Tannin containing legumes as a model for nutraceuticals against digestive parasites in livestock

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Corresponding Author: Dr. HOSTE Hervé, DVM, Phd,HDR

Corresponding Author's Institution: INRA

First Author: HOSTE Hervé, DVM, Phd,HDR

Order of Authors: HOSTE Hervé, DVM, Phd,HDR; TORRES-ACOSTA J.F.J.; SANDOVAL-CASTRO C.A.; MUELLER-HARVEY I.; SOTIRAKI S.; LOUVANDINI H.; THAMSBORG S.M.; TERRILL T.H.

Abstract: Parasitic infections with gastrointestinal nematodes (GINs) still represent a worldwide major pathological threat associated with the outdoor production of various livestock species. Because of the widespread resistance to synthetic chemical anthelmintics, there is a strong impetus to explore novel approaches for a more integrated management of the infections. The use of nutraceuticals in the control of GINs is one of the alternatives which has been widely studied for 20 years.
The objective of this review are: i) to define and illustrate the concept of 'nutraceutical' in the context of veterinary parasitology based on data obtained on the most studied GIN models in small ruminants, the tannin-containing legumes (Fabaceae); ii) to illustrate how the 'nutraceutical concept' could be expanded to other plants, other livestock production systems and other GI parasitic diseases, and iii) to explain how this concept is opening up new research fields for better understanding the interactions between the host, the digestive parasites and the environment

Cover Letter

Dr Hervé HOSTE
Unité Associée 1225 INRA / ENVT
« Interactions Hôte Agents Pathogènes »
Ecole Nationale Vétérinaire de Toulouse
23, Chemin de Capelles
31076 TOULOUSE Cedex
Tel 05 61 19 38 75 Fax 05 61 19 39 44
E mail h.hoste@envt.fr

Toulouse, the 08th June 2015.

Dear colleague,

Please find enclosed a revised version of the article "*Tannin containing legumes as a model for nutraceuticals against digestive parasites in livestock*" by Hoste H and collaborators which is submitted for publication to Veterinary Parasitology. This review was invited for the Special Issue in preparation for the next WAAVP 2015 meeting in Liverpool.

By comparison to the first version, main changes have been made on i) the main text Ii) the figure 3 and its caption and iii) 10 references have been added to address some of the referees'comments.

We wish to thank the 2 referees for their inputs and we hope to have addressed their comments. In particular, we have paid special attention to the definition of nutraceutical in the section 2 in order to take into account the comments of the 2 referees

This revised manuscript has been approved by all co-authors.

We are looking forward to receiving your comments

Yours sincerely

Hervé HOSTE

*Revision Note

Reviewer #1: This review manuscript is very well written and very thorough on the experimental approach to determine the nutraceutical effect of plants, specifically condensed tannin (CT) containing plants, on small ruminant gastrointestinal nematode (GIN) infections. It is so thorough that it could be condensed, as there is much repetitive information throughout. It would be very useful information in a textbook for veterinary students studying parasitology or animal science students studying production issues. In some instances though, the literature could have been more thoroughly researched rather than only including the authors work. An example of this can be found in Lines 180-184 and L346-348. Some papers that were missed in this discussion include those from Utah State University (even though this is not a tropical area, they do use irrigation and might consider this a model for self-medication - Villalba and Provenza, 2007; Villalba et al., 2010, 2012; Lisonbee et al., 2009; and also, Hutchings et al., 2003 to name another). Minor comments appear below.

The different references mentioned by the referee (and some others) have been now added in the section 5.3 dedicated to the subject of self medication

L80-82: I found this sentence, almost word for word on Wikipedia. I was curious because research and patent regulation concerning nutraceuticals occurred before 1998. See DeFelice, 1995 (Trends in Food Sci. Tech.) or Linko and Hayakawa, 1996 (same journal), or Mee, 1994, Hunt, 1994 concerning regulatory issues.

Because of the comments of the 2 referees, we tried to better defined and clarify in the section 2 the definition of the concept of nutraceuticals in medical and vet sciences and to make clear that we will focus on one subcategory of nutraceuticals, namely those based on PSM containing resources either when directly present or when added to animal feed.

This will be found in section 2 of the current Revised Manuscript (REVMAN)

Text throughout: There seems to be missing "t", "I" and "f" typically in either bold or italicized text throughout on my pdf copy**. We have checked these errors throughout the Revised Manuscript (REVMAN)**

L199-204: On the other hand, is it worth noting that non-native species are considered invasive by environmentalists and ecologists? **The comment has been included in section 3.1.2. of the REVMAN (see line 212-213)**

L390: (e.g., packed cell volume)**: This has been added (line 462 in the revised manuscript)**

L386-401: Should effects on toxicology be considered at some point in the experimental protocol?

As stated in the title of section 3.2, we intend and decided to focus only on the efficacy issue

L438, 617: Should non-peer reviewed papers be cited in review articles?

For the references 50 (Klongsiriwet, C., et al 2015) and the reference 85 (Quijada et al. 2015) in the Revised Manuscript = We have now replaced the previous reference to a summary for a conference by a reference to an accepted or submiited article.

For the references 19 (Castañeda-Ramírez, G.S et al. 2014); ref 22 (Covarrubias-Cárdenas, A.G. et al 2013); ref 23 (Desrues et al 2012); ref 30: (Girard et al, 2013), and ref 104 (Vargas-Magaña, J.J. et al, 2014), **we will respect the editorial rules and advices of the editor**

L450: Not just iso-proteic, but also similar in bypass protein as well. **We agree with the referee that this should be a task. However, it is difficult to assess the amount of increased by-pass proteins because of the presence of CT. We could only say that CT could alter (increase) the quantity of by-pass proteins in the diets (see section 4.1.1. (line 522) and section 4.1.2 (lines 534), in the RevMan).**

L452-459: Already been alluded to in previous paragraph.

L596: "…based on nutritional…" **CORRECTED**

L633: sporulation**. CORRECTED**

L647-648: Burke et al., 2013 fed before and at weaning. **Has been added on line 744 in the Revised Manuscript**

L661: spp. **CORRECTED (line 671)**

L666: However and ,L666-667: Is there something missing after this sentence? **We think to have clarified this sentence now (line 731 to 739 in the Revised Manuscript)**

L668-688: Does not fit the objective of the paper. **The referee is right; however, we wished to illustrate in this section 5.2 that the concept of nutraceutical can be applied to other plant families in relation with other PSM (see the title of section 5.2) (line 726 in the revised manuscript)**

References: Some are not formatted correctly and contain misspellings. **We had checked thoroughly the references and have corrected several errors.**

Figures 2, 3: Why is the male worm depicted as being larger than the female? **The referee is right**

This has been corrected now in the REVMAN

Reviewer #2: General comments are as follows:

This is an interesting review, which summarises a complicated area and makes many valid points. However, it seeks to redefine bioactive forages as nutraceuticals, and in doing so it becomes confusing. The authors defines all nutraceuticals as plant based products containing PSM, and the rest of the paper follows from th is inaccurate assumption. It is broadly accepted that a nutraceutical is a food product or a product derived from food (e.g. vitamins and minerals) which is added to the diet and is perceived to have added health benefits. Usually the beneficial compounds in nutraceuticals do not have pharmacological action against pathogens, rather they help improve host health. Hence bioactive forages presented in this context simply does not work for me. Another broad issue with the review, is that it gives few facts and figures regarding the efficacy of these 'nutraceuticals' and hence leaves you with little sense of how efficacious these plants actually are. To be of interest to a wider audience I would suggest adding in some basic information regarding CT containing forages and their efficacy. For these reasons I suggest moderate revision before acceptance. I suggest that either the definition of nutraceutical is changed, and it is acknowledged that bioactive forages are a very specific type of nutraceutical, or the term nutraceutical is removed altogether.

WE TRIED TO MAKE CLEAR in the TITLE AND in the SECTION 2, THAT IN THIS REVIEW WE WERE FOCUSSING ON BIOACTVE PLANTS USED AS NUTRACEUTICALS

IN ADDITION, TO ADRRESS ONE OF THE REFEREE, ONE CAN EXPECT THAN ANY EFFECT AGAINST PATHOGENS WILL HELP AT IMPROVING ANIMAL HEALTH

Specific points are as follows:

Line 25 - 'nutraceuticals', should be plural

NOW CORRECTED

Line 26 - 'alternatives', should be plural

NOW CORRECTED

Line 26 - suggest change 'since 20 years' to 'for 20 years' better grammar

NOW CORRECTED

Line 27 - 'objectives', should be plural

NOW CORRECTED

Line 62-63 - This sentence is unclear, consider re-phrase.

THIS HAS BEEN REPHRASED

Line 72-73 - Not sure this sentence quite makes sense - it infers the fabaceae are a GIN model. Suggest rephrase.

THE SENTENCE HAS BEEN MODIFIED

Line 80-92 - I think that the definition of a veterinary nutraceutical here is too narrow. Nutraceuticals are not all bioactive plants with PSMs. Some are derived from other biological materials e.g. glucosamine from shellfish. More accurately bioactive forages are a sub-category of nutraceuticals.

WE HAVE TAKEN INTO ACCOUNT THE REFEREE’S COMMENT AND INCLUDED IT IN THE TEXT (LINE 91-92 in the REVISED MANUSCRIPT)

Line 98-110 - Following on from the point above, referring to bioactive forage as 'nutraceuticals' could be construed as inaccurate

WE HAVE ALSO ADDED SOME PRECISION IN THE TEXT TO CLARIFY THE FACT THAT WE ARE FOCUSSING ON BIOACTIVE FORAGES AS A SUB CATEGORY OF NUTRACEUTICALS

Line 149 - What is IVDMD?

EXPLANATIONS ARE NOW PROVIDED

Line 152 - I'm not sure this is strictly true. Many nutraceuticals are added to animals feeds in specific amounts and their intake is not voluntary. E.g. joint supplements, vitamins and fatty acids

WE HAVE NOW MADE CLEAR THAT THE FOCUS WAS ON BIOACTIVE PLANTS (SEE LINE 91/92 AND 118). IN ADDITION, EVEN IN THE CASE EVOKED BY THE REFEREE, THE EFFECTS WILL DEPEND ON THE CONSUMPTION OF THE FEED BY THE ANIMALS

Line 159 - Please define acronyms (DM, OM) the first time you use them.

THIS HAS BEEN CORRECTED

Line 197-198 - This sentence is unclear - consider rephrase

WE THINK THAT THE SENTENCE IS CLEAR

Line 215 -216 - 'By the overall, logical scheme/organisation and the objectives of each step, the procedure has been adapted…' I am not sure what this means? Please rephrase more clearly

THE SENTENCE HAS BEEN MODIFIED IN ORDER TO CLARIFY THE MEANING

Line 227 - '(see point 4)' it is unclear as to what this is referring to as there are so many points and sub-points in this review

THIS HAS BEEN SUPPRESSED NOW TO AVOID ANY CONFUSION

Line 245-253 - Other studies are referred to but there are no references

REFERENCES HAVE BEEN ADDED

Line 252-253 - This sentence is unclear, do you mean CTs? or Nutraceutical plants?

WE CAN NOT ADDRESS THIS POINT BECAUSE in the SUBMITTED MANUSCRIPT, WE DID NOT FIND REFERENCE NEITHER TO CT NOR TO NUTRACEUTICAL ON LINE 252-253

Line 256-268 - This section needs more references

A REFERENCE HAS BEEN ADDED

Line 268 - Could you discuss further which tests are likely to be most biologically relevant for GINs and why?

AS SPECIFIED IN THE FIRST VERSION (LINE 226 to 228, NOW LINE 259 to 261 in the REVISED MANUSCRIPT) WE HAVE INDICATED THAT "*Since the mode of action of tannin-containing nutraceuticals differs from synthetic chemical AHs, it may be of interest to examine the effects of the same plant extract on different key stages of the GIN life cycle (egg, infective larvae, adult worms)"*

Line 270-279 - In light of this statement, what extraction technique would you recommend and why?

WHAT WE WANT TO ILLUSTRATE WITH THIS SECTION IS THE STATEMENT THAT THE EXTRACTION PROCEDURE IS IMPORTANT TO ADAPT DEPENDING ON THE TYPE OF PSM SUSPECTED AND IN FACT, THAT THERE IS NO "BEST" EXTRACTION TECHNIQUE.

HOWEVER, IT HAS BEEN SPECIFIED (LINE 282-283 in the REVISED MANSCRIPT) THAT ACETONE:WATER IS THE MOST EFFICIENT SOLVENT FOR TANNINS

Line 282-292 - This section could be more concise. Are there any references to support these observations?

WE THINK THAT THE DIFFERENT POINTS MENTIONED IN THIS SECTION ARE IMPORTANT TO UNDERLINE WHY IT IS DIFFICULT TO INFER *IN VIVO* CONDITIONS FROM THE DATA OBTAINED FROM *IN VITRO* ASSAYS

Line 295-296 - 'In that case, such an approach would not be that of a nutraceutical material' - this sentence requires rephrasing, as it suggests that the nutraceutical material is taking an approach - rather than the scientist.

THE SENTENCE HAS BEEN REPHRASED (LINE 351-356 in the REVISED MANUSCRIPT)

Line 299-300 - Again, not sure about the definition of nutraceutical here - it is generally accepted that dietary supplements containing concentrated compounds from natural sources are also considered to be nutraceuticals - e.g. vitamins, concentrated herbal supplements, glucosamine

Line 315 - The same point as above

FOR THESE 2 LAST POINTS, See COMMENTS MADE TO THE REFEREE FOR LINE 152

Line 403-410 - Not particularly keen on being referred to other studies for this information, could the authors give a brief summary seeing as this is a review?

WE THINK THAT WE HAVEN GIVEN A SUMMARY OF THE MAIN RESULTS ON THE ANTHELMINTIC EFFECTS IN THE REST OF SECTION 4

Line 440-448 - Could the apparent host resilience be a direct result of the effect of CTs on infection intensity?

