Walloon Agricultural Research Centre

DEVELOPMENT AND EVALUATION OF REAL-TIME PCR TARGETS FOR THE AUTHENTIFICATION OF INSECT FLOURS

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CONTEXT

In Europe, the processed animal proteins obtained from seven insect species have been authorised for aquaculture by Commission Regulation (EU) 2017/893 since 1 July 2017.

- Yellow Mealworm (*Tenebrio molitor*)
- Black Soldier Fly (*Hermetia illucens*)
- Common Housefly (*Musca domestica*)
- Lesser Mealworm (*Alphitobius diaperinus*)
- House Cricket (*Acheta domesticus*)
- Banded Cricket (*Gryllodes sigillatus*)
- Field Cricket (*Gryllus assimilis*)

The stakeholders are now hoping future modifications of the legislation to allow the feeding of poultry and pig.

- Many questions must be clarified concerning the presence of antinutritional compounds, the risk associated to pathogens, to residues (pesticides, antibiotics, heavy metals) and to allergens.

- Methods to detect if a product really contains insects and to authenticate insect products will also be mandatory.

- European Commission Regulation No 51/2013 added the Polymerase Chain Reaction as a reference method to determine the constituents of animal origin in feed.

- To fulfill the need in authentication methods, we started to develop real-time PCR methods specific to the insects listed above

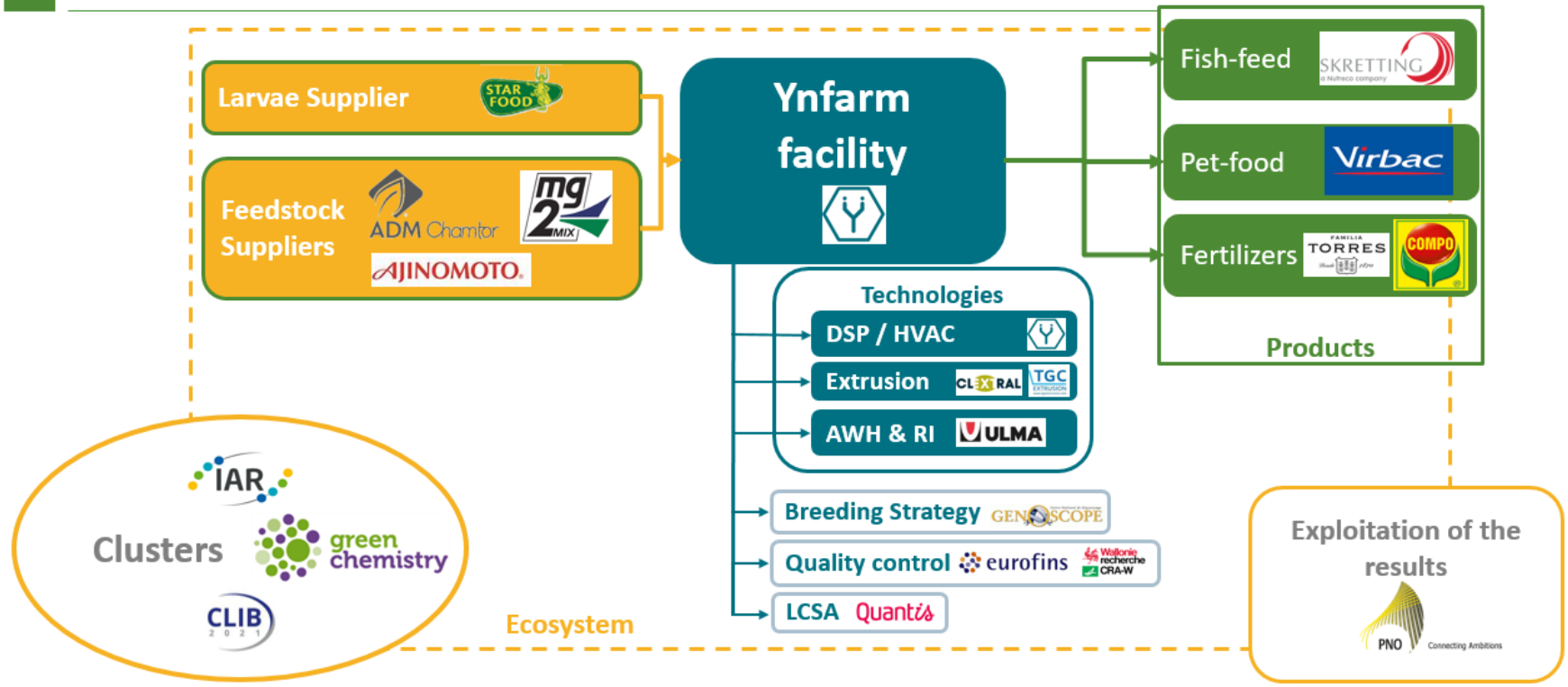
- Most of the work here was done in the framework of the FARMYNG project

FIAgship demonstration of industrial scale production of nutrient Resources from Mealworms to develop a bioeconomY New Generation

