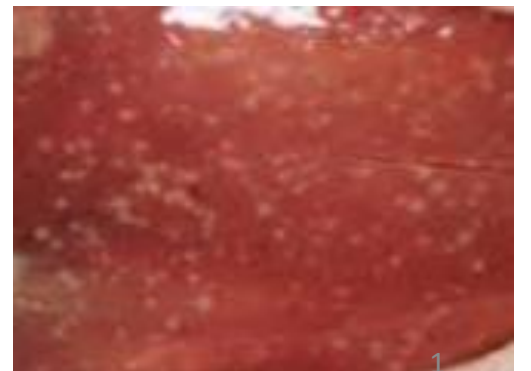




Animal &  
Plant Health  
Agency

**METhepaticus :**

**Development of tools to detect *Campylobacter hepaticus*,  
the causative agent of Spotty Liver Disease in poultry.**



Workshop *Campylobacter* / 26-27 September 2023

### Spotty liver disease (SLD)

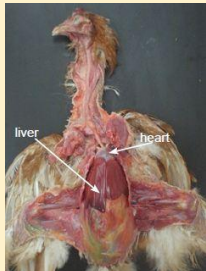
- ✓ emerging disease particularly in outdoor laying hens, causing egg laying drop and mortality with an economic impact on egg industry
- ✓ more prevalent in hot weather suggesting an increase in prevalence with global warming
- ✓ hepatitis with whitish grey spots on the surface of the liver and gallbladder infection.

### *Campylobacter hepaticus* ( in 2023, *Campylobacter bilis*)

- ✓ atypical *Campylobacter* difficult to cultivate on conventional media
- ✓ no harmonised protocols for isolation of the organism and confirmation of the disease.

### METHepaticus

- ➔ to develop efficient and harmonized protocols from farms to laboratories :
- ✓ for transporting samples to the lab without affecting bacteria survival
- ✓ for detecting of *C. hepaticus* from liver with bacteriological and molecular methods
- ✓ For the isolation and characterization of *C. hepaticus* strains
- ➔ beneficial for veterinarians, testing laboratories and research institutes.



Transport procedure

Survival of *C. hepaticus*

Direct PCR

Diagnosis

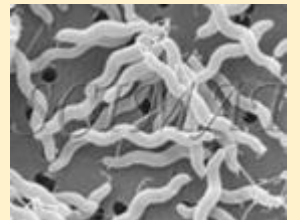
Bacteriological method

Isolation of strains

PCR / MALDI / WGS

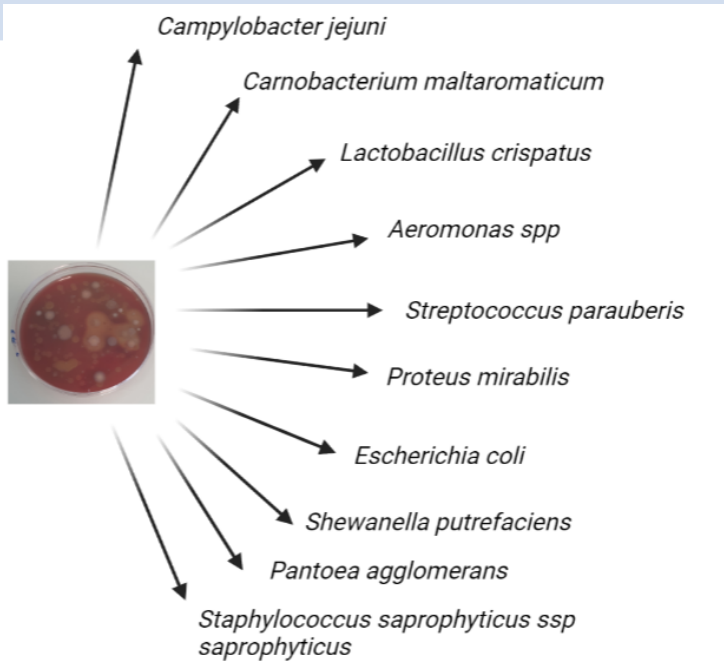
Confirmation and  
characterization of isolates

*C. hepaticus*



T1. Bacteriological method for detection of *C. hepaticus* and recovering of isolates from liver

Problems to solve	Tests carried out from artificially contaminated livers
difficult to cultivate on selective conventional <i>Campylobacter</i> media : mCCDA, Karmali, Bolton, Butzler, ....	Blood agar / blood agar + metronidazole / blood agar + bile salts / Preston agar Preston broth
slow growth / small colonies	Duration of agar and broth incubation : 4 / 8 days Temperature of incubation : 37°C vs 41.5°C Growth supplement* in broth and in agar
background flora and other <i>Campylobacter</i> on the sampled liver	Flaming livers Direct streaking vs streaking after enrichment 1/10 vs 1/50 in enrichment broth // Selective agar = Preston agar