THE HYPOTHESIS HAS NOW BEEN MENTIONED

Line 457-459 - Is there any evidence for hypothesis no.2 ?

A SECTION WITH REFERENCES HAVE NOW BEEN ADDED (line 368-375) in the REVISED VERSION

Line 545 - 'a considerable amount of results has been' suggest reword to 'a considerable amount of data has been'

CORRECTED

Line 545-549 - Could the authors give an indication of the in vivo efficacy found in the studies referred to here?

SOME VALUES (and CORRESPONDING REFERENCES) HAVE BEEN ADDED IN THE LEGEND OF FIGURE 3

Lines 551-559 - Does this not contradict what was said in lines 293-306? i.e. of you concentrate the active compound and add it to the feed it is a plant extract - not a nutraceutical. N.B. I do not agree with this point anyway - but there is some inconsistency here

AGAIN WE HOPE TO HAVE BETTER DEFINE THE CONCEPT OF NUTRACEUTICALS THAT WE TRIED TO ILLUSTRATE IN THIS REVIEW

THE FACT THAT WE WILL CONSIDER THE POSSIBLE ADDITION OF CONCENTRATED FORM WA PREVIOUSLY MENTIONNED AND IS NOW INDICATED CLEARLY IN THE REVISED MANUSCRIPT ON LINE 95-96; see ALSO TABLE 1.

Lines 570-579 - The wine analogy is not necessary to make this point, suggest omit this paragraph

THIS SECTION HAS NOW BEEN SUPPRESSED

Line 608 - suggest use 'areas' instead of 'axes'   CORRECTED

Line 609 - Grammar - suggest 'targets for' instead of 'targets with'   CORRECTED

Line 624- 625 - are not medicinal plants also technically nutraceuticals?

THE TERM “MEDICINAL PLANTS”HAS BEEN REPLACED BY “HERBAL REMEDIES” AND REFERENCE MADE TO TABLE 1 WHERE THE DIFFERENCES BETWEEN HERBAL DRUGS AND NUTRACEUTICALS ARE EXPLAINED

Line 633 - spelling – sporulation CORRECTED

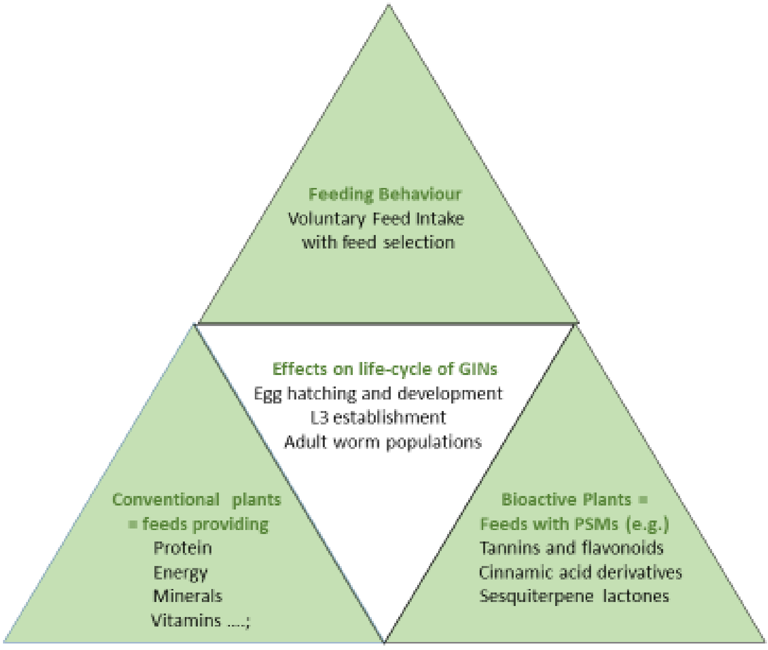
Lines 691-698 - Self-medication is mentioned a couple of times in this review, it is an interesting concept, could the authors briefly summarise the evidence for this taking place with plants containing CTs, even though there has been a recent review?

ACCORDING TO THE RECOMMENDATIONS OF THE FIRST REFEREE, WE HAVE NOW COMPLETED THIS SECTION 5.3 ON SELF MEDICATION WITH SEVERAL REFERENCES WHICH ILLUSTRATE THE DIFFERENT POINTS EVOKED. IN ADDITION, TWO REVIEWS ON THE SUBJECT ARE MENTIONNNED NOW WHERE THE READERS WILL FIND DETAILED INFORMATION. WE THINK THAT DEVELOPMENTS ON THE SELF MEDICATION IS NOT DIRECTLY RELEVANT TO THE OBJECTIVE OF THIS REVIEW.

Line 728 - spelling 'phytochemistry' CORRECTED

*Highlights (for review)

TANNIN CONTAINING LEGUMES AS A MODEL FOR NUTRACEUTICALS AGAINST DIGESTIVE PARASITES IN LIVESTOCK



A proposed model of the components defining the concept of nutraceuticals against gastrointestinal parasites in livestock

TANNIN CONTAINING LEGUMES AS A MODEL FOR NUTRACEUTICALS AGAINST DIGESTIVE PARASITES IN LIVESTOCK

H.HOSTE [1,2], J.F.J. TORRES –ACOSTA[3], C.A. SANDOVAL-CASTRO[3], I. MUELLER-HARVEY [4], S. SOTIRAKI [5], H. LOUVANDINI [6], S.M. THAMSBORG [7], T.H. TERRILL. [8]

[1/] INRA, UMR 1225 IHAP, 23 Chemin des Capelles, Toulouse F-31076, France

[2/] Université de Toulouse, ENVT, 23 Chemin des Capelles, Toulouse F-31076, France

[3/] Campus de Ciencias Biológicas y Agropecuarias, FMVZ, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, Merida, Yucatan, Mexico.

[4/] University of Reading, School of Agriculture, Policy and Development, 1 Earley Gate, P.O. Box 236, Reading RG6 6AT, United Kingdom

[5/] Veterinary Research Institute – Hellenic Agricultural Organization Demeter, 57001 Thermi, Thessaloniki, Greece

[6/] Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil,

[7/] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark,

[8/] Fort Valley State University, 1005 State University Drive Fort Valley, GA 31030, USA

Corresponding Author: (H.H.) phone/fax: + (33) 05-61-19-38-75 / + (33) 05-61-19-32-43. E-mail: h.hoste@envt.fr

ABSTRACT

Parasitic infections with gastrointestinal nematodes (GINs) still represent a worldwide major pathological threat associated with the outdoor production of various livestock species. Because of the widespread resistance to synthetic chemical anthelmintics, there is a strong impetus to explore novel approaches for a more integrated management of the infections. The use of nutraceuticals in the control of GINs is one of the alternatives which has been widely studied for ~~since~~ 20 years.

The objectives of this review are: i) to define and illustrate the concept of 'nutraceutical' in the context of veterinary parasitology based on data obtained on the most studied GIN models in small ruminants, the tannin-containing legumes (Fabaceae); ii) to illustrate how the 'nutraceutical concept' could be expanded to other plants, other livestock production systems and other GI parasitic diseases, and iii) to explain how this concept is opening up new research fields for better understanding the interactions between the host, the digestive parasites and the environment.

KEY WORDS: Nutraceuticals / Condensed tannins/ Polyphenols / Gastrointestinal nematodes / Antiparasitic effects / Small ruminants.

1. INTRODUCTION

Parasitic infections with gastrointestinal nematodes (GINs) represent a worldwide major pathological threat associated with the outdoor production of various livestock species, particularly ruminants. Up to now, the control of these parasitic helminth diseases has essentially relied on the repeated use of commercial anthelmintic (AH) drugs. However, resistance to these AH drugs is now widespread in worm populations, and the occurrence of multi-resistant strains has become a serious problem in some regions of the world (Kaplan, 2004; Torres-Acosta et al., 2012, Jackson et al., 2012). Moreover, as underscored by Waller (2006), resistance to xenobiotics develops quite rapidly and usually within 10 years. This has been illustrated since the recent launch of monepantel (Kaminsky et al., 2008), which is a drug belonging to the Amino Acetonitrile Derivatives (AADs). In spite of the novel mode of action, reports of monepantel resistant *Haemonchus contortus* have already appeared only seven years after the launch of this new drug (Van-den-Brom et al., 2015).

Therefore, modern parasite management involves an alternative approach seeking to achieve an integrated and more sustainable control of parasite infections by combining the 3 main principles for GIN control, namely **i)** management of grazing systems; **ii)** stimulation of host response and **iii)** modulation of worm biology (Hoste and Torres-Acosta, 2011). This also explains the strong impetus worldwide for exploring and adapting alternative options to different conditions. Among these novel approaches, the long tradition of using bioactive plants for their anti-parasitic properties has been re-explored. It is now ~~and~~ considered ~~identified~~ as one option for improving GIN control, and therefore, to counteract the negative pathophysiological consequences in hosts (Rochfort et al., 2008). Plants are widely used as phytotherapeutic drugs or herbal remedies, and their administration is based on a long tradition of ethno-veterinary or ethno-medicinal applications on all continents (Hammond et al., 1997; Sandoval-Castro et al., 2012). However, over the last 20 years, a novel, overall concept of nutraceutical plants (plants combining positive effects for both animal nutrition and health) has emerged in veterinary science and helminthology for improved controlling livestock parasites (Waller and Thamsborg, 2004; Hoste et al., 2006; Alonso-Diaz et al., 2010b).

The aim of this review is

1/ to illustrate ~~define~~ the concept of 'nutraceutical' based on resources containing Plant Secondary Metabolites (PSMs) in the context of veterinary parasitology. We will mainly refer to the tannin-containing legumes (Fabaceae) and other plants, which is ~~,~~one of ~~by evaluating~~ the most studied ~~GIN~~ models to control GIN in ~~of~~ small ruminants~~, i.e.~~. ~~the tannin-containing legumes (Fabaceae) and other plants.~~

2/ to show~~illustrate~~ how the 'nutraceutical concept' could be expanded to other plants, other livestock production systems and other parasitic diseases of the digestive tract.

3/ to explain how this concept is opening up new research fields for probing the interactions between the host, the parasites of the digestive system, and the environment.

2. DEFINITION OF NUTRACEUTICALS

The term "nutraceutical" results from a portmanteau between the words "nutrition" and "pharmaceutical". It ~~and~~ has sometimes also been described as "functional food", which was first coined in the context of medical health (Hasler, 1998). ~~Nutraceuticals are nutritional products that provide health and medicinal benefits.~~ According to Andlauer and Fürst (2002), a nutraceutical is defined as "any substance that may be considered a food or part of a food which provides health benefits, including the prevention and treatment of disease". In a similar way, a nutraceutical in veterinary science can be defined as a livestock feed which combines nutritional value with beneficial effects on animal health. In contrast to pharmaceutical drugs, nutraceuticals are not synthetic compounds formulated for specific indications. ~~Instead,~~

Here, we will focus on a sub-category of nutraceuticals, i.e. ~~they are~~ plant-based products that contain nutrients and bioactive compounds that can be used **either directly** as part of the animal~~plant~~ diet or **added in a concentrated form** to another diet after extraction from the bioactive plant or resource~~. Simply put, a nutraceutical in veterinary science can be defined as a livestock feed which combines nutritional value with beneficial effects on animal health~~. This double action is suspected to rely~~based~~ on the presence of various plant secondary metabolites (PSM) or bioactive compounds.

Although the nutraceutical concept sounds simple, it is important to recognise that there are several key differences compared to synthetic AH and phytotherapeutic drugs (Table 1). This makes studying their efficacy a rather complex task. The key points and differences between nutraceuticals and the other options for interfering with the parasite life cycle will be discussed below.

1/Nutrition: Compared to herbal drugs (phytotherapeutical remedies) or synthetic chemicals, a nutraceutical based on bio active plants is not imposed, but offered to animals over a relatively long period (from several days to weeks or months). Therefore, the efficacy of this sort of~~a~~ nutraceuticals will directly depend on the Voluntary Feed Intake (VFI) of the animal~~. The interaction between PSM ingested from different plants needs also be considered when nutraceutical feeds are supplemented, especially when animals are freely grazing/browsing on natural pastures or woodlands.~~.

2/ Nature of the bioactive compounds (PSM): The bioactivity against the parasites is dependent on the presence of natural chemical compounds in plants, which are usually described as Plant Secondary Compounds (PSM). The concentrations of these compounds can be highly variable and may depend on environmental growing conditions, on the plant cultivar, or chemotype. In addition, technological factors (harvesting, processing methods) can also interfere with the preservation of the active compounds.

3/. **Effect on animal health:** The effects of nutraceutical feeds and of the associated bioactive compounds against parasitic nematodes differ from the mode of action of synthetic chemicals (see figure 3). This statement implies to develop specific methodologies of measurements of the AH effects.~~.~~

3. TANNIN CONTAINING LEGUMES WITH ANTHELMINTIC PROPERTIES AGAINST GINS IN SHEEP AND GOATS: THE MOST STUDIED MODEL OF NUTRACEUTICALS IN VETERINARY PARASITOLOGY.

It is only recently (in the last 20 years) that researchers have obtained enough evidence that supports the use of plants as nutraceutical feeds against GIN. The first evidence originated from research in New Zealand based on empirical results that reported significant reduction of GIN faecal egg counts in sheep when grazing pastures with different legumes, i.e. birdsfoot trefoil and big trefoil (*Lotus corniculatus, L. pedunculatus*), sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychis viciifolia*), which contain condensed tannins (CTs) (Niezen et al., 1995, 1998, 2002). The information generated by these early field trials started the search for new legumes (Fabaceae) and for other plant candidates elsewhere in the world against GINs in small ruminants. This has become the subject of extensive studies and still represents an important model aiming to explore the concept of using nutraceutical plants against other parasites, with other hosts, and to identify other botanical families with nutraceutical properties (Sandoval-Castro et al., 2012).

