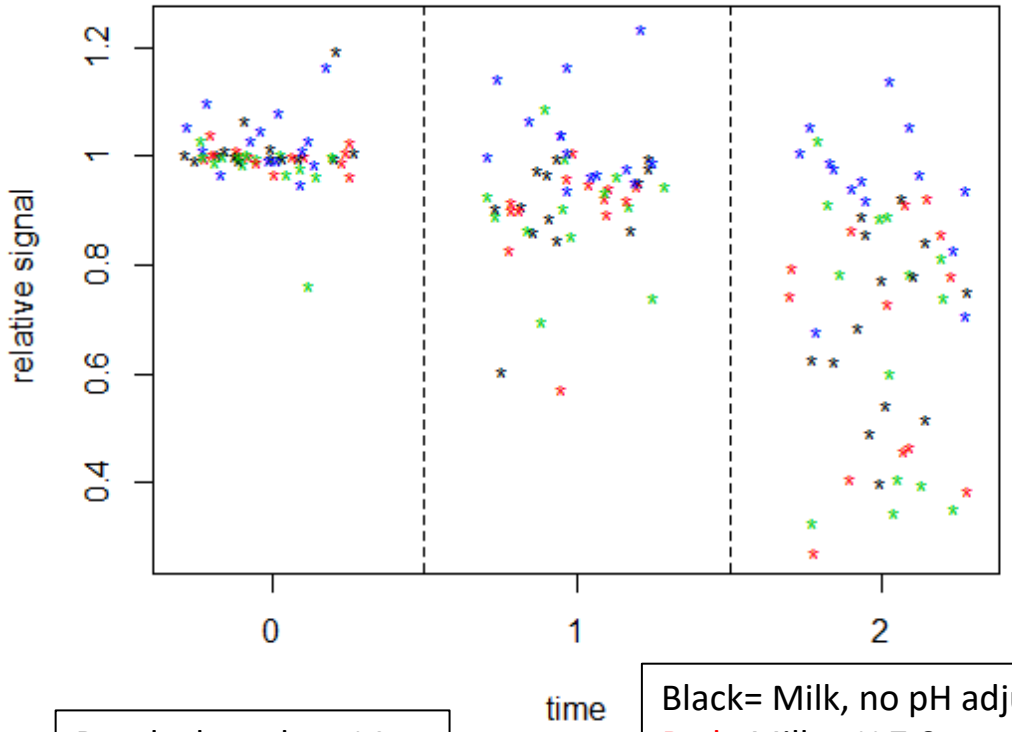


*Also controls to check for absence of natural contamination, and for viable counts of the inoculums

Participation

- Protocol 1 (survival over time and effect of pH)
  - 6 laboratories (in 5 countries) and 14 milk samples
- Protocol 2 (enrichment broth)
  - 8 laboratories (in 7 countries) and 16 milk samples
- Protocol 3 (viability qPCR)
  - no participants

Results protocol 1 (the effect of storage and pH on culturable *Campylobacter*)



Results based on 14 milk samples

Black= Milk, no pH adjustment
Red= Milk, pH 7.0
Green= Milk, pH 7.6
Blue= BPW, no pH adjustment

Statistical calculations: linear regression using a mixed effect model
Protocol or time = fixed effect
Milk sample = random effect

The study observed:

- a significant difference in decrease of culturable bacteria in raw milk compared to BPW.
- a significantly bigger decline of culturable bacteria between 24-48 hours than between 0-24 hours.
- the rate of decrease of culturable bacteria in milk is dependent on the milk sample

The study did not observe:

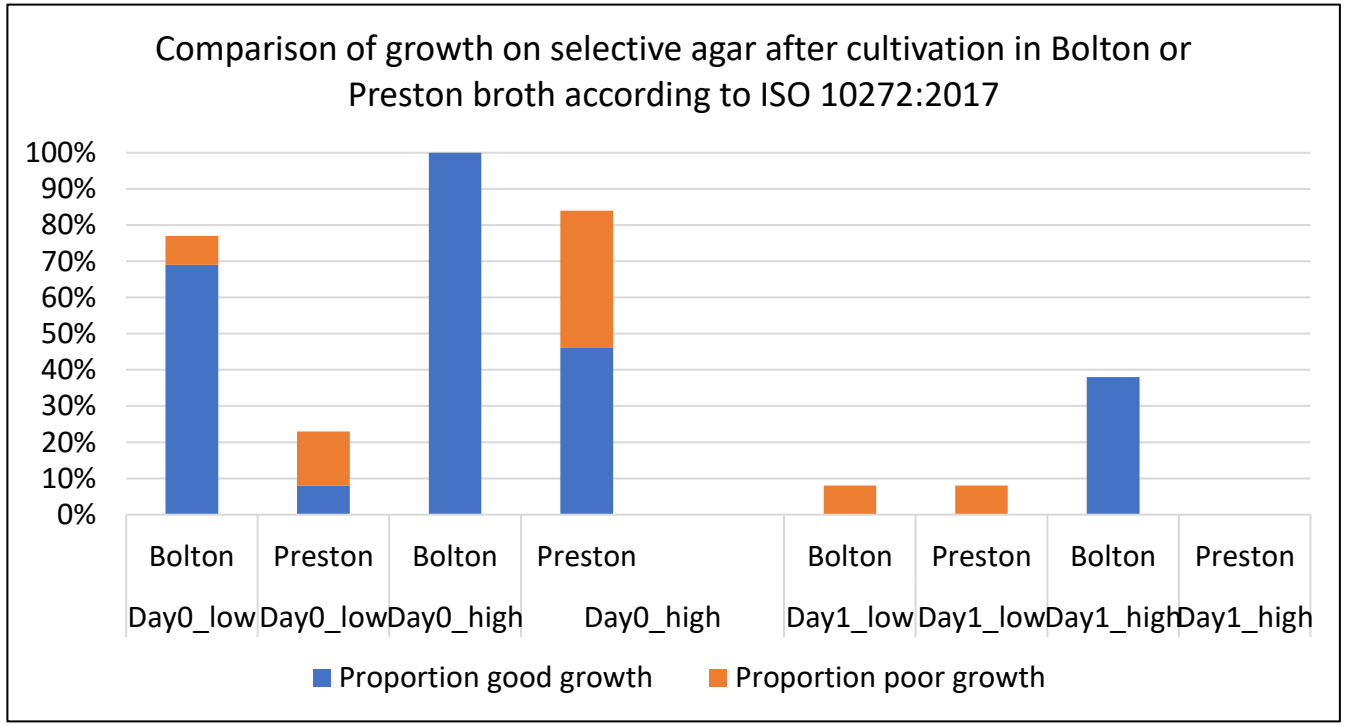
- a statistically significant difference when adjusting pH to 7 or 7.6 compared to not adjusting pH.

Result protocol 2 (the effect of enrichment broth on detection of *Campylobacter*)

			ISO 10272:2017			Growth supp. in Preston		
Time point	Vial	Broth	Total	Total detect*	Sensitivity (%)	Total	Total detect*	Sensitivity (%)
Day 0	Low	Bolton	13	10	77	3	3	100
	Low	Preston	13	3	23	3	3	100
	High	Bolton	12	12	100	3	3	100
	High	Preston	13	11	85	3	3	100
Day 1	Low	Bolton	13	1	8	3	0	0
	Low	Preston	13	1	8	3	0	0
	High	Bolton	13	5	38	3	0	0
	High	Preston	13	0	0	3	1	33

*At least on one of the plates mCCDA or Preston (Same result in 92% of sub-samples (90% only ISO 10272)).
Median value innoculation Low: 11 CFU/milk sample, median value innoculation High: 2513 CFU/milk sample

Comparison of growth on plates



*Best result on mCCDA or Preston used. Same result in 91% of samples.

When growth supplement had been added to Preston broth – only good growth (same result with Bolton broth for the same laboratories).

Summary of the study

1. How does storage of raw milk at refrigerated temperature affect the recovery of CFU of the *Campylobacter* strain over time (up to 48 hours)?

Storage reduced recovery of CFU over time, especially after 24 hours. If using cultivation-based methods, we recommend to analyse the milk as fast as possible, preferably within the first 24 hours after sampling.

2. Does pH adjustment to 7.0 or 7.6 have a positive impact on the detection of the *Campylobacter* strain in raw milk compared to no adjustment of pH?

No, we could not observe a positive impact of pH adjustment on the culturability of the strain in the study.

3. Does the choice of enrichment broth, Preston or Bolton, have an impact on the detection of the *Campylobacter* strain?

Yes, Bolton broth caused a higher sensitivity than Preston broth in detection of *Campylobacter* when present in low numbers, and it resulted in better growth on the selective plates.

Possibly, the addition of growth supplement to Preston broth increases the sensitivity of detecting *Campylobacter*.

Future plans at SVA

- Repeat Protocol 1 with additional strains
- Repeat Protocol 1 with stressed strains
- Repeat Protocol 1 and include protocol 3 (viability qPCR)

Acknowledgements:

NRLs in Estonia, Finland, France, Germany, Greece, Ireland and Sweden

To Gunnar Andersson, SVA for statistical analysis

To EURL colleagues, especially Linda Svensson, Sevinc Ferrari and Ásgeir Ástvaldsson

Questions or comments?