Flora on poultry livers purchased in supermarkets

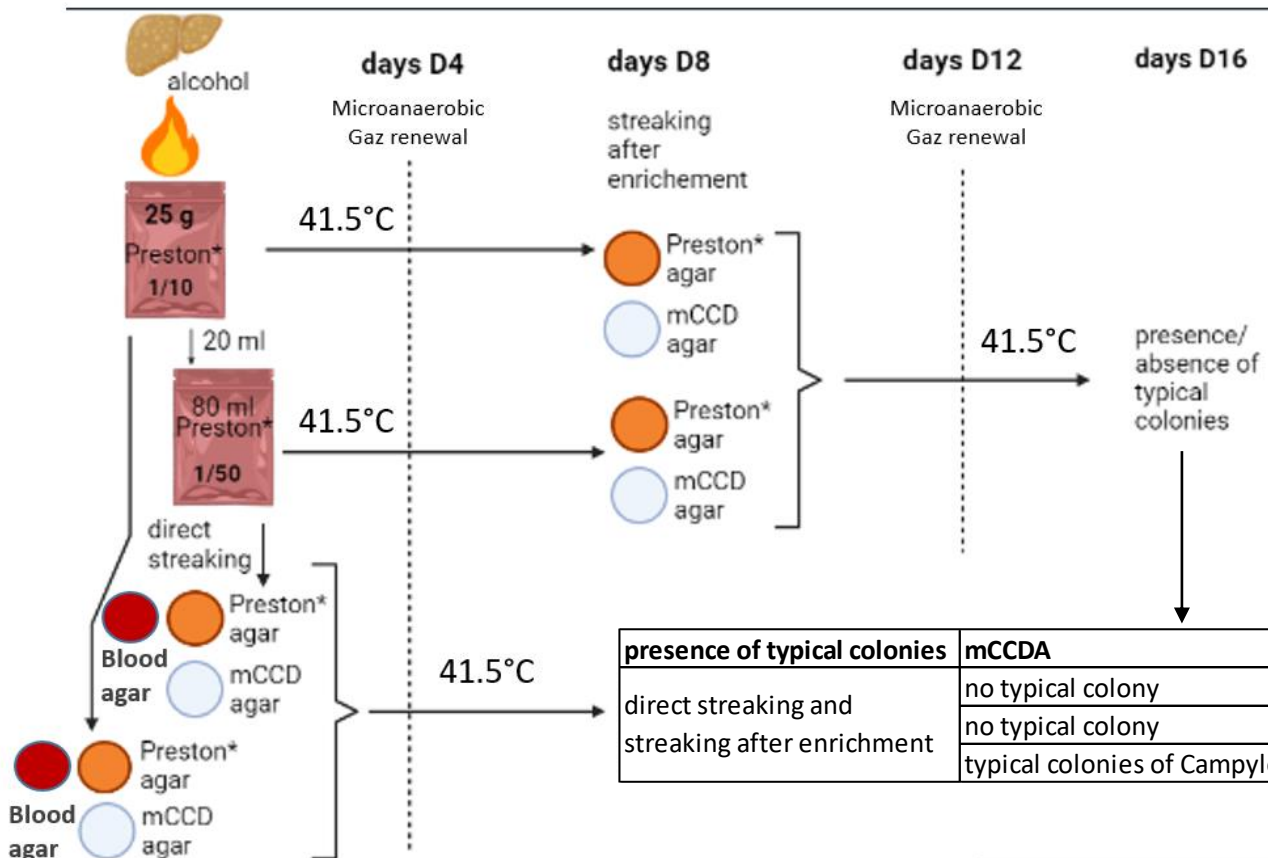
- streaking on Columbia agar with blood (GCS) (non-selective agar)
- identification of isolates by MALDI-TOF



soaking the liver in alcohol, then flaming

**T1. Bacteriological method for detection of *C. hepaticus* and recovering of isolates from liver**

**First method : streaking before and after enrichment in Preston broth**



**Sensibility of the method :**

- 700 UFC/g at direct streaking
- and 80 UFC/g after enrichment at 1/50th



Confirmation of typical colonies

	mCCDA	Preston* agar	conclusion on Preston* agar
presence of typical colonies			
direct streaking and streaking after enrichment	no typical colony	no typical colony	no <i>C. hepaticus</i> on Preston* agar
	no typical colony	typical colonies of <i>Campylobacter</i>	high suspicion of <i>C. hepaticus</i>
	typical colonies of <i>Campylobacter</i>	typical colonies of <i>Campylobacter</i>	presence of other <i>Campylobacter</i> with or without <i>C. hepaticus</i>