These studies on tannin-containing legumes have led to:

1/ the development of several important methodological procedures that are based on *in vitro* and *in vivo* methods to evaluate plants as potential nutraceuticals;

2/ a better definition of the difference in effects between nutraceuticals, phytotherapeutic drugs, and synthetic AH chemicals.

3.1. Identification/selection of future potential candidates of nutraceuticals against GINs.

As illustrated in Figure 1, the effects of a nutraceutical against GIN depend on complex interactions. Hence, the selection of a plant candidate as a potential nutraceutical requires good knowledge and interaction between different scientific disciplines, namely livestock nutrition and production, phytochemistry, parasitology, and to some extent, ethology and toxicology. However, the presence of bioactive PSM is a key issue.

Identifying viable nutraceutical candidates against parasites is a complex task, and the following steps need to be considered in the selection of nutraceutical materials. This procedure is intended to help in identifying candidate plant materials that can subsequently be explored under *in vivo* conditions.

3.1.1 Nutritional issues.

Even before the plant materials are tested *in vitro* or *in vivo* for AH activity, information should be gathered (or evaluated) in terms of nutritional value and palatability for the hosts. This information should include:

***a~~A~~**) A good macronutrient profile.*

Ideally, the feed should have medium to high levels of protein or energy and low lignin. However, not all plants with tannins may have an optimal “nutritional”profile. Therefore, initial selection of such plants should not be based solely on their macronutrient profile, especially if such plants are selected by ruminants during grazing/browsing. The nutritional quality of the plant material that is intended for nutraceutical use should include the determination of its *In Vitro* Dry Matter Digestibility (IVDMD)~~IVDMD~~. This information can help to identify if the plant material could be useful under *in vivo* conditions.

b~~B~~) A feed readily consumed by the animal host.

Since the general definition of a nutraceutical is linked to VFI~~voluntary feed intake~~ by animals, this key issue needs to be first considered and then measured during *in vivo* assays. Short intake or preference trials should be carried out before engaging with *in vivo* evaluation of the plant. If the plant is not eaten, it might still be useful in other ways, as PSM ~~-~~could be extracted and then dosed (Phytotherapy).

c~~C~~) An acceptable in vivo apparent digestibility.

Any plant material that will be used in large quantities or during long periods of time will require an evaluation of its impact on the apparent *in vivo* digestibility, including at least measurements of the dry matter (DM) and the organic matter (OM) ~~measurements~~. These data may help to ensure that the nutraceutical material will not cause a severe digestibility reduction.

***d~~D~~)** Little or no negative known impact on animal production.*

Before selecting a nutraceutical plant for AH effects, it is important to collect information on the possible negative impact on production (i.e. growth, milk production, reproduction) or even clinical signs (e.g. abortions or foetus deformities, such as arthrogryposis due to phytoestrogens) or photosensitisation. In such cases, the plant material should not be considered as a viable nutraceutical.

3.1.2 PSM issues

Since the early results in NZ, several tannin containing legumes (Fabaceae) have been subjected to a wide range of studies as potential nutraceuticals. The focus on these species was not based on prior ethno-botanic information about the action against signs of GIN parasitism (i.e. diarrhoea), or de-wormers used for humans, as in both cases the information could be inadequate for ruminant species (Sandoval-Castro et al., 2012).

In contrast, they were mainly supported by knowledge and principles of pharmacognosy, based on the assumption that the presence of similar PSMs in the same botanical families (Fabaceae) should achieve similar antiparasitic effects. However, a few results have shown that some plant candidates, which were retained because of the presence of CTs, also corresponded to plants that had been identified based on ethnoveterinary knowledge. This double-sided approach can be of particular interest in many tropical countries.

A possible third approach could be based on detailed observational studies to identify plant selection (González-Pech et al, 2014), feeding behaviour, resource selection and possibly self-medication under grazing or browsing conditions. However, such studies are laborious and few exist from tropical regions, where there is a large plant diversity (Novelo-Chi et al, 2014, Retama-Flores et al, 2010 and Ventura-Cordero et al, 2014a, 2014b).

The Fabaceae family is one of the richest botanical families in terms of species numbers (nearly 20,000) and tannin-containing legume and plant resources occur worldwide, just like GINs, and, therefore, the search for possible nutraceuticals has included temperate and tropical tannin-containing plants. These include herbaceous plants, trees, and shrubs, which are perennials or annuals, and which are used by small ruminants in a wide range of feeding systems.

In temperate areas, two legume species of the sub family Faboideae, have received special attention ~~and~~ and have been the focus of a number of studies. These are ~~, namely~~ sainfoin (*Onobrychis viciifoliae*) in Europe (Paolini et al., 2003a, 2005b; Heckendorn et al., 2006, 2007; Manolaraki et al., 2010; http://sainfoin.eu; www.legumeplus.eu) and Chinese bush clover or sericea lespedeza (*Lespedeza cuneata*) in the USA and South Africa (Shaik et al., 2006; Joshi et al., 2011; Kommuru et al., 2014; Terrill et al., 2009; 2012 http://www.acsrpc.org/). This special attention on these two herbaceous plants is explained by their ability to produce seeds, to be grown (cultivated) efficiently, and to organise their production for farm application on a large scale (see below).

It is worth underscoring that this scientific interest is also explained by their other beneficial effects in regards to nutrition and health of ruminants and environmental issues (see reviews by Rochfort et al., 2008, Waghorn, 2008, Mueller Harvey, 2006): i) a reduced use of chemical fertilisers because of biological nitrogen fixation; ii) high palatability for ruminants and feeding values; iii) positive effects to reduce ruminal methane emission and GHG; iv) anti bloat effects; v) switch of nitrogen excretion from urine to faeces. In contrast, it is also worth noting that some non-native species exploited for these properties are considered invasive by environmentalists and ecologists

In tropical areas in Africa and Latin America, several genera (shrubs or trees) of the Fabaceae family have been particularly under focus and identified as possible nutraceuticals because of *in vitro* results or *in vivo* reductions in egg excretion; e.g. *Leucaena leucocephala* (Ademola et al., 2006), *Lysiloma latisiliquum* (Matínez-Ortíz de Montellano et al., 2010), *Havardia albicans* (Galicia-Aguilar et al., 2012; Méndez-Ortíz et al., 2012), or different Acacia species: *Acacia pennatula* , A. *molissima* , *A. karroo, A. mearnsii, Mimosa tenuiflora* (Kahiya et al., 2003; Alonso Diaz et al., 2008a; Minho et al., 2008; Cenci et al., 2007; Oliveira et al., 2013, Murare et al., 2012).

3.2 A methodological procedure to confirm the antiparasitic effects.

A methodological procedure has progressively emerged from scientific results from different studies to validate the possible efficiency of a wide range of tannin-containing plants as nutraceuticals against GINs. By its~~the~~ overall, logical scheme ~~/organisation~~ and~~-~~ by the objectives defined for ~~of~~ each step, the procedure has been widely adapted from the WAAVP guidelines to examine the efficiency of synthetic AHs (Wood et al., 1995).

The three successive steps are **1/** the *in vitro* screening of plant extracts based on a wide range of assays (Figure 2); **2/** controlled *in vivo* studies on experimentally infected animals to confirm the efficiency of the selected plant candidates; **3/** holistic studies to examine how and when to apply the nutraceuticals in breeding/feeding systems for small ruminants.

However, because of the complexity and intrinsic variability of nutraceuticals, which are based on naturally occurring chemicals rather than synthetic anthelmintics (see Table 1, Quality of the compounds and mode of administration), this scheme requires adaptations and critical comments to interpret the results.

Since the mode of action of tannin-containing nutraceuticals differs from synthetic chemical AHs, ~~(see point 4)~~ it may be of interest to examine the effects of the same plant extract on different key stages of the GIN life cycle (egg, infective larvae, adult worms) (Figure 2).

3.2.1 T*he in vitro* screening of plant extracts.

Most *in vitro* assays for the primary screening of plant products have been adapted from those developed to assess the efficacy of synthetic AHs against GINs in ruminants (Wood et al., 1995). One exception is the Larval Exsheathment Inhibition Assay, which has been set up specifically (Bahuaud et al., 2006) to reproduce artificially, under *in vitro* conditions, disturbances related to the establishment of infective larvae as measured *in vivo* in sainfoin-fed sheep (Brunet et al., 2007). The detailed methodologies of these different assays, when adapted to plant materials, can be found in Jackson and Hoste (2010). In addition, it is worth mentioning that the model represented by the free living nematode, *Caenorhabditis elegans*, is also increasingly being exploited to screen the AH properties of different plant resources (Katiki et al., 2012, 2013).

It is important to emphasise that these different *in vitro* assays, first used to screen potential nutraceutical candidates, have also been exploited for a second main objective: to better understand the mode of action of PSM compounds against GINs. This has been achieved **i)** by applying either isolated and purified tannins, which were selected because of their -different structural features (Molan et al., 2004, Novobilsky et al., 2013, Quijada et al., 2015), or pure flavonoid compounds (Molan et al, 2003a; Brunet and Hoste, 2006), and **ii)** by performing detailed studies of the functional and structural changes induced in nematodes (Hoste et al., 2012). This allowed testing of the "direct" hypothesis (see section 4.1).

Most research groups started screening the AH activity of different plant materials under *in vitro* conditions. In most cases, the screening process was developed with plants that had been extracted with different solvents. When tannins are suspected as the main bioactive compounds, extraction with acetone/water (70:30) is the most common and efficient solvent in regard of the yield of extraction. We note that several studies reported drying plant materials at high temperatures and that this may have reduced their AH activity. It is highly recommended that all plant samples should either be freeze-dried or dried below 40 °C in order to preserve polyphenols and tannins, as these are easily oxidised at higher temperatures.

In addition, because of the intrinsic variability of the active compounds and/or mode of actions against GINs, many misunderstandings need to be clarified:

***a~~a~~)** If a plant extract shows an AH effect against a given life stage, it will also show a clear AH effect against other life stages of the same parasite, at roughly the same concentrations.*

This is not the case. If the same extract from a single plant material is applied to different life stages of a nematode~~parasite~~ species under the respective *in vitro* conditions, the AH effect may differ between different life stages (Paolini et al, 2004). For instance, a plant extract may show a clear AH effect against *H. contortus* on a given life stage (i.e. L_3 larvae) and show a mild or no AH effect against another stage (i.e. eggs). Furthermore, the AH effect may only be evident on a certain aspect of the same life stage, such as inhibition of the L_3 exsheathment process, but have no clear effect on another aspect, such as the motility of the same L_3 larvae (Alonso-Díaz et al., 2011). This differential effect on different life stages of GIN has also been recorded for conventional anthelmintics, such as levamisole, that show a clear effect on L_3 motility but fail to show any AH effect on the exsheathment of L_3 or the eclosion of GIN eggs. The latter imply that the selection of candidates for further *in vivo* tests could be judged either on the overall effective concentration (EC 50, 90 or 99%) of different *in vitro* tests with the different life stages tested.~~, or,~~ A~~a~~lternatively, the candidate should be the one showing the lowest EC in the most biologically relevant *in vitro* test chosen by the research group on valid biological grounds, depending on the stage of interest.

***b)** If the plant extract is obtained from the same plant material with a solvent of similar polarity, the potential AH effect against GIN will be similar.*

This is also not the case. Different extraction procedures, even with solvents of similar polarity (i.e. methanol vs. acetone:water (70:30) applied to the same plant material, will result in different PSM being extracted. As a result, the extracts of the same plant material will show different AH activities even when applied to the same life stage. For example, acetone-water (70:30) extracts of Annonaceae leaves showed limited ovicidal activity and a clear exsheathment inhibition activity, while the methanolic extraction of the same plant material showed good ovicidal activity and less clear exsheathment inhibition activity (Castañeda-Ramírez et al., 2014). Thus, the extraction procedure, even with solvents of similar polarity, can result in marked differences in the extracted PSM.

c*) *An in vitro screening test will help to decide on the dose or concentration of the nutraceutical material that needs to be consumed by the infected animals in order to control GIN infections.

The extrapolation of *in vitro* doses to *in vivo* conditions is more difficult to achieve for plants when used against parasites dwelling in the lumen of the gastrointestinal tract. A plant extract used *in vitro* contains a concentrated quantity of PSM from the experimental plant material. Those PSM will be present in the *in vitro* assay~~system~~ at a certain quantity and quality that may not be comparable with the *in vivo* conditions, and also during a time span difficult to replicate under *in vivo* conditions. Besides, under *in vivo* conditions, the digestion processes and the conditions occurring in the digestive tract may affect the liberation of the PSM from the plant material. Also, the quantity or structure of PSM may be affected along the digestive tract after the plant is consumed, chewed, ruminated and digested by the ruminant host or gut microflora. Thus, research groups must remember that *in vitro* assays can help at identifying candidates with potential AH activities, but will not produce a dose level that can be used as a starting point for the *in vivo* dose level that should be applied to the various hosts.

With the limitations mentioned above, one may think that it might be easier to find a plant extract with excellent *in vitro* activity, validate its ~~'s~~ *in vivo* AH activity against GIN in the relevant host, and then determine~~identify~~ a dose that can be used to control their GIN populations. S~~In that case, s~~uch an approach would not be pertinent for ~~that of~~ a nutraceutical plant material but can~~. It might~~ be considered for an herbal remedy or phytotherapeutical medicine.~~, and,~~ A~~a~~s such, this will need to be tested as a drug and its efficacy validated using the relevant guidelines for efficacy against GIN (Wood et al., 1995).

Why can these extracts not be considered as a nutraceutical? The reason is simple: if the plant material is not providing macronutrients for the animal, then it is not a nutraceutical material. In reality, there may be a more straightforward method for identifying plant materials with potential nutraceutical effects and for investigating the dose level required to control the GIN population: letting the hosts eat the plant material, while evaluating the faecal GIN egg excretion. If animals are able to eat sufficient plant material in the chosen presentation and form and produce a visible AH effect, then such level of ingestion (or dosage) may be suggested against parasites in the test animals.

