Efficacy of herbal and aromatic components in vitro suggests prevention of histomonosis and enteritis problems in turkeys.

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Abstract: Histomonosis is the main disease facing all turkey producers since the ban on specific antihistomonal drugs in 2003. Moreover, coccidiosis and non-specific enteritis are diseases that can exacerbate blackhead disease because of the intestinal health problems they cause. Therefore, it appeared pertinent to develop a solution that can prevent all these issues at the same time. Recent publications underlined the reemergence of the blackhead disease not only in turkeys but also in chicken parent stock and free-range laying hens. Because of the stop or the limited access to specific therapeutics and the difficulty in manufacturing a vaccine, it appeared that a plant-based solution would be a possible way forward. Different *in vitro* experimentations, in collaboration with some research institutes, indicated that a pertinent choice of plant extracts and essential oils can lead to the design of an effective product capable of preventing all these gut disorders at the same time.

Introduction

The main challenge for the turkey gut health is to fight against the main parasitic threats, mainly histomonosis and coccidiosis, against bacterial infections like *E. coli* and *Clostridium* and at the same time preserving the positive microflora. The right balance of this microflora is very valuable for the preservation of the gut epithelium integrity and consequently for a positive digestibility trend.

Anti-histomonosis drugs were banned for a long time and the turkey producers are facing since that time regular black-head diseases outbreaks with different levels of mortality and with degraded performances in all cases of outcome (clinical or sub-clinical courses of the disea).

Coccidiosis prophylaxis relies for more than 50 years on the use of anticoccidials. Subsequently, the emergence of parasite populations resistant to anticoccidials was unavoidable due to their widespread use as a feed additive in poultry feed.

The research works carried out to design a phytoproduct for the chicken coccidiosis prevention and the dysbiosis that occurred at the same time will be also presented here. For this purpose, we worked with INRAE team which is able to cultivate Eimeria parasites on specific cells culture.

Together with INRAE (National Research Institute for Agriculture, Food and Environment), we evaluated 150 active ingredients, mainly of natural origin (plant extracts or essential oils) but also synthetic aromatic compounds naturally present in the environment. These compounds were selected for their direct cytotoxic effect on the dissemination stage of the parasite (oocyst) or for their ability, alone or in combination, to limit invasion and/or development in epithelial cells.

In another side, we have studied with another research partner (Clinic for Poultry and Fish Medicine, University of Veterinary Medicine, Vienna) a way to test specific bioactives, in the same spirit than the previous study. The goal of this study was to investigate the effect of plant substances on the growth and viability of *in vitro* cultivated *Histomonas meleagridis.*

Finally, some bioactive mixtures were *in vitro* tested against a wide range of bacteria, *Clostridia, Enterococcus and Lactobacillus* in particular, in collaboration with the University of Lille (France).

Besides these three experimental studies with scientific partners, we have added the specific research results coming from the R&D department from IDENA (final choice of compounds, choice of adjuvants, choice of emulsifiers to make the microemulsion).

Specific experimental designs for each part of the project

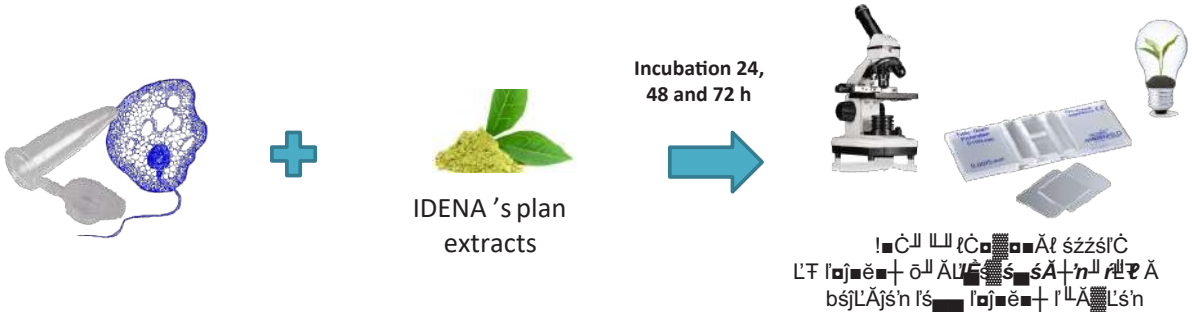
Histomonads study part

The aim of the project was to investigate the effect of plant substances on the growth of *H. meleagridis in vitro*.