**T1. Bacteriological method for detection of *C. hepaticus* and recovering of isolates from liver**

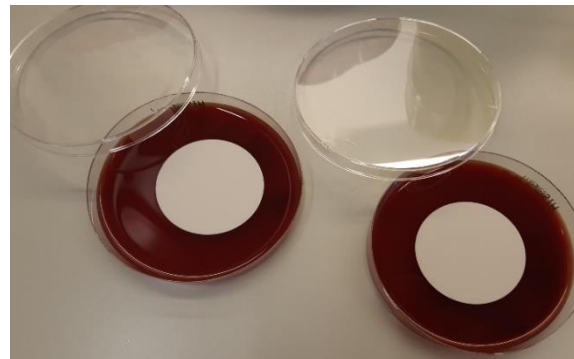
**Second method with filter**

**chicken livers spiked  
with different levels of *C.  
hepaticus***



the liver is diluted  
and ground

one ml is placed  
on the filter

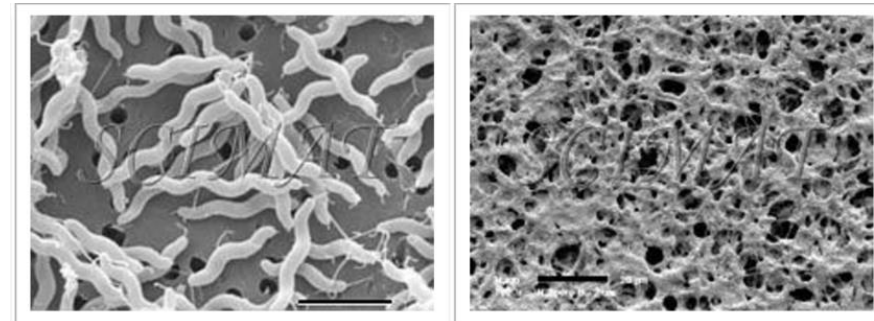


Incubation of  
the plates  
***C. hepaticus* passes  
through the filter**

**Low sensitivity of the  
method : High levels of  
*C.hepaticus* are required  
for a positive result**

- MF-Millipore™ Membrane Filter (DAWP04700), 0.65 µm pore size, mixed cellulose esters (MCE) membrane (nitrocellulose) (NC)
- Isopore Membrane Filter (DTTP04700), 0.6 µm pore size, hydrophilic polycarbonate membrane (PC)

**not a suitable method for  
detection of  
*C. hepaticus* from field  
samples**



T2. Confirmation of isolates as *C. hepaticus*.

Morphology under microscope



*Campylobacter*

PCR sybergreen from Van et al. (2017)

Amplification of glycerol kinase gene  
PCR which also amplifies *C. bilis*  
but does not allow differentiation between *C. hepaticus* and *C. bilis*

MALDI-TOF

Comparison of the MSP (Maldi spectra ) with reference MSPs  
But not reference MSPs of *C. hepaticus* and *C. bilis* in the Bruker data base



Generate reference MSP



T2. Confirmation of isolates as *C. hepaticus* : MALDI-TOF

29 Strains

(METHepaticus/ENOVAT)

Reference (2) / France (6) / UK (6)