3.2. 2 Confirmation of potential nutraceutical candidates, under *in vivo* controlled conditions, based on *in vitro* results.

As for the 1st step (i.e. *in vitro* screening), the *in vivo* procedures to assess the efficacy of nutraceuticals against GINs are also derived from the general guidelines described for synthetic chemical AHs (Wood et al., 1995). However; it is important to take into account several specificities of the nutraceutical concept (see Figure 1).

a) *A nutraceutical is at first a feed*

Therefore, it is essential to evaluate nutritional value by using conventional methodologies. The information should include the macronutrient content of crude protein (CP), metabolisable energy (ME), neutral detergent fibre (NDF), acid detergent fibre (FDA) and lignin. This information may help to identify plant materials fitting the nutritional characteristics of an edible ruminant feedstuff. The information generated from conventional nutritional methodologies could be correlated with the near infra-red (NIR) spectra. With time, when a large dataset of plant samples are tested, the NIR spectra can be linked with nutritional quality, presence of PSM and related AH effects. This may be automated with other NIR tools in the future. One key aspect of these measurements for controlled *in vivo* studies is to achieve iso-proteic and iso-energetic diets in the groups of animals receiving the control or the nutraceutical diets, in order to really evaluate the role of the PSM and to avoid any confounding effects because of differences in the amount of macronutrients (protein, energy or their balance) (Coop and Kyriazakis, 1999). However, differences in palatability may result in different feeding levels when feed on offer is not controlled.

Alternatively, assessing the trade-off of plant selection and intake could be an alternative for when iso-energetic and iso-proteic diets are not attainable. The working hypothesis should be carefully stated in all cases. Nutrient balance studies are needed to contribute to the understanding of parasitism and nutrient costs (for the host) and proper assessment of trade-off of nutraceutical plant intake.

b) *A nutraceutical should be readily consumed by the relevant host. The effects on health depend on the animal feeding behaviour*

Most plants materials meant to be used for nutraceutical purposes are not normally included in the diet of the relevant host. Since the general impact of nutraceuticals on animal health stems from the consumption of these materials, it is important: (1) to include in the experimental design of controlled *in vivo* studies a period for animal adaptation to the novel feed, lasting from 10 to 15 days, until experimental animals reach a plateau of consumption; and (2) once this plateau of consumption is reached, to estimate regularly the animal VFI. The evaluation can be as simple as measuring the plant material offered and refused, to determine intake. Under some circumstances, the ingestion of the plant material can be enhanced by means of some additives, such as sugar cane molasses. In such cases, the future use of these plant materials will depend on the availability of the plant *per se*, as well as the existence of the potential feed additive. This could mean an added difficulty for on-farm applications. If possible, plant intake should be evaluated with both infected and non-infected animals with the objective to provide information on self-medication (Martinez-Ortiz-de-Montellano et al, 2010).

c) The impact of nutraceuticals against the parasite depends on the presence of PSM

It is essential to obtain a measurement of the potential bioactive PSM before implementing the *in vivo* controlled studies. It is also important to describe the analytical methods used. In the case of condensed tannins, which cover the core of the current review, a critical description of the different analytical methods has been reviewed previously (Mueller-Harvey, 2006). One simple recommendation, when possible, is to try to obtain two different evaluations (usually one chemical assay and one biological assay in order to estimate the ability of tannins to complex proteins) (Makkar et al., 2003).

d) The effects of tannin-containing nutraceuticals differ from synthetic AH

And this requires adapted experimental designs and measurements. Once the information to characterise the nutritional value and the PSM content of a nutraceutical have been obtained, valid *in vivo* assays in controlled conditions can be implemented. Such studies could consider animals with natural GIN infections. However, artificial infection trials are preferred that focus on the most prevalent GIN genera and species in the areas of interest.

Within this general experimental context, several factors need to be explored which are specific to nutraceuticals

- ~~1/~~ *The multivalent effects on a range of GIN species:*

  As a general recommendation, combined infections with at least one species of the abomasal and one species of the small intestinal species should be encouraged. The reason for this is that i) as part of the overall objective, as for synthetic AHs, a multivalent effect can be expected from nutraceuticals and also, ii) variations in the effect of tannin-containing resources against different GIN species have been mentioned on several occasions (see Hoste et al., 2006, 2012). Of course, the choice of the GIN models should be based on epidemiological information, which needs to identify the most prevalent and pathogenic species in the regional areas.

- ~~2/~~ *The multivalent effects on different GIN stages:*

As shown~~illustrated~~ in ~~below (section 4,~~ Figure 3~~)~~, thee -consequences of -nutraceuticals and related PSMs on GINs (at least for the tannin-containing legume models), result from a combination of effects on 3 key stages of the GIN life cycle. Therefore, when possible, it is important to design experimental studies that examine the effects on these different stages (Castañeda-Ramírez et al., 2014; Vargas-Magaña et al., 2014a, 2014b).

- ~~3/~~ *The role of quantity and/or time period for offering tannin-containing resources in the diet to optimise effects against GINs in small ruminants*

After completing initial simple experimental designs that validate the effects of tannin-containing nutraceuticals against different genera and/or stages of GINs in small ruminants, some additional studies are worth designing in order to help in evaluating the effect of two factors which can influence the efficacy of nutraceuticals against GINs, namely the proportions of nutraceuticals and the related concentration of CTs in the feed (Athanasiadou et al., 2001, Brunet et al., 2007, Terrill et al., 2009) and the length of distribution.

- ~~4/~~ *Specificities in the evaluation of the parasitological and pathophysiological consequences*

During the whole evaluation period in studies based on experimental infections, animals should be monitored regularly in terms of **i)** repeated parasitological measurements (faecal excretion of GIN eggs), ii) pathophysiological measurements to establish the effects on host resilience based on quantitative and/or semi-quantitative measurements of the (sub)clinical effects of infection (e.g. Packed Cell Volume = PCV).

Because of the variations in the PSM effects, which can depend on the nematode species and/or stages (see Figure 3), two experimental measurements are worth considering to complete the information obtained from experimental *in vivo* studies: 1/ it is recommended that these methodologies need to differentiate effects from different GIN species in case of multispecific experimental infections; and 2/ whenever possible, to measure also effects on egg hatching and development.

Ideally, the studies in controlled conditions should be complemented with post-mortem evaluations of the worm populations. The latter will help to strengthen the parasitological protocol by defining if the effect on GIN egg excretion is due to the reduction of GIN populations, proportions of species, sex ratio, or reduction in female worm fecundity (Martinez-Ortiz de Montellano et al., 2010; Galicia-Aguilar et al., 2012).

4 TANNIN-CONTAINING LEGUME NUTRACEUTICALS AGAINST GINs~~S~~ IN RUMINANTS

Several (recent) reviews have summarised the existing results on the potential of tannin containing legumes as nutraceuticals. Their focus concerns three different aspects:

- the overall effects on ruminant nutrition, health and production (e.g. Waghorn, 2008; Mueller-Harvey, 2006; Rochfort et al., 2008 ; Wang et al., 2015)
- the specific AH effects (Min and Hart, 2003; Hoste et al., 2006, 2012)
- the mode of action against the GINs by understanding the nature of active compounds (tannins and flavonoids) (Mueller-Harvey, 2006) and their possible effects on the worms (Hoste et al., 2012).

The readers are invited to refer to these different reviews. The aims of this section will be **i)** to provide an updated summary of the main results of basic research studies that describe the antiparasitic bioactivity of tannin-containing legumes against GIN nematodes in small ruminants and the current hypotheses on their mechanisms of action; and **ii)** to illustrate possible on-farm applications, which are starting to be developed.

4.1 Updated summary of the main effects on the GIN life cycle and hypotheses on their modes of action.

4.1.1. Impact on the GIN life cycle

Three main potential impacts have been linked to the intake of tanniniferous plants by infected ruminants (Hoste et al., 2012) on the GIN life cycle (illustrated in Figure 3)

1/ lower establishment of the infective third-stage larvae in the host.

2/ lower excretion of nematode eggs by adult worms, related either to a reduction in worm numbers or lower fertility of female worms; and

3/ impaired development of eggs into third-stage larvae.

Point 1 leads to reduced host invasion by third stage larvae. Steps 2 and 3 both contribute to reducing the environmental contamination with parasitic elements and thus to reduce the risks for hosts.

To summarise, tanniniferous nutraceuticals do not lead to a 100 % elimination of worms, but they can interfere with the life cycle; whereas the main goal of synthetic AHs is to completely break the parasitic life cycle. The role of nutraceuticals can be linked to severely impairing several key biological stages of the life cycle (eggs, infective larvae, adult worms). The potential combined effect of these separate impacts contributes to slowing down the dynamics of the infection and to lowering the rate of infection to levels that enable acceptable productivity and animal welfare. This also provides an opportunity for the animal to develop its own immunity to the parasites.

Similar effects have been observed with the main nematode genera that infect the abomasum and small intestine of small ruminants (*Haemonchus* spp, *Teladorsagia* spp, *Trichostrongylus* spp). Some recent results (Gaudin et al, 2015 Abstract to WAAVP 2015) have demonstrated that significant reductions in egg excretion also occurred with a multi-resistant isolate of *Haemonchus contortus* in the presence of nutraceutical plants. This confirmed that tanniniferous nutraceuticals do indeed represent an alternative solution to AH resistance that is worth exploring further.

It is worth mentioning that these AH effects that affect the worm biology have also repeatedly been linked to positive effects on host resilience (e.g. better production parameters, less severe signs of diarrhoea or anaemia, lower mortality under parasitic challenge) ever since the early studies in NZ

(Niezen et al, 1996). The mechanisms explaining this improved resilience remain obscure but can partly be explained by the effects on reducing the worm population as well as by the nutritive value of legumes (i.e. higher protein content) and the rumen-escape effect that occurs in the presence of certain types of tannins, which are able to protect dietary proteins (Waghorn, 2008). Identification of which particular tannins are best able to improve host resilience remains an outstanding research goal. Therefore, it is of paramount importance to underline the need to achieve iso-proteic and iso-energetic diets in control vs nutraceutical diets that are fed to sheep or goats in controlled *in vivo* studies.

4.1.2. Hypotheses on the modes of action

Two non-exclusive general hypotheses have been proposed to explain the activity of bioactive tannin containing feeds against gastrointestinal parasitic worms (Hoste et al., 2012):

1/ The " direct " hypothesis is based on pharmacological-type of interactions between the various polyphenols and the different stages of gastrointestinal nematodes.

2/ The "indirect" hypotheses assumes a possible improvement of the host resistance (i.e. an immunologically based response) because of the effects of tannin-containing feeds that can improve the overall host protein nutrition by increasing the amount of by-pass proteins .

It is clear that the bulk of *in vitro* data obtained so far with different tannin-containing extracts support the hypothesis of "pharmacological-like" effects.

As previously stated (Hoste et al, 2012), numerous studies on GIN from small ruminants strongly suggest that both CTs **and** various flavonoids (at least some flavanols) contribute to the AH action (Molan et al., 2004, Brunet and Hoste, 2006). This has been supported also by recent results on another nematode model (e.g. *Ascaris suum* in pigs; Williams et al., 2014b). New evidence is also starting to emerge that tannins and other flavonoids in combination can generate either favourable (synergistic) or unfavourable (e.g. antagonistic) interactions (Klongsiriwet et al., 2014; Vargas-Magana et al., 2014a).

A dose-dependent relationship between tannins and/or flavonoid monomers and their *in vitro* effects has been found on several occasions with different assays and GIN species (see review by Hoste et al., 2012). A few *in vivo* studies have also described such relationships, where effects of a tanniniferous source on infective L3 larvae (Brunet et al., 2007) or on adult worm populations (Athanasiadou et al, 2001, Terrill et al., 2009) were examined. Taken together, it would appear that a CT threshold needs to be reached in the diet in order to achieve AH effects against the worms.

In addition, evidence is now accumulating from various *in vitro* studies which have sought to unravel the mode of action by tannins on the worms, that, besides quantitative factors (i.e. concentrations), qualitative factors (i.e. compound structures) can also modulate the bioactivity. Recent data obtained with GIN models from cattle, small ruminants, and pigs (Novobilsky et al., 2011, 2013; Quijada et al., 2014; Williams et al., 2014a) lend support to the hypothesis first raised by Molan et al. (2003) that bioactivity is linked to the prodelphinidin/procyanidin (PD/PC) ratio in plants. These recent studies have also revealed that tannin size is an important structural feature that contributes to the extent of the AH effects. As CTs usually occur in complex mixtures of closely related tannin compounds, their average polymer size is measured and reported as their mean degree of polymerisation (Gea et al., 2011).

Less studies have examined the “indirect” hypothesis, based on long –term studies and by measuring different effectors cells (mast cells, globule leucocytes, eosinophils, goblet cells) along the digestive tract in sheep (Tzamaloukas et al., 2006a and b; Martínez-Ortíz-De-Montellano et al., 2010; Rios de Alvarez et al., 2010) and goats (Paolini et al., 2003) when receiving different TR resources.Some of the most convincing evidence to support the “indirect hypothesis” is that some measurements indicating an enhanced local immune response (significantly higher numbers of mast cells and globule leucocytes) (Tzamaloukas et al.,2006) has been shown when lambs were consuming sulla or chicory and had a reduced development of worms.

~~**Figure 3:** Three key stages of the GIN life cycle have been identified as possible targets when tanniniferous plants are consumed by infected small ruminants: 1/ a reduced excretion of nematode eggs by the adult worms; 2/ a reduced establishment of the infective third-stage larvae in the host; and 3/ a reduced development of eggs to third-stage larvae.~~

4.2 Strategies to apply tannin-containing nutraceutical materials on-farm

The bulk of current data on potential nutraceuticals stems from *in vitro* assays. These results provided preliminary information on the future use of various plants or plant resources. However, gaps remain between *in vitro* results and on-farm application and these are outlined in section 3.