For that, ten substances (aromatic and synthetic substances and mix of different substances) with two different concentrations (0.5% and 2.5%, respectively 100 and 500 ppm for bioactive concentration) were selected and provided by IDENA (Sautron, France).

The *in vitro* tests were performed at the Clinic for Poultry and Fish Medicine, University of Veterinary Medicine Vienna.

The research process was the following:



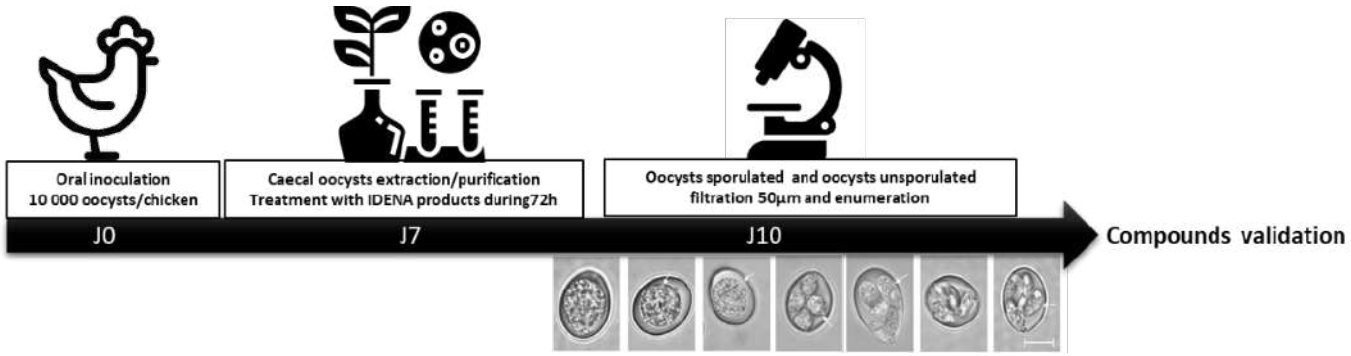
Coccidia study part

In collaboration with INRAE, IDENA evaluated a wide range of bioactives against avian coccidiosis agents by carrying out an *in vitro* screening. It aimed at evaluating their capacity to limit the invasion and or the replication of the parasites in intestinal epithelial cells.

For the adaptation of this study to the specificity of turkeys Eimeria, we consider that the mode of action of the bioactives on Eimeria in this study (*E. tenella* here) is not specific to one Eimeria species but common to the Apicomplexa phylum and thus those affecting the turkey species.

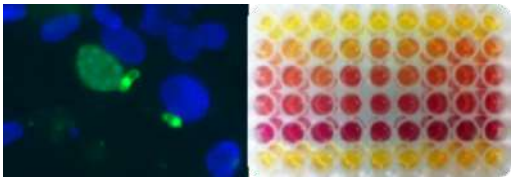
Effect of IDENA compounds on sporulation of Oocysts:

Infected animal release unsporulated oocysts in their feces, in order to be infectious for other congeners, need to sporulate. Several IDENA compounds significantly reduced 90% of sporulation of *Eimeria tenella* oocysts with dilutions up to 30 ppm.



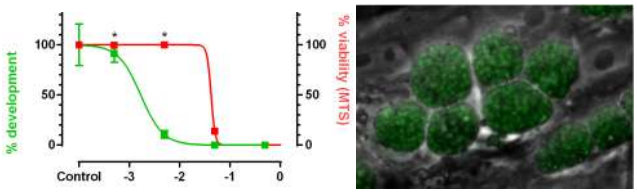
Effect of IDENA compounds on the invasion of sporozoites in cell culture:

Transgenic parasites expressing β-galactosidase (β-Gal) were developed by INRAE team to facilitate the evaluation of this process. A range of doses of IDENA compounds were preincubated with sporozoites (invasive stage) and their ability to invade monolayers of epithelial cells was quantified by β-Gal activity. Some compounds have been further selected for their ability to reduce cell invasion by 50%.



Effect of IDENA compounds on the parasite development in cell culture:

Specific *E. tenella* transgenic INRAE strain were produced to investigate parasite development *in vitro* and precisely determine the half maximal inhibitory concentration (IC50) for each compound.



Bacterial control part

To design effective bioactives, we carried out an extensive screening of active ingredients in partnership with the University of Pharmacy in Lille (France).

The aim was to determine the Minimum Inhibitory Concentration (MIC) using agar dilution, in the same way as antibiotic molecules.

These trials enabled us to test around thirty combinations of active ingredients for their ability to inhibit bacterial strains.