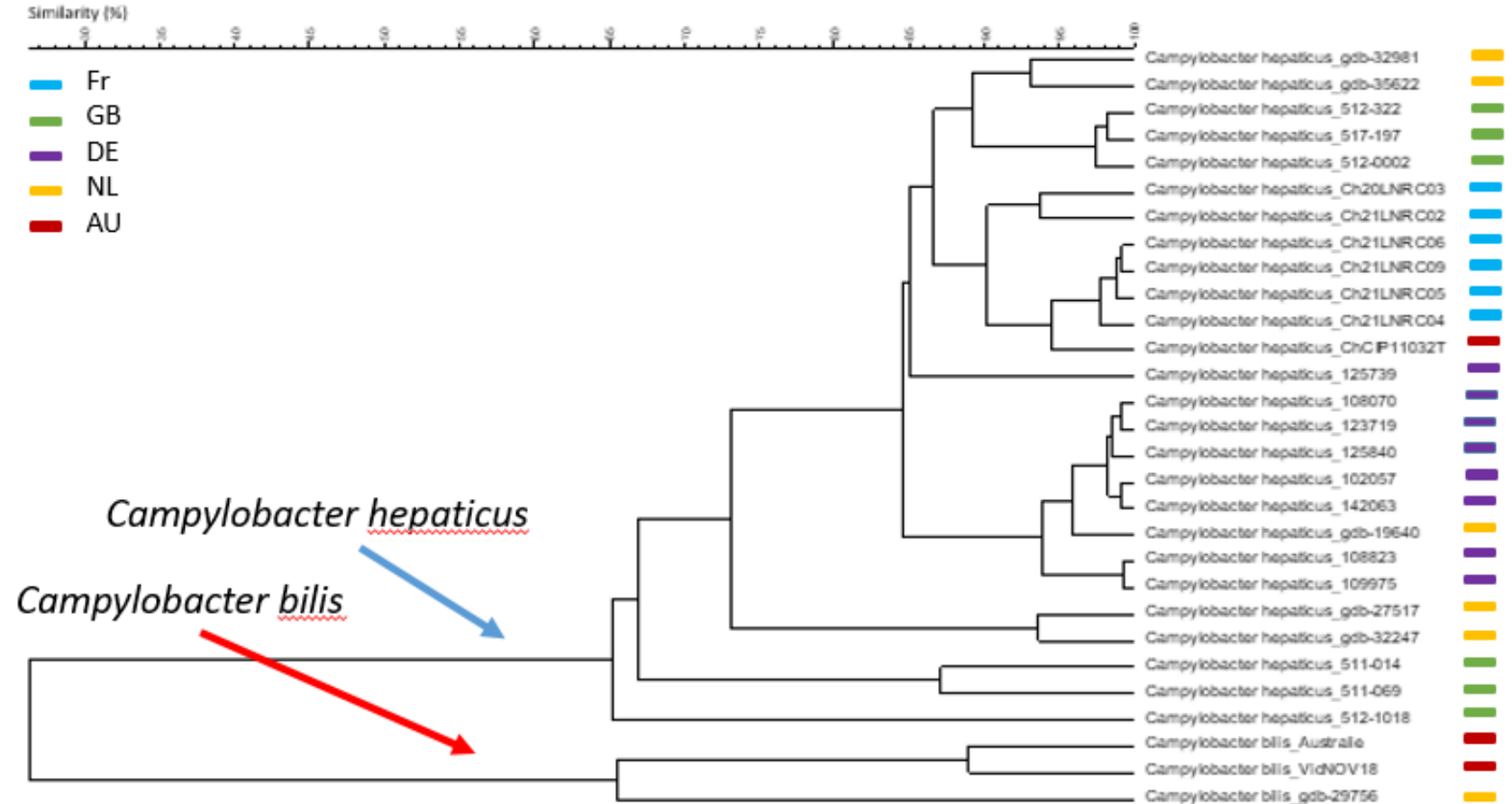
Germany (8) / Netherlands (6)

Australian MSP (3 *C. bilis*)

➔ reference MSP (Maldi spectra)

*C. hepaticus* and *C. bilis*  
can be identified by MALDI

and differentiated  
from the others *Campylobacter*



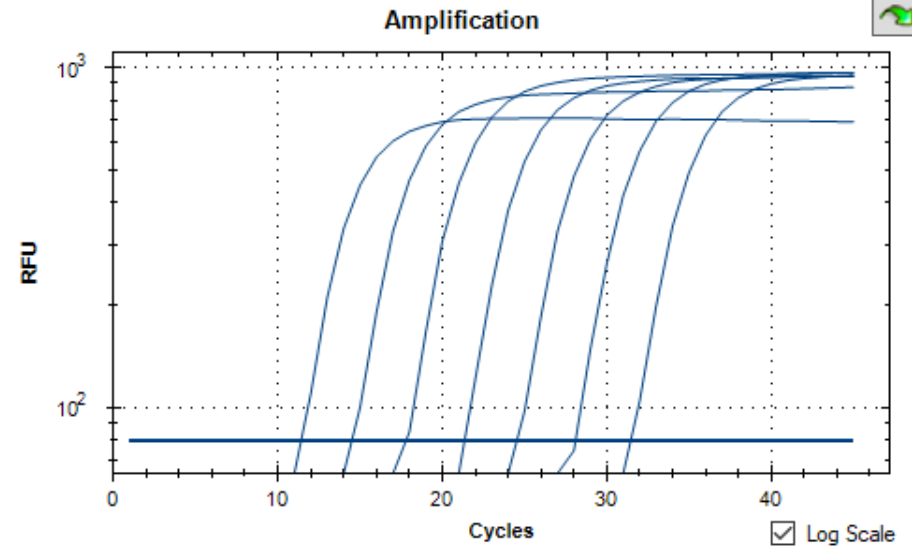
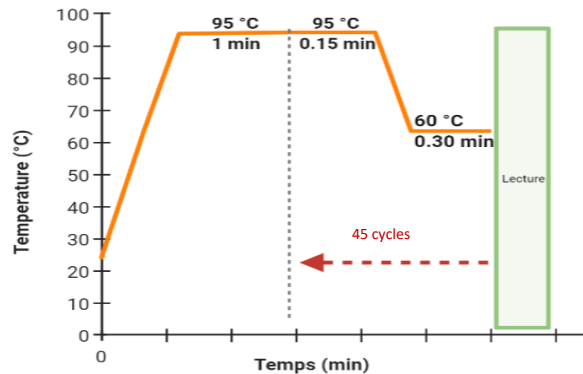
The species of the strains were also confirmed by Whole Genome Sequencing