A few resources have been investigated more thoroughly through extensive approaches that included a succession or combination of *in vitro* assays, controlled *in vivo* studies and sometimes holistic studies in farming systems in order to prepare for implementation under field conditions. Fortunately, these specific plants illustrate various options that can be used to exploit tannin-containing resources as nutraceuticals.

4.2.1 Cultivating legumes as nutraceuticals

The availability of the plant material can be considered as the main constraint for using a nutraceutical material on-farm. If the plant material is not widely available, or the cost and effort of producing such material is high, then there will be insufficient quantities and it will be unlikely that farmers can adopt it. Up to now, very few plants exist that have potential for nutraceutical exploitations and that benefit from the availability of solid agronomic information suitable for large-scale production.

In some parts of the world, where nutraceutical legume plants can be sown in conventional pastures, animals may consume the nutraceutical plant together with the normal grass or herbs. In other areas, farmers may not be able to overseed their paddocks or may decide to dedicate a certain paddock to produce only the nutraceutical plant (i.e. monoculture). In that case, the nutraceutical material can be offered in the form of hay, silage or pellets. These types of R&D studies have been developed or seem best suited for farmers who are looking for alternative GIN control measures. This is particularly the case of organic farming or milk production systems based on small ruminants.

In the temperate areas, as mentioned above, two legume forages have been the subject of extensive studies, namely sainfoin (*Onobrychis viciifolia*) in Europe and sericea lespedeza (*Lespedeza cuneata*) in South Africa and USA. These studies have involved a wide range of disciplines: phytochemistry, agronomy, animal production, ruminant physiology and parasitology. Both of these tanniniferous legume species have also been the preferred model for exploring the mode of action of polyphenols against the different stages of GINs (see Hoste et al., 2012). In addition, R&D studies for nearly 20 years on *O. vicifolia* or *L. cuneata* have examined and compared different forms of exploitation, namely direct grazing, conservation as hay or silage (Paolini et al, 2005, Heckendorn et al, 2006, Shaik et al., 2006, Werne et al, 2013) or more recently with dehydrated pellets (Terrill et al., 2007, Gujja et al., 2013, Girard et al., 2013, Kommuru et al., 2014).

4.2.2 Exploiting rangelands and their biodiversity of plant resources

In most tropical parts of the world, the ecosystems present a wide plant biodiversity and include several plant species with nutraceutical potential as natural forage. However, for many of those plant species, there is no information on their agronomy or on possible propagation methods. There are, however, a few exceptions, such as *Leucaena leucocephala, Arachis pintoi, Gliricidia sepium or Cratylia argentea (*von son de Ferneix et al., 2014*);* all are from the Fabaceae family and are exploited i) in plantations, ii) in silvo-pastoral systems that consist of rows of grass and trees, or iii) in so-called “fodder banks” that are tree plantations in a very dense system that are not allowed to grow beyond approximately 2 m in height. Thus, nutraceutical forages in tropical areas could offer a variety of situations for their production. However, most farmers currently are relying on the natural availability of nutraceutical plants. Meanwhile, the few people who can invest money in their farms may also be able to build silvo-pastoral systems or fodder banks. Irrespective of the production strategy, such plants may be used for direct browsing or in a cut-and-carry system.

At the moment, most farmers let their animals browse the natural vegetation. Under such conditions, it is not possible to determine whether animals have consumed enough nutraceutical material to obtain the desired AH effect. Cut-and-carry systems could instead be the answer to ensure that animals are exposed to sufficient nutraceutical material. However, these systems are constrained by time spent by farmers to harvest enough plant material for all animals, the need for a vehicle to move sufficient plant material, and difficulties with harvesting (e.g. thorns). In any case, such management strategies could eventually deplete the resource. Thus, the creation of low-cost fodder banks represents a more sustainable option, but it poses considerable challenges in terms of investment and technical knowledge that is beyond the capability of most small farmers at present.

For the tropical areas, a considerable amount of data~~results~~ has been acquired in Mexico for tannin-containing leguminous trees, such as *Havardia albicans* and *Lysiloma latisiliquum*. These materials were tested first under *in vitro* conditions (Alonso-Díaz et al., 2008a, 2008b; Hernández-Orduño et al., 2008) and their promising results led to subsequent *in vivo* studies with sheep (Martínez-Ortíz de Montellano et al., 2010; Galicia-Aguilar et al., 2012; Mendez-Ortíz et al., 2012) (see section 3.1.2).

4.2.3 Exploring the value of agro industrial by-products

In different areas of the world, interest has also been growing in exploring the potential of tannin-containing 'waste' or by-products from agro-industries. These represent an alternative option to "natural" nutraceuticals, in the sense that the PSM can be extracted from the by-products and then added to an existing feed (see Table 1). Transforming 'waste' tannin-rich materials into nutraceutical feeds with antiparasitic properties could have several advantages. First, this represents a viable alternative to add value to agro-industrial waste products; secondly, it may help to solve the problem of the inherent variability of PSM content (see below) in nutraceutical plants, as it would allow adjusting the bioactive PSM concentration(s) in feeds (Girard et al., 2013). Consequently, it may also help to avoid any negative consequences caused by an excess of PSM in the feed.

Some examples of tannin-containing plant by-products that have been under recent investigation for their AH activities are: 1/ by products from the nut industry in temperate areas (Desrues et al., 2012), 2/ carob pods (Manolaraki et al., 2010; Arroyo-Lopez et al., 2014) in the Mediterranean region, and 3/ coffee by-products and cocoa fruit husks and leaves in Yucatan, Mexico (Covarrubias-Cárdenas et al., 2013; Vargas-Magaña et al., 2014a, 2014b).

4.2.4 Inherent variability of nutraceutical plant materials

Whatever the mode of exploitation (grown, browsed, or by-products), once a plant species has been identified through *in vitro* and *in vivo* studies as a potential candidate for use as a nutraceutical, it is still important to consider the inherent variability caused by several factors which can influence the quantity and/or quality of PSMs~~ content~~ and hence the~~ir~~ antiparasitic effects of nutraceutical candidates.

~~Nutraceutical plants should be considered in the same manner as grapes that are produced for wine-making. It is evident that certain types of grapes result, generally speaking, in good wines. However, wine-makers and wine-connoisseurs are aware of the existence of many factors that affect the quality of the wine, some of which are not in the control of the producer. Thus, variation can result from the climate, amount of sun radiation and rainfall, timing of the rainfall, agronomic conditions of the vineyard, etc. This will result in varying contents of macronutrients, e.g. sugar in the case of grapes, which is a relatively stable feature of feeds, and variation of the content and composition of plant secondary compounds (e.g. tannins), which can be prone to a wide range of variation. Even the best vineyards in the world, with a long tradition of wine-making, accept that PSM variability will result in an excellent wine, a good wine or an average wine depending on the year.~~

Several data are now available, which show that similar questions also apply to nutraceutical candidates. Sainfoin (*O. viciifolia*) has been extensively studied to explore the factors that influence PSM contents and composition and related AH activities (Manolaraki et al., 2011). Three main factors have been identified: environmental conditions (e.g. phenological stages, areas or soil conditions for growth, climate and seasons), genetic factors (cultivars or chemotypes) (Azuhnwi et al., 2013; Stringano et al., 2012) and also technological processes (e.g. fresh versus hay, silage and pellet samples).

It is possible that this issue of variability caused by the different factors is also important for growing sericea lespedeza (Muir et al., 2014), or for the leaves of *L. leucocephala* or *Manihot esculenta* that originate from plantations in tropical zones. Variation of PSM contents may be even more evident in plants that grow in the native vegetation. Under such conditions, variation of PSM content is significant between individuals even during the same season in the geographical region (Alonso-Díaz et al., 2010b).

Further factors, which are frequently not considered include variation in harvesting methods by farmers, such as the leaf:stem ratio that results from pruning only young leaves (branch tip) or complete branches (often based on biomass feed requirement), and post-harvest practices, such as sun drying, wilting, which are often based on nutritional advice, but may not have considered the effects on the bioactive compounds, such as their reduced content or activity in the feed. As the common and traditional view is that PSMs are anti-nutritional factors, this type of advice will need to be modified for nutraceuticals.

However, as far as we are aware, hardly any research has been conducted on selecting or breeding for bioactive plants with stable tannin or other PSM compositions. This seems to be an opportunity that would be worth exploring as we have shown that a few relatively 'robust' sainfoin and sorghum chemovars chemotypes used earlier exist, where the PSM composition was much less dependent on the environment (Azuhnwi et al., 2013a; Mueller-Harvey and Dhanoa, 1991).

5. PERSPECTIVES

Based on the previous description of tannin-containing legumes against GIN in small ruminants and the development of methodological approaches for exploring the nutraceutical concept, several areas~~xes~~ for future research have been identified that focus on on-farm applications.

5.1 What else? Novel parasitic "targets" for~~with~~ tannin-containing legumes

5.1.1 Against GINs in other host species.

Several recent *in vitro* results have shown that tanniniferous legumes can also regulate worm biology of the main genera of GINs in cattle (see review by Sandoval-Castro et al., 2012). Significant results were obtained with extracts of sainfoin (*O. viciifolia*); *L. corniculatus* and *L. pedunculatus* against *Ostertagia ostertagii* and *Cooperia oncophora* (Novobilsky et al, 2011, 2013). These data are offering opportunities for *in vivo* studies and to promote the combined control of bloat and GINs in cattle production. Recently, a 50% reduction in worm burden was obtained in calves infected with *O. ostertagi* and fed sainfoin pellets (Desrues et al, 2015 WAAVP 2015 abstract).

In fact, attempts have been made in South Africa to regularly include wattle (*A. karroo*) as a supplementary feed for cattle that are infected with *Haemonchus* sp and *Oesophagostomum columbianum*. These studies reported a reduction in egg excretion (Xhomfulana et al., 2009). Two recent studies, based on *in vitro* assays, have also underscored that different tanniniferous plant sources could be used against GIN in monogastric livestock hosts given the positive assessment of AH effects against *Oesophagostomum dentatum* and *Ascaris suum* in pigs (Williams et al., 2014a and b). However, it remains to be seen whether these tannin resources will be used as herbal remedies ~~medicinal plants~~ or as nutraceuticals (see Table 1). However, these *in vitro* data are supported by a field observation study that examined the effects of acorns (*Quercus robur*) fed to outdoor pigs which had been raised with a natural nematode infection. The results indicated a dramatic reduction (> 90 %) in GIN faecal egg count (Salajpal et al., 2004).

5.1.2. Against other digestive parasites

Recent investigations have also assessed the effects of tannin-containing plants against *Eimeria* infections in small ruminants. Although results were disappointing from an *in vitro* assay that examined a wide range of sainfoin extracts for their capacity to inhibit oocyst sporulation (Saratsis et al., 2012), the results of *in vivo* studies on natural infection were much more encouraging. These studies evaluated sainfoin fed to lambs (Saratsis et al., 2012), and sericea lespedeza (*Lespedeza cuneata*) fed to either lambs (Burke et al., 2013) fed before and at weaning or kids (Kommuru et al., 2014). Moreover, some early results are also available that examined the effect of *Pistacia lentiscus* in young goats (Markovics et al., 2012).

In most of these trials, significant reductions in oocyst excretion were measured when young animals were fed with legumes, in particular, kids fed with *L. cuneata* showed reductions greater than 90 %. In addition, there was some evidence that sericea lespedeza had a positive effect on host resilience, as it led to a lower requirement for anti-coccidian treatments. These promising results suggest that tannin containing legumes, in particular pellets of sericea lespedeza, represent an option for a plant-based control of coccidiosis in small ruminants around weaning time.

The ability lambs and kids to consume sufficient nutraceutical amounts to prevent coccidiosis around weaning could be a limiting factor that will need to be considered. However, results of a recent experiment with calves, which were under 15-day old, are promising: supplementation of milk with concentrated pomegranate extract may, depending on the concentration, reduce faecal oocyst count and diarrhoea intensity and duration because of *Cryptosporidium parvum* infection (Weyl-Feinstein et al., 2014) [it should be noted, however, that pomegranate contains mostly ellagitannins – and not CTs].

5.2. Exploring plant resources as nutraceuticals: example of other plant families, other bioactive PSMs.

The logical and successive steps described in section 3 have helped to identify other botanical resources, which could be exploited as nutraceuticals. Two examples that meet the general criteria of nutraceuticals as defined in Table 1 will be mentioned here to illustrate these issues:

An impressive range of studies has been performed in Spain that investigated the AH effects of different heather species that belong to three *Erica spp* and one *Caluna sp* (Ericeacae) and are browsed by Cashmere goats (Moreno-Gonzalo et al., 2012). This is a first example for which a whole series of results were obtained based on a range of studies that covered *in vitro* assays (e.g. Moreno-Gonzalo et al., 2013), controlled *in vivo* studies with experimental infections in confined conditions (Frutos et al., 2008; Moreno-Gonzalo et al., 2014) and systemic studies with natural infections (Osoro et al., 2009). ~~These studies confirmed their AH effects. However. Caluna is known to contain CTs and other flavonoids and these are likely to contribute to their bioactivity.~~

Chicory (*Cichorium intybus*) (Asteraceae), when used as a forage for ruminants, represents another AH example, which has illustrated the “robustness” of the methodological approach described in section 3. Chicory has been shown to possess AH properties based on experiments that ranged from *in vitro* assays (Molan et al., 2003b; Foster et al., 2011 a,b), simple *in vivo* studies (Peña-Espinoza et al., 2015 WAAVP2015 abstract), to systemic studies (Athanasiadou et al., 2007; Tzamaloukas et al., 2006 a,b; Nielsen et al., 2009).

Two other points are worth mentioning in comparison of chicory and the legume models and illustrate which additional studies are required to expand the nutraceutical concept for tackling digestive parasites in infected livestock.