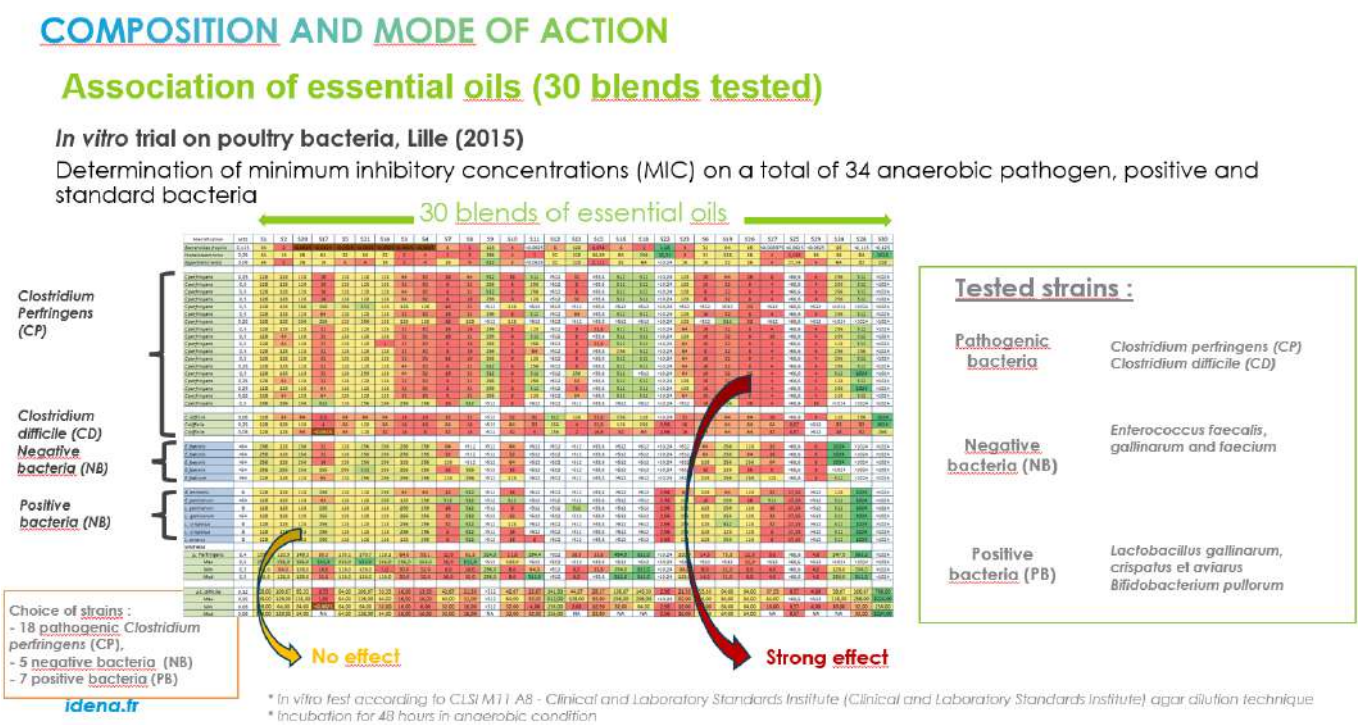
We tested several active ingredients (essential oils, plant extracts or mixtures of active ingredients) on different strains of Clostridium and on positive flora such as lactobacilli.

The study generated 4 main results.

Thanks to these tests, we were able to select 3 interesting essential oils and calculate their effective incorporation dose.

Gelose diffusion: in vitro test on poultry germs, Lille (2015)

The determination of the Minimum Inhibitory Concentrations (MICs) on pathogenic and beneficial anaerobic bacteria was performed at the University of Lille (France). These different strains were tested according to this way:



Part of the other component properties:

Because we know that the infection and infestation processes always generate inflammation as part of the infection, we have added some well-chosen plant extracts that have famous anti-oxidant and anti-inflammatory properties.

Other processing ways to improve the efficiency of the components:

To decrease the drawbacks of the essential oils present in the final product, a specific technology was implemented based on the choice of emulsifiers and the application of a high-shear emulsion. That specific process leads to an optimal bioavailability of active ingredients, a better penetration of coccidia (*Eimeria*), *Histomonas* and bacteria by active ingredients and a longer product stability without demixing.

Results:

Efficacy on *Histomonas*:

In this *in vitro* study, the MLC (Minimum Lethal Concentration) showed a significant reduction ($p<0.05$) of live *H. meleagridis* cells without re-growing in a further culture with fresh medium. That was noticed by adding FORKEY and substance #6 at respectively a concentration of 500 and 50 ppm of active ingredients.