The reference MSP generated were shared to all the METhepaticus partners

T3. Molecular method for detection of *C. hepaticus*

➔ quantitative PCR method for the detection (diagnosis) and quantification of *C. hepaticus*

- Design of a PCR primers and probes targeting the glycerol kinase gene (Van et al., 2016): Amplicon size 191 bp
- Optimal conditions for the amplification :
- PCR Efficiency: **98.8%** over a **wide dynamic range of 7 log**



• Specificity:

- Inclusivity on 6 *C. hepaticus* isolated by the French NRL
- Exclusivity on 83 *Campylobacter* strains belonging to 8 species + 19 non *Campylobacter* strains

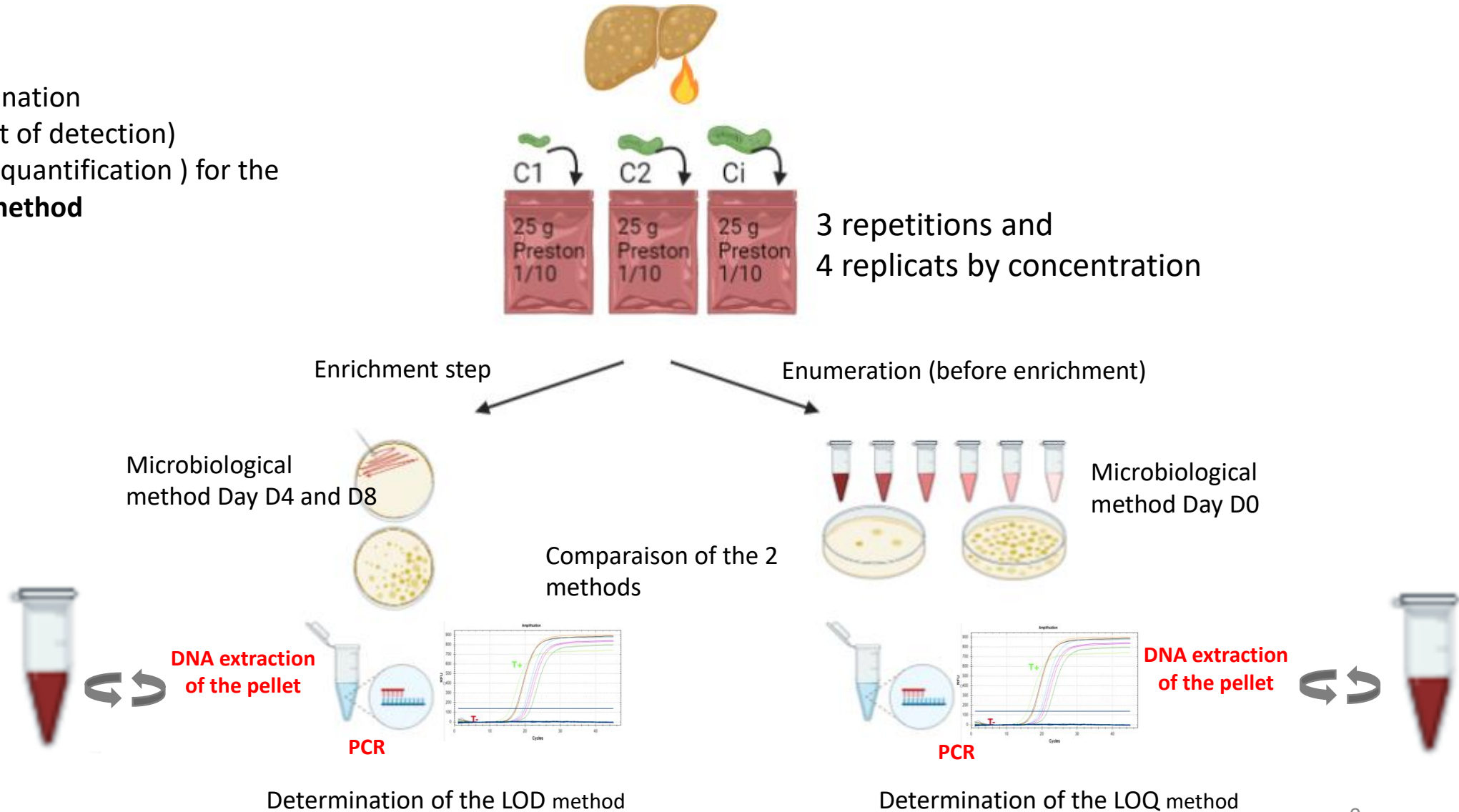
• Sensibility :