EU project gathering 20 partners

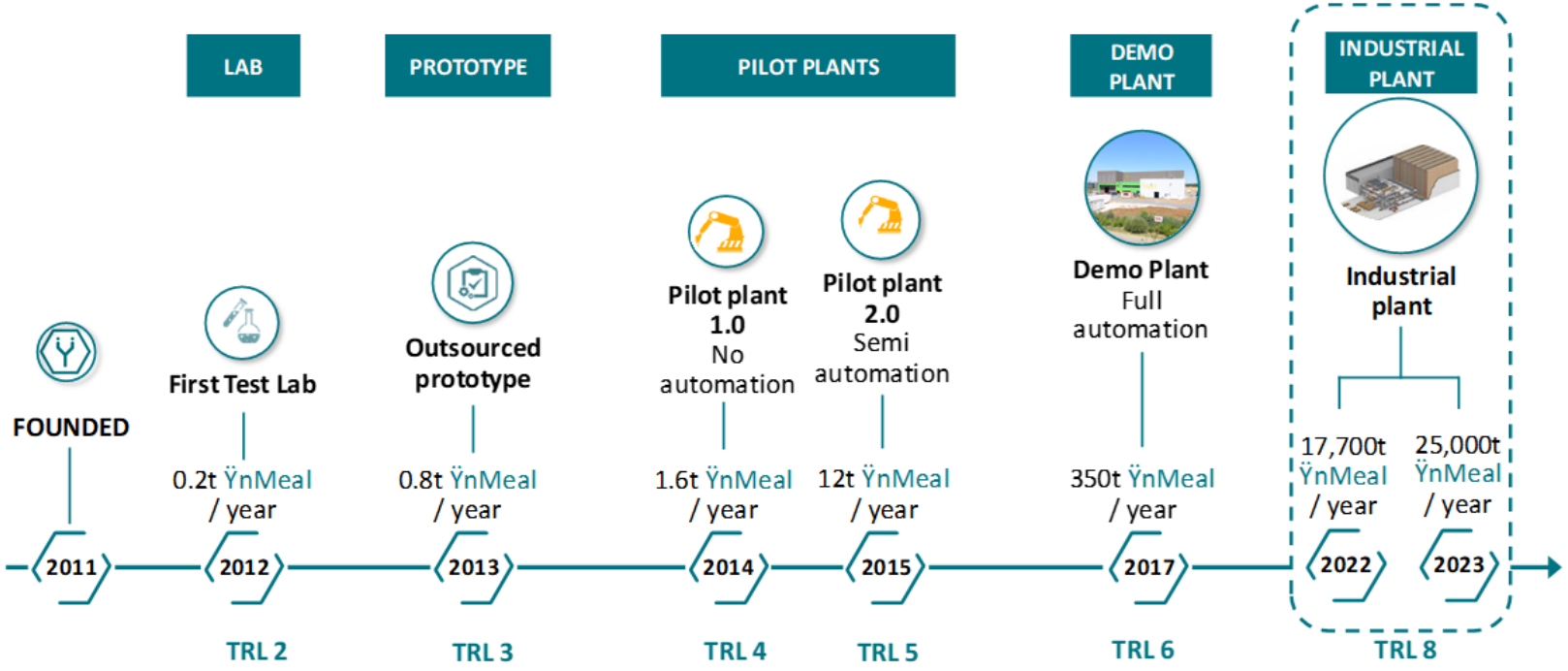
FARMYNG: a full value chain consortium

An integrated biorefinery valorising existing side streams from crops biorefineries into premium products



YNSECT in FARMYNG

An innovative and emerging agro-industrial leader dedicated to premium insect-based resources



FARMYNG: *Tenebrio molitor* insect

Tenebrio molitor is the most important insect species farmed in the feed application sector

- ✓ High protein content: *72%*
- ✓ *significant content of fat : 20%*
- ✓ *a very low proportion of ashes.*

- ✓ Non-flying insect
- ✓ Endemic to many countries
- ✓ Scalability

- ✓ No light required
- ✓ Omnivorous diet and low water requirements

Wallonie recherche CRA-W is active in the Work Package 6 in collaboration with eurofins

WP6 is based on Quality, Safety and Authenticity of insects based-products

This WP includes real-time PCR and High Troughput Sequencing (HTS) methods for the authentication of insect derived products

Step 1 : Searching for sequences of interest, design of primers and probes, *in silico* testing

Insect	Selected gene	Amplicon size (bp)	Reference
All insects	18S	81	Debode *et al.*, 2017
Yellow Mealworm (*Tenebrio molitor*)	*wingless* and *cadherin*	87	Debode *et al.*, 2017
Black Soldier Fly (*Hermetia illucens*)	*COX*3	67	Marien *et al.*, 2018
Common Housefly (*Musca domestica*)	*white*	72	/
Lesser Mealworm (*Alphitobius diaperinus*)	*cadherin*	134	/
House Cricket (*Acheta domesticus*)	*cyt*B	150	/
Field Cricket (*Gryllus assimilis*)	*juvenile hormone esterase*	87	/
Silkworm (*Bombyx Mori*)	*cadherin*	98	/