- Based on several *in vitro* results, the nature of the AH PSMs of chicory seems to stem from sesquiterpene lactones and not from CTs and related polyphenols (Molan et al., 2003b, Foster et al., 2006, 2011a and b).

- Despite the different PSMs, results acquired with chicory forages also illustrated that environmental and genetic factors affected the anti-parasitic activity and were linked to variations in PSM quantity or composition. This is evidenced by marked differences between cultivars (Miguel Peña-Espinoza, personal communication, 2015). Therefore, these factors require addressing before to seeking on-farm implementations for such nutraceuticals (Foster et al., 2006, 2011a).

5.3. Self-medication and nutraceuticals: a novel field for basic researches on the host-gastro intestinal nematode interactions.

Given that the antiparasitic effects of nutraceuticals depend on the PSM concentration in a feed and length of consumption by the infected host, the tannin-containing legumes provide a valuable model to explore the host-parasite (GIN) interactions in regard of regulation related to the host feeding behaviour and /or to assess the balance between host immunity and behaviour (Hoste et al., 2010) (especially when testing conserved forms, which can be used under controlled conditions).). Interesting studies can and have yet been performed that examine 1/ the ability of the host to select a feed with AH properties when hosts are infected or not with GINs (=self-medication) (Lisonbee et al, 2009; Villalba et al, 2013, Junkhe et al, 2012); **2/** the influence of various host or parasite factors on the self-medication behaviour (Amit et al, 2013); and **3**/ the trade-offs between negative nutritional effects and beneficial health effects that accrue from the consumption of nutraceuticals (Frutos et al, 2008).

It is important to indicate, however, that the influence of GIN on the ingestion of nutraceutical plant materials is not easy to evaluate in regard of methodological issues. Attempts to evaluate this phenomenon using cafeteria studies, where animals are exposed to different types of plant materials, or direct observation methods in the field, that compare animals with and without parasites, will be influenced by the quantity of parasites present in the animals (light to heavy burdens), the time that animals had been naturally infected with GIN before the study began (naïve or immune competent animals), the existing feeding experience , physiological adaptations (e.g. tannin binding saliva) of the animals investigated, amongst several other aspects that are difficult to control (Alonso-Díaz et al., 2010a; Vargas-Magaña et al., 2013). Because self-medication has been the subject of 2 ~~a~~ recent review (Villalba and Provenza, 2007; Villalba et al., 2014), this issue will not be developed further.

6. CONCLUSIONS.

- Because of the constant, worldwide, rapid development of resistance to synthetic chemical AHs, especially, and also because of societal demand, there is nowadays a clear and urgent need to explore and validate alternative options for specific livestock systems (e.g. organic farming systems, small dairy ruminant systems in EU or in caprine breeding).
- For these reasons, in regard of the control of GINs in livestock, as previously evoked (Thamsborg et al., 1999) in the 21st century, we are now probably entering the post-anthelmintic era and are moving from relatively simple solutions for on-farm applications (use of synthetic AHs) towards much more complex options.
- The development of nutraceutical products with real potential for the control of GIN in ruminants is a possibility that is well underway of becoming a reality in different parts of the world for different livestock breeding systems and relying on different plant materials.
- The complexity of the scientific questions which need to be addressed are intrinsic to nutraceuticals. Therefore, the possibility of developing on-farm applications against digestive

parasites requires a multidisciplinary approach between scientists with expertise in parasitology, but also phytochemistry animal production, digestive physiology, ethology and others.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 1: The criteria defining the main concepts of xenobiotics and plant secondary metabolites for controlling gastrointestinal nematodes of livestock (VFI = voluntary feed intake)**.**

Nature of the bioactive compounds	**Synthetic chemical compounds**		**Natural chemical compounds (Plant secondary metabolites)**	
Formulations	**Therapeutic drugs**	**Chemical additives (supplements)**	**Herbal drugs**	**Nutraceuticals**
Mode of administration	Forced Administration	Added to the feed	Forced administration	PSMs Included in and/or added to the feed
	Independent of VFI	Dependent of VFI	Independent of VFI	Dependent of Voluntary Feed Intake (VFI)
	Short term Well defined posology	Long term Posology defined by a range within the feed	Short term	Long term
Objective	**Curative /** (preventive)	**Preventive**	**Curative/** (preventive)	**Preventive/** (curative)
Quality of the active compounds	Standardised Identified	Standardised Identified	Variable Usually non identified	Variable Identification of the family of phytochemical compounds

Mode of action	Usually, well identified	Usually, well identified	Unknown	Hypotheses
Development of resistance	High	High	Unknown	Suspected

TANNIN CONTAINING LEGUMES AS A MODEL FOR NUTRACEUTICALS AGAINST DIGESTIVE PARASITES IN LIVESTOCK

H.HOSTE [1,2], J.F.J. TORRES –ACOSTA[3], C.A. SANDOVAL-CASTRO[3], I. MUELLER-HARVEY [4], S. SOTIRAKI [5], H. LOUVANDINI [6], S.M. THAMSBORG [7], T.H. TERRILL. [8]

[1/] INRA, UMR 1225 IHAP, 23 Chemin des Capelles, Toulouse F-31076, France

[2/] Université de Toulouse, ENVT, 23 Chemin des Capelles, Toulouse F-31076, France

[3/] Campus de Ciencias Biológicas y Agropecuarias, FMVZ, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, Merida, Yucatan, Mexico.

[4/] University of Reading, School of Agriculture, Policy and Development, 1 Earley Gate, P.O. Box 236, Reading RG6 6AT, United Kingdom

[5/] Veterinary Research Institute – Hellenic Agricultural Organization Demeter, 57001 Thermi, Thessaloniki, Greece

[6/] Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil,

[7/] Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark,

[8/] Fort Valley State University, 1005 State University Drive Fort Valley, GA 31030, USA

Corresponding Author: (H.H.) phone/fax: + (33) 05-61-19-38-75 / + (33) 05-61-19-32-43. E-mail: h.hoste@envt.fr

ABSTRACT

Parasitic infections with gastrointestinal nematodes (GINs) still represent a worldwide major pathological threat associated with the outdoor production of various livestock species. Because of the widespread resistance to synthetic chemical anthelmintics, there is a strong impetus to explore novel approaches for a more integrated management of these infections. The use of nutraceuticals in the control of GINs is one of the alternatives which has been widely studied for 20 years.

The objectives of this review are: **i)** to define and illustrate the concept of 'nutraceutical' in the context of veterinary parasitology based on data obtained on the most studied models to control GINs in small ruminants, the tannin-containing legumes (Fabaceae); **ii)** to illustrate how the 'nutraceutical concept' could be expanded to other plants, other livestock production systems and other GI parasitic diseases, and iii) to explain how this concept is opening up new research fields for better understanding the interactions between the host, the digestive parasites and the environment.

KEY WORDS: Nutraceuticals / Condensed tannins/ Polyphenols / Gastrointestinal nematodes / Antiparasitic effects / Small ruminants.

1. INTRODUCTION

Parasitic infections with gastrointestinal nematodes (GINs) represent a worldwide major pathological threat associated with the outdoor production of various livestock species, particularly ruminants. Up to now, the control of these parasitic helminth diseases has essentially relied on the repeated use of commercial anthelmintic (AH) drugs. However, resistance to these AH drugs is now widespread in worm populations, and the occurrence of multi-resistant strains has become a serious problem in some regions of the world (Kaplan, 2004; Torres-Acosta et al., 2012, Jackson et al., 2012). Moreover, as underscored by Waller (2006), resistance to xenobiotics develops quite rapidly in pathogens and usually within 10 years. This has been illustrated since the recent launch of monepantel (Kaminsky et al., 2008), which is a drug belonging to the Amino Acetonitrile Derivatives (AADs). In spite of the novel mode of action, reports of monepantel resistant *Haemonchus contortus* have already appeared only seven years after the launch of this new drug (Van-den-Brom et al., 2015).

Therefore, modern parasite management involves alternative approaches seeking to achieve an integrated and more sustainable control of helminth infections by combining the 3 main principles for GIN control, namely **i)** management of grazing systems; **ii)** stimulation of host response and **iii)** modulation of worm biology (Hoste and Torres-Acosta, 2011). This also explains the strong impetus worldwide for exploring and adapting alternative options to different conditions. Among these novel approaches, the long tradition of using bioactive plants for their anti-parasitic properties has been re-explored. It is now considered as one option for improving GIN control, and therefore, to counteract the negative pathophysiological consequences in hosts (Rochfort et al., 2008). Plants are widely used as phytotherapeutic drugs or herbal remedies, and their administration is based on a long tradition of ethno-veterinary or ethno-medicinal applications on all continents (Hammond et al., 1997; Sandoval-Castro et al., 2012). However, over the last 20 years, a novel, overall concept of nutraceutical plants (plants combining positive effects for both animal nutrition and health) has emerged in veterinary science and helminthology for improved controlling livestock parasites (Waller and Thamsborg, 2004; Hoste et al., 2006; Alonso-Diaz et al., 2010b).

The aim of this review is

1/ to illustrate the concept of 'nutraceutical' based on resources containing Plant Secondary Metabolites (PSMs) in the context of veterinary parasitology. We will mainly refer to the tannin-containing legumes (Fabaceae) and other plants, which is one of the most studied models of nutraceuticals to control GIN in small ruminants.

2/ to show how the 'nutraceutical concept' could be expanded to other plants, other livestock production systems and other parasitic diseases of the digestive tract.

3/ to explain how this concept is opening up new research fields for probing the interactions between the host, the parasites of the digestive system, and the environment.

2. DEFINITION OF NUTRACEUTICALS

The term "nutraceutical" results from a portmanteau between the words "nutrition" and "pharmaceutical". It has sometimes also been described as "functional food", which was first coined in the context of medical health (Hasler, 1998). According to Andlauer and Fürst (2002), a nutraceutical is defined as "any substance that may be considered a food or part of a food which provides health benefits, including the prevention and treatment of disease".

In a similar way, a nutraceutical in veterinary science can be defined as a livestock feed which combines nutritional value with beneficial effects on animal health. This double action is suspected to rely on the presence of various plant secondary metabolites (PSM) or bioactive compounds. In contrast to pharmaceutical drugs, nutraceuticals are not synthetic compounds formulated for specific indications.

Here, we will focus on a sub-category of nutraceuticals, i.e. plant-based products that contain nutrients and bioactive compounds that can be used **either directly** as part of the animal diet or **added in a concentrated form** to another diet after extraction from the bioactive plant or resource. Although the nutraceutical concept sounds simple, it is important to recognise that there are several key differences compared to synthetic AH and phytotherapeutic drugs (Table 1). This makes studying their efficacy a rather complex task. The key points and differences between nutraceuticals and the other options for interfering with the parasite life cycle will be discussed below.

1/Nutrition: Compared to herbal drugs (phytotherapeutical remedies) or synthetic chemicals, a nutraceutical based on bio active plants is not imposed, but offered to animals over a relatively long period (from several days to weeks or months). Therefore, the efficacy of this sort of nutraceuticals will directly depend on the Voluntary Feed Intake (VFI) of the animal.

2/ Nature of the bioactive compounds (PSM): The bioactivity against the parasites is dependent on the presence of natural chemical compounds in plants, which are usually Plant Secondary Compounds (PSM). The concentrations of these compounds can be highly variable and may depend on environmental growing conditions, on the plant cultivar, or chemotypes. In addition, technological factors (harvesting, processing methods) can also interfere with the preservation of the active compounds.

3/ Effect on animal health: The effects of nutraceutical feeds and of the associated bioactive compounds against parasitic nematodes differ from the mode of action of synthetic chemicals (see figure 3). This statement implies to develop specific methodologies of measurements of the AH effects.

3. TANNIN CONTAINING LEGUMES WITH ANTHELMINTIC PROPERTIES AGAINST GINS IN SHEEP AND GOATS: THE MOST STUDIED MODEL OF NUTRACEUTICALS IN VETERINARY PARASITOLOGY.

It is only recently (in the last 20 years) that researchers have obtained enough evidence that supports the use of plants as nutraceutical feeds against GIN. The first evidence originated from research in New Zealand based on empirical results that reported significant reduction of GIN faecal egg counts in sheep when grazing pastures with different legumes, i.e. birdsfoot trefoil and big trefoil (*Lotus corniculatus, L. pedunculatus*), sulla (*Hedysarum coronarium*) and sainfoin (*Onobrychis viciifolia*), which contain condensed tannins (CTs) (Niezen et al., 1995, 1998, 2002). The information generated by these early field trials started the search for new legumes (Fabaceae) containing the same PSMs and for other plant candidates elsewhere in the world against GINs in small ruminants. This has become the subject of extensive studies and still represents an important model aiming to explore the concept of using nutraceutical plants against other parasites, with other hosts, and to identify other botanical families with nutraceutical properties (Sandoval-Castro et al., 2012).

These studies on tannin-containing legumes have led to:

1/ the development of several important methodological procedures that are based on *in vitro* and *in vivo* methods to evaluate plants as potential nutraceuticals;

2/ a better definition of the difference in effects between nutraceuticals, phytotherapeutic remedies, and synthetic AH chemical drugs.

3.1. Identification/selection of future potential candidates of nutraceuticals against GINs.

As illustrated in Figure 1, the effects of a nutraceutical against GIN depend on complex interactions. Hence, the selection of a plant candidate as a potential nutraceutical requires good knowledge and interaction between different scientific disciplines, namely livestock nutrition and production, phytochemistry, parasitology, and to some extent, ethology and toxicology. However, the presence of bioactive PSM is a key issue.

Identifying viable nutraceutical candidates against parasites is a complex task, and the following steps need to be considered in the selection of potential materials. This procedure is intended to help in identifying candidate plant materials that can subsequently be explored under *in vivo* conditions.