RESULTS

FORKEY
LA SOLUTION NATURELLE POUR LA SANTÉ DIGESTIVE

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Mix	Bioactive conc.(ppm)	x 10^3 histomonas 0H	24H	48H	72H	Mann- Whitney U	Regrowth
1	100*	100	169	223	343		
	500	100	149	226	284		
2	100	100	36	16	8	p< 0,05	
	500	100	3	0	0	p< 0,05	
3	10	100	151	175	241		
	50	100	96	117	138		
FORKEY 1 Kg/T	100 (Incl. 10 EO)	100	32	103	241		
	500 (Incl. 50 EO)	100	0	0	0	p< 0,05	NO
6	10	100	89	167	236		
	50	100	0	0	0	p< 0,05	NO
7	10	100	80	191	151		
	50	100	12	34	67	p< 0,05	
8	100 (Incl. 10 EO)	100	94	97	99		
	500 (Incl. 50 EO)	100	0	0	0	p< 0,05	
9	10	100	128	198	339		
	50	100	102	367	574		
10	60 (Incl. 10 ppm EO)	100	9	55	147		
	300 (Incl. 50 ppm EO)	100	0,6	0	0	p< 0,05	
DMZ	0,4	100	0	0	0	p< 0,05	
Negative control		100	154	163	301		

* mean number of live cells of three independent experiments, each performed in triplicate

Table 1 : List of substances with the applied concentrations and mean count *of H. meleagridis* before and after 24, 48 and 72 H of incubation

No regrowth of the parasite in a further incubation in fresh medium

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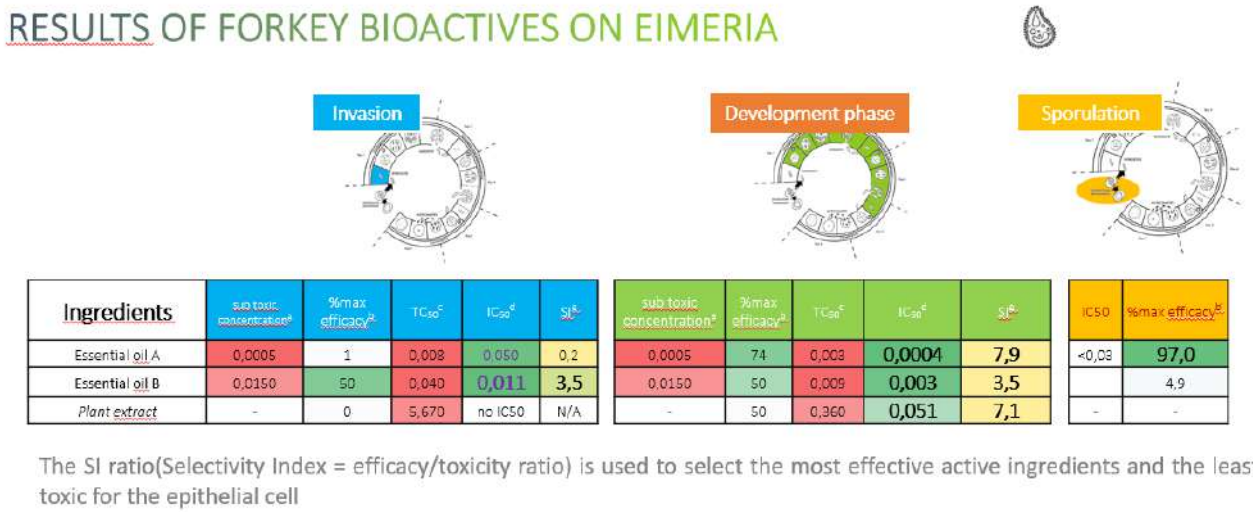
Efficacy on coccidia

Three recombinant *E. tenella* parasite strains were generated to screen the efficacy of the 150 IDENA library compounds against all stages of the parasite life cycle.

Six compounds were able to restrict *E. tenella* oocyst sporulation by more than 90%. Three compounds limited invasion by at least 50% with a Selectivity Index (S.I. =efficacy/toxicity ratio) up to 3.5. Finally, 15 compounds were capable of inhibiting parasite development in epithelial cells with an efficacy ranging from 50% to 100% and a S.I. (Selectivity Index) that can be as high as 8.