Note that some methods are based on low copy targets and other on high copy targets : both are interesting

Step 2 : Evaluation of the performances of the PCR tests

Amplificability
Check that the PCR test is able to amplify the targeted DNA with a good efficiency

Specificity
Test against other insect species (DNA of 52 insect species available in the laboratory) but also mammals, crustacean, fish, birds, mollusca plant species

Applicability
Able to work on real-life samples as processed products or industrial samples

Step 2 : Evaluation of the performances of the PCR tests

Sensitivity

Check that the PCR test is able to detect low amounts of the target

Tests : LOD_6 or LOD_{10}, $LOD_{95\%}$

Minimal performance criteria : 20 copies of the target

Robustness

Check that the test is still working with slight deviations to the standard experimental conditions (concentrations of primers and probes, volume of master mix, annealing temperature, different real-time PCR machine, different commercial master mixes)

Acceptance criterion : all deviations to the standard protocol must give a positive result at a level of 20 copies of the target

(Digital PCR)

Better control of copy numbers used in PCR reactions

Check the absence of similar sequences (that could generate a second PCR product)

Step 2 : Evaluation of performance - Results

Insect	Efficiency	Specificity	Applicability	Sensitivity	Robustness
All insects	V	V (due to plant species)	V	/	/
Tenebrio molitor	V	V*	V	LOD_6 = 20	/
Hermetia illucens	V	V*	V	LOD_6 = 5 $LOD_{95\%}$ = 5	
Musca domestica	V	/ (?)	/	/	/
Alphitobius diaperinus	V	V*	V	LOD_6 = 5 $LOD_{95\%}$ =10	V
Acheta domesticus	V	V*	V	LOD_{10} = 5 $LOD_{95\%}$ =10	V
Gryllus assimilis	V	V*	V	LOD_6 = 10 $LOD_{95\%}$ =20	V
Bombyx Mori	V	V*	V	LOD_6 = 10 $LOD_{95\%}$ =10	V

(*) late signals for few insect species are sometimes encountered

- Real-time PCR methods : targeted method, we only find what we are looking for

- Need to develop untergated methods : High Troughput Sequencing (HTS) is a solution

  → Development of approach by metagenomics / barcoding

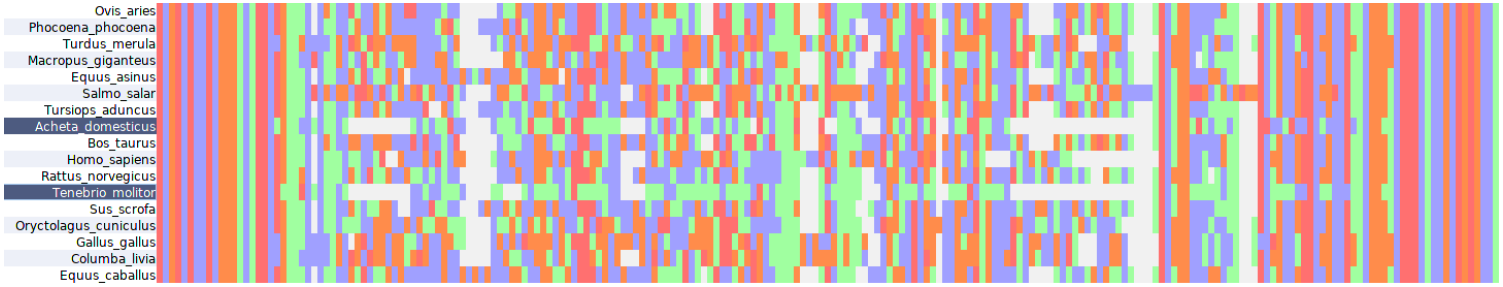
  → CRA-W is already working on approaches for animals and plants detection

  → Need of bioinformatic skills (available at CRA-W)

High Troughput Sequencing (HTS)

First tests realised on products containing insects with a 16S DNA region used for detection and distinction of animal species

16S DNA



Variable region

borded by

conserved regions

High Troughput Sequencing (HTS)

Pasta with Tenebrio meal (commercial food product)

Compounds :

- **Tenebrior molitor** (10%)
- Chickpea (43%)
- Brown rice (43%)
- Egg whites (4%)

All animal reads (> 35000) are assigned to *Tenebrio molitor*

With the vegetal targets, chickpea and rice were also detected

CONCLUSIONS on DNA based methods

Real-time PCR

- Real-time PCR methods are developed for several insect species authorised as feed
- Low and high copy number targets have both an interest
- General target for insect must be improved
- Concerning *Musca domestica*, many close species are erroneously considered to be *Musca domestica*

High Troughput Sequencing (HTS)

- Research is only starting
- Promising method
- Several targets will be necessary to confirm the results

Wallonie recherche CRA-W

Thanks for your attention

HEPL Haute Ecole de la Province de Liège

HELHa Haute École Louvain en Hainaut

eurofins

Strategisch Platform Insecten

ipiff International Platform of Insects for Food and Feed

UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

Gembloux Agro-Bio Tech Université de Liège

EURL Animal Proteins