- Limit of Detection LOD<sub>PCR</sub>: **5 copy / μL**
- Limit of Quantification LOQ<sub>PCR</sub>: **100 copy / μL**



T3. Molecular method for detection of *C. hepaticus*

Determination of the LOD (limit of detection) and of the LOQ (limit of quantification) for the whole method



➔ to test the robustness of the protocol “Detection and confirmation of *Campylobacter hepaticus* / *Campylobacter bilis* in chicken liver” following SOP produced from Anses

First, SVA had to produce stable *C. hepaticus* and *C. bilis* reference materials

**ILS performed April – May 2023 :**

Four institutes participated to ILS (APHA, Anses, WBVR, Royal GD).

The SOP included background information to spread awareness about the disease

- Contents of the ILS packages:
  - One bag of approx. 170 g whole chicken livers
  - 5 vials with freeze-dried sample (with or without *Campylobacter*)
  - All reagents required for qPCR (Van et al. 2017)
- The SOP method included
  - Detection (by parallel procedures for enrichment / direct streak)
  - confirmation by PCR (Van et al. 2017) and MALDI-TOF (optional)



## ILS results

<p><b>2 samples containing <i>C. hepaticus</i></b></p>	<p>correctly reported as positive for <i>C. hepaticus</i> or <i>C. bilis</i> by all 4 participants</p>
<p><b>1 sample containing <i>C. bilis</i></b></p>	<p>reported as positive for <i>C. hepaticus</i> or <i>C. bilis</i> by <b>2 participants</b>          reported as negative for <i>C. hepaticus</i> and <i>C. bilis</i> by <b>2 participants</b>          (suspected <i>Campylobacter</i> colonies negative at PCR confirmation)</p>
<p><b>2 samples not containing <i>C. hepaticus</i> or <i>C. bilis</i></b></p>	<p>correctly reported as negative for <i>C. hepaticus</i> and <i>C. bilis</i> by all <b>4 participants</b></p>

The laboratory procedure (enrichment, direct streak, medium) producing the final results varied between samples and participants highlighting the need for parallel procedure.