3.1.1 Nutritional issues.

Even before the plant materials are tested *in vitro* or *in vivo* for AH activity, information should be gathered in terms of nutritional value and palatability for the hosts. This information should include:

a*) A good macronutrient profile.*

Ideally, the feed should have medium to high levels of protein or energy and low lignin. However, not all plants with tannins may have an optimal “nutritional” profile. Therefore, initial selection of such plants should not be based solely on their macronutrient profile, especially if such plants are selected by ruminants during grazing/browsing. The nutritional quality of the plant material that is intended for nutraceutical use should include the determination of its *In Vitro* Dry Matter Digestibility (IVDMD). This information can help to identify if the plant material could be further useful under *in vivo* conditions.

b) *A feed readily consumed by the animal host.*

Since the general definition of a nutraceutical is linked to VFI by animals, this key issue needs to be first considered and then measured during *in vivo* assays. Short intake or preference trials should be carried out before engaging with *in vivo* evaluation of the plant. If the plant is not eaten, it might still be useful in other ways, as the active PSMs could be extracted and then dosed to animals (Phytotherapy).

c) *An acceptable in vivo apparent digestibility.*

Any plant material that will be used in large quantities or during long periods of time will require an evaluation of its impact on the apparent *in vivo* digestibility, including at least measurements of the dry matter (DM) and the organic matter (OM). These data may help to ensure that the nutraceutical material will not cause a severe reduction of digestibility.

d) *Little or no negative known impact on animal production.*

Before selecting a nutraceutical plant for AH effects, it is important to collect information on the possible negative impact on production (i.e. growth, milk production, reproduction) or even clinical signs (e.g. abortions or foetus deformities, such as arthrogryposis due to phytoestrogens) or photosensitisation. In such cases, the plant material should not be considered as a viable nutraceutical.

3.1.2. PSM issues

Since the early results in NZ, several tannin containing legumes (Fabaceae) have been subjected to a wide range of studies as potential nutraceuticals. The focus on these species was usually not based on prior ethno-botanic information about the action against signs of GIN parasitism (i.e. diarrhoea), or de-wormers used for humans, as in both cases the information could be inadequate for ruminant species (Sandoval-Castro et al., 2012).

In contrast, they were mainly supported by knowledge and principles of pharmacognosy, based on the assumption that the presence of similar PSMs in the same botanical families (Fabaceae) should achieve similar antiparasitic effects. However, a few results have shown that some plant candidates, which were retained because of the presence of CTs, also corresponded to plants that had been identified based on ethnoveterinary knowledge. This double-sided approach can be of particular interest in many tropical countries.

A possible third approach could be based on detailed observational studies to identify plant selection (González-Pech et al, 2014), feeding behaviour, resource selection and possibly self-medication under grazing or browsing conditions. However, such studies are laborious and few exist from tropical regions, where there is a large plant diversity (Novelo-Chi et al, 2014, Retama-Flores et al, 2010 and Ventura-Cordero et al, 2014a, 2014b).

The Fabaceae family is one of the richest botanical families in terms of species numbers (nearly 20,000) and tannin-containing legume and plant resources occur worldwide, just like GINs. Therefore, the search for possible nutraceuticals has included temperate and tropical tannin-containing plants. These include herbaceous plants, trees, and shrubs, which are perennials or annuals, and which are used by small ruminants in a wide range of feeding systems.

In temperate areas, two legume species of the sub family Faboideae, have received special attention and have been the focus of a number of studies. These are the sainfoin (*Onobrychis viciifoliae*) in Europe (Paolini et al., 2003a, 2005b; Heckendorn et al., 2006, 2007; Manolaraki et al., 2010; http://sainfoin.eu; www.legumeplus.eu) and the Chinese bush clover or sericea lespedeza (*Lespedeza cuneata*) in the USA and South Africa (Shaik et al., 2006; Joshi et al., 2011; Kommuru et al., 2014; Terrill et al., 2009; 2012 http://www.acsrpc.org/). This special attention on these two herbaceous plants is explained by their ability to produce seeds, to be grown (cultivated) efficiently, and to organise their production for farm application on a large scale (see below).

It is worth underscoring that this scientific interest is also explained by their other beneficial effects in regards to nutrition and health of ruminants and environmental issues (see reviews by Rochfort et al., 2008, Waghorn, 2008, Mueller Harvey, 2006) which are : **i)** a reduced use of chemical fertilisers because of biological nitrogen fixation; **ii)** high palatability for ruminants and feeding values; **iii)** positive effects to reduce ruminal methane emission and GHG**; iv**) anti bloat effects; **v**) switch of nitrogen excretion from urine to faeces. In contrast, it is also worth noting that some non-native species exploited for these properties are considered invasive by environmentalists and ecologists

In tropical areas in Africa and Latin America, several genera (shrubs or trees) of the Fabaceae family have been particularly under focus and identified as possible nutraceuticals because of *in vitro* results or *in vivo* data indicating reductions in egg excretion; e.g. *Leucaena leucocephala* (Ademola et al., 2006), *Lysiloma latisiliquum* (Matínez-Ortíz de Montellano et al., 2010), *Havardia albicans* (Galicia-Aguilar et al., 2012; Méndez-Ortíz et al., 2012), or different Acacia species: *Acacia pennatula* , A.

molissima , *A. karroo, A. mearnsii, Mimosa tenuiflora* (Kahiya et al., 2003; Alonso Diaz et al., 2008a; Minho et al., 2008; Cenci et al., 2007; Oliveira et al., 2013, Murare et al., 2012).

3.2. A methodological procedure to confirm the antiparasitic effects.

An overall methodological procedure has progressively emerged from scientific results from different studies to validate the possible efficiency of a wide range of tannin-containing plants as nutraceuticals against GINs. By its logical scheme and by the objectives defined for each step, the procedure has been widely derived from the WAAVP guidelines to examine the efficiency of synthetic AHs (Wood et al., 1995).

The three successive steps are **1/** the *in vitro* screening of plant extracts based on a wide range of assays (Figure 2); **2/** controlled *in vivo* studies on experimentally infected animals in confined conditions to confirm the efficiency of the selected plant candidates; **3/** holistic studies to examine how and when to apply the nutraceuticals in breeding/feeding systems for small ruminants.

However, because of the complexity and intrinsic variability of nutraceuticals, which are based on naturally occurring chemicals rather than synthetic anthelmintics (see Table 1, Quality of the compounds and mode of administration), this scheme requires adaptations and critical comments to interpret the results.

Since the mode of action of tannin-containing nutraceuticals differs from synthetic chemical AHs, it is of interest to examine the effects of the same plant extract on different key stages of the GIN life cycle (egg, infective larvae, adult worms) (Figure 2).

3.2.1 T*he in vitro* screening of plant extracts.

Most *in vitro* assays for the primary screening of plant products have been adapted from those developed to assess the efficacy of synthetic AHs against GINs in ruminants (Wood et al., 1995). One exception is the Larval Exsheathment Inhibition Assay, which has been set up specifically (Bahuaud et al., 2006) to reproduce artificially, under *in vitro* conditions, the disturbances related to the establishment of infective larvae as measured *in vivo* in sainfoin-fed sheep (Brunet et al., 2007).

The detailed methodologies of these different assays, when adapted to plant materials, can be found in Jackson and Hoste (2010). In addition, it is worth mentioning that the model represented by the free living nematode, *Caenorhabditis elegans*, is also increasingly being exploited to screen the AH properties of different plant resources (Katiki et al., 2012, 2013).

It is important to emphasise that these different *in vitro* assays, first used to screen potential nutraceutical candidates, have also been exploited for a second main objective: to better understand the mode of action of PSM compounds against GINs. This has been achieved **i)** by applying either isolated and purified tannins, which were selected because of their different structural features (Molan et al., 2004, Novobilsky et al., 2013, Quijada et al., 2015), or pure flavonoid compounds (Molan et al, 2003a; Brunet and Hoste, 2006), and **ii)** by performing detailed studies of the functional and structural changes induced in nematodes (Hoste et al., 2012). This allowed testing of the "direct" hypothesis (see section 4.1).

Most research groups started screening the AH activity of different plant materials under *in vitro* conditions. In most cases, the screening process was developed with plants that had been extracted with different solvents. When tannins are suspected as the main bioactive compounds, extraction with acetone/water (70:30) is the most common and efficient solvent in regard of the yield of extraction. We note that several studies reported drying plant materials at high temperatures and that this may have reduced the AH activity. It is highly recommended that all plant samples should either be freeze-dried or dried below 40 ^{o}C in order to preserve polyphenols and tannins, as these are easily oxidised at higher temperatures.

In addition, because of the intrinsic variability of the active compounds and/or mode of actions against GINs, many misunderstandings need to be clarified:

a) *If a plant extract shows an AH effect against a given life stage, it will also show a clear AH effect against other life stages of the same parasite, at roughly the same concentrations.*

This is not the case ! If the same extract from a single plant material is applied to different life stages of a nematode species under the respective *in vitro* conditions, the AH effect may differ between different life stages (Paolini et al, 2004). For instance, a plant extract may show a clear AH effect against *H. contortus* on a given life stage (i.e. L_3 larvae) and show a mild or no AH effect against another stage (i.e. eggs). Furthermore, the AH effect may only be evident on a certain aspect of the same life stage, such as inhibition of the L_3 exsheathment process, but have no clear effect on another aspect, such as the motility of the same L_3 larvae (Alonso-Díaz et al., 2011).

Such differential effects depending on the life stages of GIN have also been recorded for conventional anthelmintics, such as levamisole, that shows a clear effect on L_3 motility but fails to show any AH effect on the exsheathment of L_3 or the eclosion of GIN eggs.

These comments imply that the selection of candidates for further *in vivo* tests could be judged either on the overall effective concentration (EC 50, 90 or 99%) of different *in vitro* tests with the different life stages tested or alternatively, that the candidate should be the one showing the lowest EC in the most biologically relevant *in vitro* test chosen based on valid biological grounds, depending on the stage of interest for the designed study.

b) *If the plant extract is obtained from the same plant material with a solvent of similar polarity, the potential AH effect against GIN will be similar.*

Again, this is not the case ! Different extraction procedures, even with solvents of similar polarity [i.e. methanol vs. acetone:water (70:30)] applied to the same plant material, can result in different profiles of PSMs being extracted. As a result, the extracts of the same plant material will show different AH activities when applied to the same life stage. For example, acetone-water (70:30) extracts of Annonaceae leaves showed limited ovicidal activity and a clear exsheathment inhibition activity, while the methanolic extracts of the same plant material showed significant ovicidal activity but was less efficient to inhibit the exsheathment of larvae (Castañeda-Ramírez et al., 2014). Thus, the extraction procedure and the choice of solvent, even with similar polarity, can result in marked differences in the extracted PSMs.

***c**) An in vitro screening test will help to decide on the dose or concentration of the nutraceutical material that needs to be consumed by the infected animals in order to control GIN infections.*

The extrapolation of *in vitro* doses to *in vivo* conditions is more difficult to achieve for plants when used against parasites dwelling in the lumen of the gastrointestinal tract. A plant extract prepared for *in vitro* use contains a concentrated quantity of PSM from the experimental plant material. Hence, those PSMs will be present in the *in vitro* assay at a certain quantity and quality that may not be comparable with the *in vivo* conditions, and also during a time span difficult to replicate under *in vivo* conditions. Moreover, under *in vivo* conditions, the digestion processes and the conditions occurring in the digestive tract may affect the liberation of the bioactive PSMs from the plant material. Also, the quantity or structure of PSM may be affected along the digestive tract after the plant is consumed, chewed, ruminated and digested by the ruminant host or gut microflora. Thus, it should be remembered that *in vitro* assays can help at identifying candidates with potential AH activities, but will not produce indications on a dose level that can be used as a starting point for the *in vivo* dose level that should be applied to the various hosts.

With the limitations mentioned above, one may think that it might be easier to find a plant extract with excellent *in vitro* activity, validate its *in vivo* AH activity against GIN in the relevant host, and then determine a dose that can be used to control their GIN populations. Such an approach would not be pertinent for a nutraceutical plant material but can be considered for an herbal remedy or phytotherapeutical medicine. As such, this will need to be tested as a drug and its efficacy validated using the relevant guidelines for efficacy against GIN (Wood et al., 1995).

Why can these extracts not be considered as a nutraceutical? The reason is simple: if the plant material is not providing macronutrients for the animal, then it is not a <u>nutra</u>ceutical material. In reality, there may be a more straightforward method for identifying plant materials with potential nutraceutical effects and for investigating the dose level required to control the GIN population: letting the hosts eat the plant material, while evaluating the faecal GIN egg excretion. If animals are able to eat sufficient plant material in the chosen presentation and form and produce a visible AH effect, then such level of ingestion (or dosage) may be suggested against parasites in the test animals.

3.2. 2 Confirmation of potential nutraceutical candidates, under *in vivo* controlled conditions, based on *in vitro* results.

As for the 1st step (i.e. *in vitro* screening), the *in vivo* procedures to assess the efficacy of nutraceuticals against GINs are also derived, to some extent, from the general guidelines described for synthetic chemical AHs (Wood et al., 1995). However; it is important to take into account several specificities of the nutraceutical concept (see Figure 1).

a) *A nutraceutical is at first a feed*

Therefore, it is essential to evaluate its nutritional value by using conventional methodologies. The information should include the macronutrient content of crude protein (CP), metabolisable energy (ME), neutral detergent fibre (NDF), acid detergent fibre (FDA) and lignin. This information may help to identify plant materials fitting the nutritional characteristics of an edible ruminant feedstuff. The information generated from conventional nutritional methodologies could be correlated with near infra-red (NIR) spectra. With time, when a large dataset of plant samples are tested, the NIR spectra can be linked with nutritional quality, presence of PSM and related AH effects. This may be automated with other NIR tools in the future.

One key aspect of these measurements for controlled *in vivo* studies is to achieve iso-proteic and iso-energetic diets in the groups of animals receiving the control or the nutraceutical diets, in order to specifically evaluate the role of the PSMs and to avoid any confounding effects because of differences in the amount of macronutrients (protein, energy or their balance) (Coop and Kyriazakis, 1999). However, differences in palatability may result in different feeding levels when feed on offer is not controlled.