Results on invasion, development and sporulation stage are summarized in the table 1 below:



Ingredients	sub toxic concentration[a]	%max efficacy[b]	TC_{50}[c]	IC_{50}[d]	SI[e]	sub toxic concentration[a]	%max efficacy[b]	TC_{50}[c]	IC_{50}[d]	SI[e]	IC50	%max efficacy[b]
Essential oil A	0,0005	1	0,008	0,050	0,2	0,0005	74	0,003	0,0004	7,9	<0,03	97,0
Essential oil B	0,0150	50	0,040	0,011	3,5	0,0150	50	0,009	0,003	3,5		4,9
Plant extract	-	0	5,670	no IC50	N/A	-	50	0,360	0,051	7,1	-	-

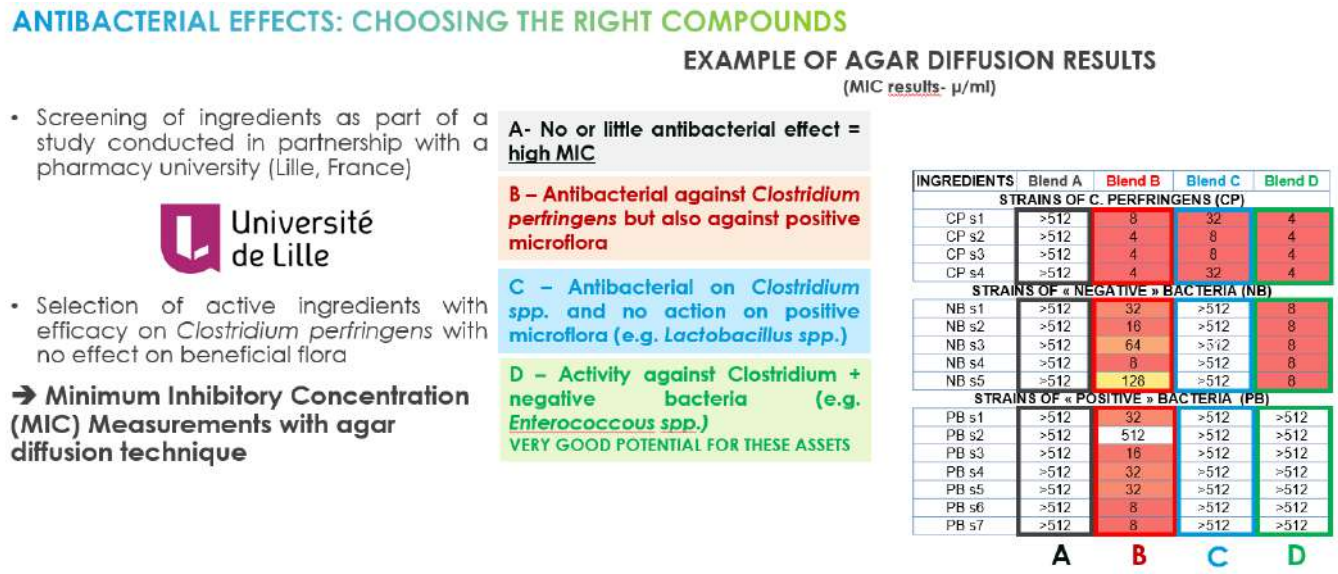
The SI ratio(Selectivity Index = efficacy/toxicity ratio) is used to select the most effective active ingredients and the least toxic for the epithelial cell

Table 1 : effects of Idena Compounds (alone or mixed) on invasion, development, and sporulation of Eimeria tenella on epithelial cells

Antibacterial effects:

The table below summarises the results of the screening carried out at the University of Lille, showing the inhibitory activities (in MIC) on selected strains of *Clostridium perfringens*, "negative" bacteria (*Clostridium, Enterococcus*) and positive bacteria (such as lactobacilli), leading to a relevant choice of a mixture with the active ingredients.

In conclusion, we select a mix of ingredients (blend #4) with a strong antibacterial effect against *Clostridum spp.* and weak detrimental effect against beneficial bacteria, with average minimum inhibitory concentration of 6.2 ppm and more than 512 ppm.



Conclusion:

All these experimental studies indicate that it is possible to formulate an alternative and specific solution able to prevent at the same time the following threats that affect the turkey health: an anti-parasitic effect to stop development of *H. meleagridis*, to break the parasitic cycle of Eimeria, and an intestinal antiseptic effect to fight bacterial infections (mainly *C. perfringens*).

By using this product for several years, some turkey production companies have demonstrated the efficiency of feeding turkeys with alternative in-feed solutions rather than the old anti-histomonosis drugs that are now banned, without encountering any clinical cases of histomonosis.

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