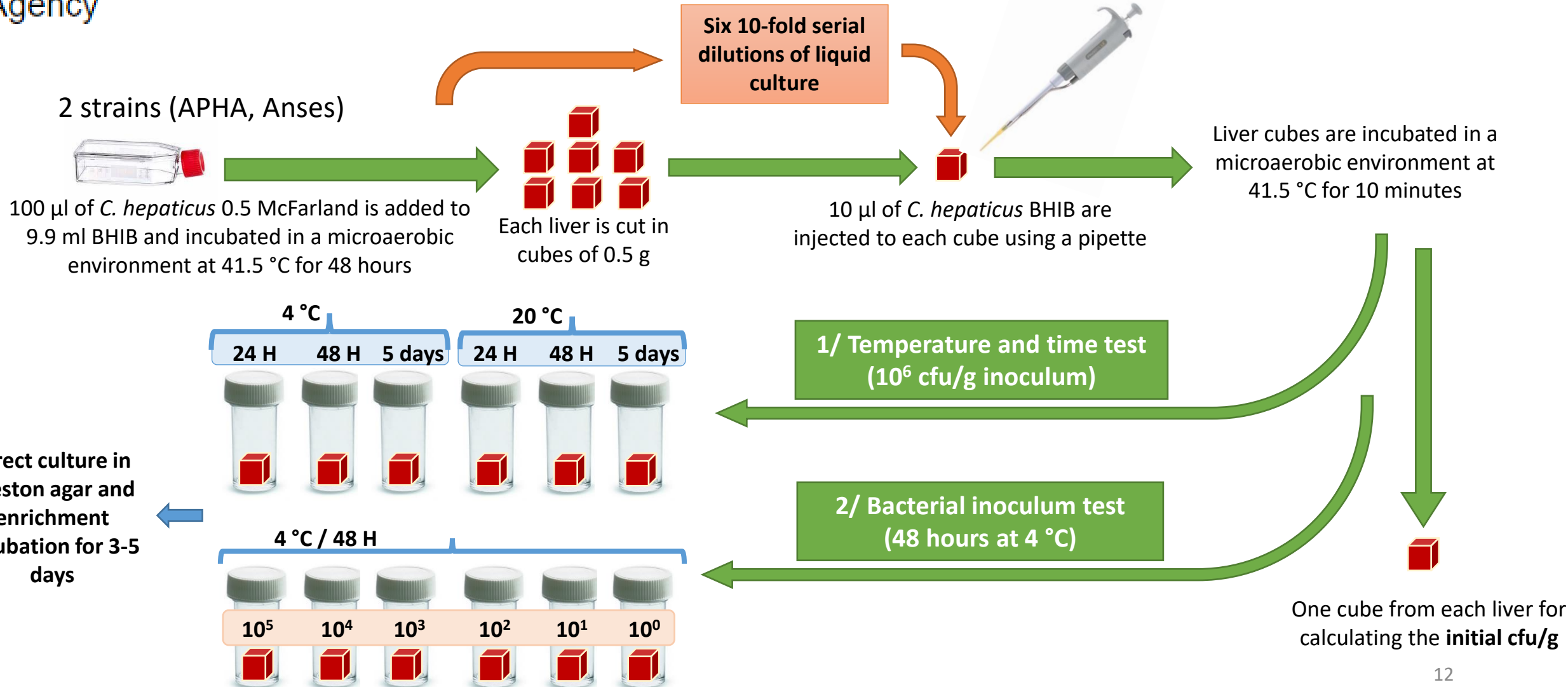


# WP3: SAMPLING AND TRANSPORTATION PROCEDURES

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## T1. Survival studies / T2. Production of sampling protocols

Variables	Conditions tested
Temperature	4 °C and 20 °C
Time	24, 48 and 120 hours
Bacterial inoculum	10 <sup>5</sup> , 10 <sup>4</sup> , 10 <sup>3</sup> , 10 <sup>2</sup> , 10 <sup>1</sup> and 10 <sup>0</sup> cfu/g





# WP3: SAMPLING AND TRANSPORTATION PROCEDURES

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Bacterial inoculum test  
(48 hours at 4 °C)

Temperature and time test  
(10<sup>6</sup> cfu/g inoculum)

S12/1018

	4 °C			20 °C		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
	24 hours	Confluent	Confluent	Confluent	Confluent	Confluent
48 hours	Confluent	Confluent	Single colonies uncountable	Confluent	Confluent	Single colonies uncountable
5 days	Confluent	Confluent	Single colonies uncountable	Single colonies uncountable	Single colonies uncountable	Single colonies countable

Ch21LNRC04

	4 °C			20 °C		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
	24 hours	Confluent	Confluent	Single colonies uncountable	Confluent	Single colonies uncountable
48 hours	Confluent	Confluent	Single colonies uncountable	Confluent	Single colonies uncountable	Single colonies uncountable
5 days	Confluent	Confluent	Single colonies uncountable	Single colonies uncountable	Single colonies uncountable	Single colonies countable

Growth of *C. hepaticus*



# T1 Survival studies: results

S12/1018

Inoculum (cfu/g)	Direct culture			Enrichment		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
10 <sup>5</sup>	Single colonies uncountable	Confluent	Single colonies uncountable	Confluent	Confluent	Single colonies uncountable
10 <sup>4</sup>	Single colonies countable	Single colonies uncountable	Single colonies countable	Confluent	Confluent	Single colonies uncountable
10 <sup>3</sup>	Single colonies countable	Single colonies countable	Single colonies countable	Confluent	Confluent	Single colonies uncountable
10 <sup>2</sup>	Single colonies countable	Single colonies countable	Single colonies countable	Confluent	Confluent	Single colonies uncountable
10 <sup>1</sup>	One or no colonies	One or no colonies	One or no colonies	Confluent	Confluent	One or no colonies
10 <sup>0</sup>	No colonies	No colonies	No colonies	Contaminated	Confluent	Single colonies uncountable

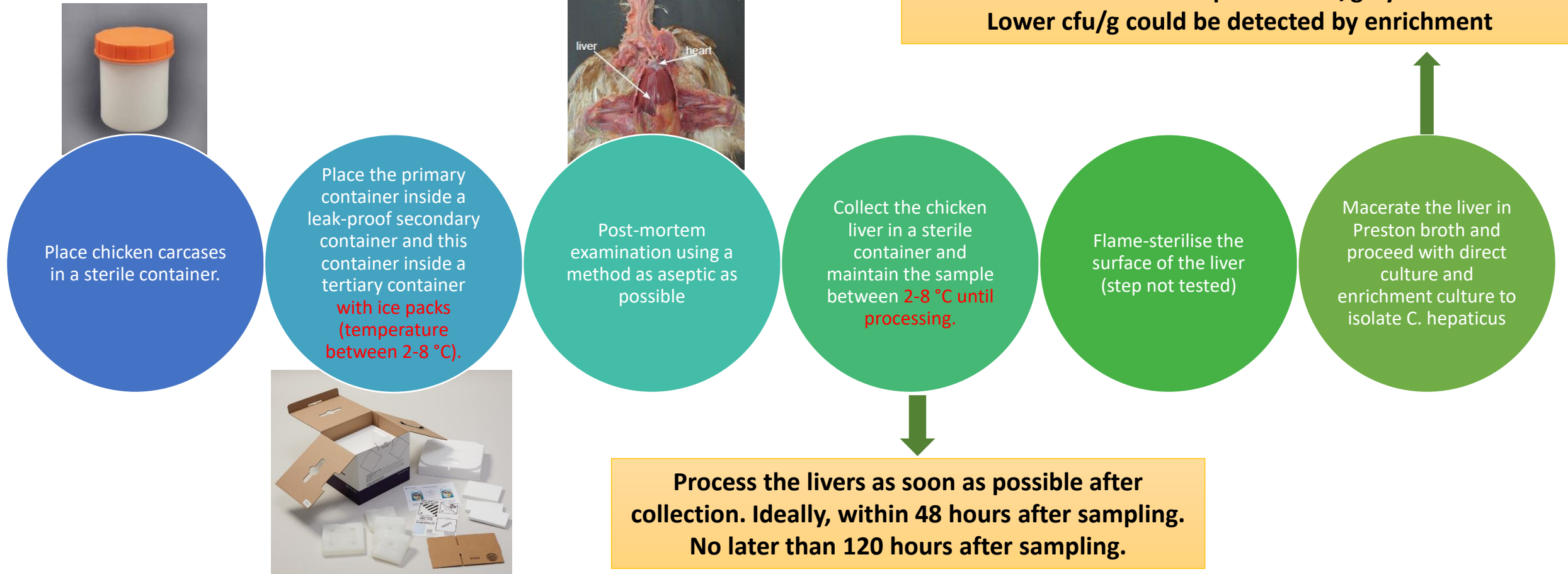
Ch21LNRC04

Inoculum (cfu/g)	Direct culture			Enrichment		
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
10 <sup>5</sup>	Single colonies uncountable	Confluent	Confluent	Single colonies uncountable	Confluent	Confluent
10 <sup>4</sup>	Single colonies countable	Single colonies uncountable	Single colonies countable	Single colonies uncountable	Confluent	Confluent
10 <sup>3</sup>	Single colonies countable	Single colonies countable	Single colonies countable	Single colonies uncountable	Confluent	Single colonies uncountable
10 <sup>2</sup>	Single colonies countable	Single colonies countable	One or no colonies	Single colonies uncountable	Confluent	Single colonies uncountable
10 <sup>1</sup>	One or no colonies	Single colonies countable	One or no colonies	One or no colonies	Confluent	Confluent
10 <sup>0</sup>	No colonies	No colonies	No colonies	Contaminated	Confluent	Confluent



## WP3: SAMPLING AND TRANSPORTATION PROCEDURES

### T2 Production of sampling and transport protocols → recommendations



Thanks to all our partners for this successful collaboration



Muriel Guyard  
Bérendère Nagard  
Ségolène Quesne  
Camille Lucas  
Elisabeth Repérant  
Martine Denis



Hanna Skarin  
Helena Höök  
Sevinc Ferrari



Miriam Koene  
Conny Van Solt  
Alieda van Essen



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Miranda Kirchner

CoVetLab