Alternatively, assessing the trade-off of plant selection and intake could be an alternative for when iso-energetic and iso-proteic diets are not attainable. The working hypothesis should be carefully stated in all cases. Nutrient balance studies are needed to contribute to the understanding of parasitism and nutrient costs (for the host) and proper assessment of trade-off of nutraceutical plant intake.

b) *A nutraceutical should be readily consumed by the relevant host since the effects on health depend on the animal feeding behaviour*

Most plants materials meant to be used for nutraceutical purposes are not normally included in the diet of the relevant host. Since the general impact of nutraceuticals on animal health stems from the consumption of these materials, it is important: (1) to include in the experimental design of controlled *in vivo* studies a period for animal adaptation to the novel feed, lasting from 10 to 15 days, until experimental animals reach a plateau of consumption; and (2) once this plateau of consumption is reached, to estimate regularly the animal VFI. The evaluation can be as simple as measuring the plant material offered and refused, to determine intake. Under some circumstances, the ingestion of the plant material can be enhanced by means of some additives, such as sugar cane molasses. In such cases, the future use of these plant materials will depend on the availability of the plant *per se*, as well as the existence of the potential feed additive. This could mean an added difficulty for on-farm applications. If possible, plant intake should be evaluated with both infected and non-infected animals with the objective to provide information on self-medication (Martinez-Ortiz-de-Montellano et al, 2010).

c) *The impact of nutraceuticals against the parasite depends on the presence of PSM*

It is essential to obtain a measurement of the potential bioactive PSM before implementing the *in vivo* controlled studies. It is also important to describe the analytical methods used. In the case of condensed tannins, which cover the core of the current review, a critical description of the different analytical methods has been reviewed previously (Mueller-Harvey, 2006). One simple recommendation, when possible, is to try to obtain two different evaluations (usually one chemical assay and one biological assay in order to estimate the ability of tannins to complex proteins) (Makkar et al., 2003).

d) *The effects of tannin-containing nutraceuticals differ from synthetic AH*

This requires adapted experimental designs and measurements. Once the information to characterise the nutritional value and the PSM content of a nutraceutical have been obtained, valid *in vivo* assays in controlled conditions can be implemented. Such studies could consider animals with natural GIN infections. However, artificial infection trials are preferred that focus on the most prevalent GIN genera and species in the areas of interest.

Within this general experimental context, several factors need to be explored which are specific to nutraceuticals

- *The multivalent effects on a range of GIN species:*

As a general recommendation, combined infections with at least one species of the abomasal plus one species of the small intestinal species should be encouraged. The reason for this is that **i**) as part of the overall objective, as for synthetic AHs, a multivalent effect can be expected from nutraceuticals and also, **ii**) variations in the effect of tannin-containing resources against different GIN species have been mentioned on several occasions (see Hoste et al., 2006, 2012). Of course, the choice of the GIN models should be based on epidemiological information, which needs to identify the most prevalent and pathogenic species in the regional areas.

- *The multivalent effects on different GIN stages:*

As shown in Figure 3, the consequences of nutraceuticals and related PSMs on GINs (at least for the tannin-containing legume models), result from a combination of effects on 3 key stages of the GIN life cycle. Therefore, when possible, it is important to design experimental studies that examine the effects on these different stages (Castañeda-Ramírez et al., 2014; Vargas-Magaña et al., 2014a, 2014b).

- *The role of quantity and/or time period for offering tannin-containing resources in the diet to optimise effects against GINs in small ruminants*

After completing initial simple experimental designs that validate the effects of tannin-containing nutraceuticals against different genera and/or stages of GINs in small ruminants, some additional studies are worth designing in order to help in evaluating the effect of two factors which can influence the efficacy of nutraceuticals against GINs, namely the proportions of nutraceuticals and the related concentration of CTs in the feed (Athanasiadou et al., 2001, Brunet et al., 2007, Terrill et al., 2009) and the length of distribution.

- *Specificities in the evaluation of the parasitological and pathophysiological consequences*

During the whole evaluation period in studies based on experimental infections, animals should be monitored regularly in terms of **i)** repeated parasitological measurements (faecal excretion of GIN eggs), **ii)** pathophysiological measurements to establish the effects on host resilience based on quantitative and/or semi-quantitative measurements of the (sub)clinical effects of infection (e.g. Packed Cell Volume = PCV, FAMACHA Score).

Because of the variations in the PSM effects, which can depend on the nematode species and/or stages (see Figure 3), two experimental measurements are worth considering to complete the information obtained from experimental *in vivo* studies: 1/ it is recommended that these methodologies need to differentiate effects from different GIN species in case of multispecific experimental infections; and 2/ whenever possible, to measure also effects on egg hatching and development.

Ideally, the studies in controlled conditions should be complemented with post-mortem evaluations of the worm populations. The latter will help to strengthen the parasitological protocol by defining if the effect on GIN egg excretion is due to the reduction of GIN populations, proportions of species, sex ratio, or reduction in female worm fecundity (Martinez-Ortiz de Montellano et al., 2010; Galicia-Aguilar et al., 2012).

4 . TANNIN-CONTAINING LEGUME NUTRACEUTICALS AGAINST GINs IN RUMINANTS

Several (recent) reviews have summarised the existing results on the potential of tannin containing legumes as nutraceuticals. Their focus concerns three different aspects:

- the overall effects on ruminant nutrition, health and production (e.g. Waghorn, 2008; Mueller-Harvey, 2006; Rochfort et al., 2008 ; Wang et al., 2015)
- the specific AH effects (Min and Hart, 2003; Hoste et al., 2006, 2012)
- the mode of action against the GINs by understanding the nature of active compounds (tannins and flavonoids) (Mueller-Harvey, 2006) and their possible effects on the worms (Hoste et al., 2012).

The readers are invited to refer to these different reviews. The aims of the current section will be **i)** to provide an updated summary of the main results of basic research studies that describe the antiparasitic bioactivity of tannin-containing legumes against GIN nematodes in small ruminants and the current hypotheses on their mechanisms of action; and **ii)** to illustrate possible on-farm applications, which are starting to be developed.

4.1 Updated summary of the main effects on the GIN life cycle and hypotheses on their modes of action.

4.1.1. Impact on the GIN life cycle

Three main potential impacts have been linked to the intake of tanniniferous plants by infected ruminants (Hoste et al., 2012) on the GIN life cycle (illustrated in Figure 3)

1/ lower establishment of the infective third-stage larvae (L3) in the host.

2/ lower excretion of nematode eggs by adult worms, related either to a reduction in worm numbers or lower fertility of female worms; and

3/ impaired development of eggs into third-stage larvae.

Point 1 leads to reduced host invasion by L3. Steps 2 and 3 both contribute to reducing the environmental contamination with parasitic elements and thus to reduce the risks for hosts.

To summarise, tanniniferous nutraceuticals do not lead to a 100 % elimination of worms, but they can interfere with the life cycle; whereas the main goal of synthetic AHs is to completely break the parasitic life cycle. The role of nutraceuticals can be linked to severely impairing several key biological stages of the life cycle (eggs, infective larvae, adult worms). The potential combined effect of these separate impacts contributes to slowing down the dynamics of the infection and to lowering the rate of infection to levels that enable acceptable productivity and animal welfare. This also provides an opportunity for the animal to develop its own immunity to the parasites. Similar effects have been observed with the main nematode genera that infect the abomasum and small intestine of small ruminants (*Haemonchus* spp, *Teladorsagia* spp, *Trichostrongylus* spp). Some recent results (Gaudin et al, 2015 Abstract to WAAVP 2015) have demonstrated that significant reductions in egg excretion also occurred with a multi-resistant isolate of *Haemonchus contortus* in the presence of nutraceutical plants. This confirmed that tanniniferous nutraceuticals do indeed represent an alternative solution to AH resistance that is worth exploring further.

It is worth mentioning that these AH effects that affect the worm biology have also repeatedly been linked to positive effects on host resilience (e.g. better production parameters, less severe signs of diarrhoea or anaemia, lower mortality under parasitic challenge) ever since the early studies in NZ (Niezen et al, 1996). The mechanisms explaining this improved resilience remain obscure but can partly be explained by the effects on reducing the worm population as well as by the nutritive value of legumes (i.e. higher protein content) and the rumen-escape effect that occurs in the presence of certain types of tannins, which are able to protect dietary proteins (Waghorn, 2008). Identification of which particular tannins are best able to improve host resilience remains an outstanding research goal. Therefore, it is of paramount importance to underline the need to achieve iso-proteic and iso-energetic diets in control vs nutraceutical diets that are fed to sheep or goats in controlled *in vivo* studies.

4.1.2. Hypotheses on the modes of action

Two non-exclusive general hypotheses have been proposed to explain the activity of bioactive tannin containing feeds against gastrointestinal parasitic worms (Hoste et al., 2012):

1/ The " direct " hypothesis is based on pharmacological-type of interactions between the various polyphenols and the different stages of gastrointestinal nematodes.

2/ The "indirect" hypotheses assumes a possible improvement of the host resistance (i.e. an immunologically based response) because of the effects of tannin-containing feeds that can improve the overall host protein nutrition by increasing the amount of by-pass proteins

It is clear that the bulk of *in vitro* data obtained so far with different tannin-containing extracts support the hypothesis of "pharmacological-like" effects.

As previously stated (Hoste et al, 2012), numerous studies on GIN from small ruminants strongly suggest that both CTs and various flavonoids (at least some flavanols) contribute to the AH action (Molan et al., 2004, Brunet and Hoste, 2006). This has been supported also by recent results on another nematode model (e.g. *Ascaris suum* in pigs; Williams et al., 2014b). New evidence is also starting to emerge that tannins and other flavonoids in combination can generate either favourable (synergistic) or unfavourable (e.g. antagonistic) interactions (Klongsiriwet et al., 2014; Vargas-Magana et al., 2014a).

A dose-dependent relationship between tannins and/or flavonoid monomers and their *in vitro* effects has been found on several occasions with different assays and GIN species (see review by Hoste et al., 2012). A few *in vivo* studies have also described such relationships, where effects of a tanniniferous source on infective L3 larvae (Brunet et al., 2007) or on adult worm populations (Athanasiadou et al, 2001, Terrill et al., 2009) were examined. Taken together, it would appear that a CT threshold needs to be reached in the diet in order to achieve AH effects against the worms.

In addition, evidence is now accumulating from various *in vitro* studies which have sought to unravel the mode of action by tannins on the worms, that, besides quantitative factors (i.e. concentrations), qualitative factors (i.e. compound structures) can also modulate the bioactivity. Recent data obtained with GIN models from cattle, small ruminants, and pigs (Novobilsky et al., 2011, 2013; Quijada et al., 2015; Williams et al., 2014a) lend support to the hypothesis first raised by Molan et al. (2003) that bioactivity is linked to the prodelphinidin/procyanidin (PD/PC) ratio in plants. These recent studies have also revealed that tannin size is an important structural feature that contributes to the extent of the AH effects. As CTs usually occur in complex mixtures of closely related tannin compounds, their average polymer size is measured and reported as their mean degree of polymerisation (Gea et al., 2011).

Less studies have examined the “indirect” hypothesis, based on long –term studies and by measuring different effectors cells (mast cells, globule leucocytes, eosinophils, goblet cells) along the digestive tract in sheep (Tzamaloukas et al., 2006a and b; Martínez-Ortíz-De-Montellano et al., 2010; Rios de Alvarez et al., 2010) and goats (Paolini et al., 2003) when receiving different TR resources. Some of the most convincing evidence to support this “indirect hypothesis” is that some measurements indicating an enhanced local immune response (significantly higher numbers of mast cells and globule leucocytes) (Tzamaloukas et al.,2006) has been shown when lambs were consuming sulla or chicory and had a reduced development of worms.

4.2 Strategies to apply tannin-containing nutraceutical materials on-farm

The bulk of current data on potential nutraceuticals stems from *in vitro* assays. These results provided preliminary information on the future use of various plants or plant resources. However, gaps remain between *in vitro* results and on-farm application. These are outlined in section 3.

A few resources have been investigated more thoroughly through extensive approaches that included a succession or combination of *in vitro* assays, controlled *in vivo* studies and sometimes holistic studies in farming systems in order to prepare for implementation under field conditions. Fortunately, these specific plants illustrate various options that can be used to exploit tannin-containing resources as nutraceuticals.

4.2.1 Cultivating legumes as nutraceuticals

The availability of the plant material can be considered as the main constraint for using a nutraceutical material on-farm. If the plant material is not widely available, or the cost and effort of producing such material is high, then there will be insufficient quantities and it will be unlikely that farmers can adopt it. Up to now, very few plants exist that have potential for nutraceutical exploitations and that benefit from the availability of solid agronomic information suitable for large-scale production.

In some parts of the world, where nutraceutical legume plants can be sown in conventional pastures, animals may consume the nutraceutical plant together with the normal grass or herbs. In other areas, farmers may not be able to overseed their paddocks or may decide to dedicate a certain paddock to produce only the nutraceutical plant (i.e. monoculture). In that case, the nutraceutical material can be offered in the form of hay, silage or pellets. These types of R&D studies have been developed or seem best suited for farmers who are looking for alternative GIN control measures. This is particularly the case of organic farming or milk production systems based on small ruminants.

In the temperate areas, as mentioned above, two legume forages have been the subject of extensive studies, namely sainfoin (*Onobrychis viciifolia*) in Europe and sericea lespedeza (*Lespedeza cuneata*) in South Africa and USA. These studies have involved a wide range of disciplines: phytochemistry, agronomy, animal production, ruminant physiology and parasitology. Both of these tanniniferous legume species have also been the preferred model for exploring the mode of action of polyphenols against the different stages of GINs (see Hoste et al., 2012). In addition, R&D studies for nearly 20 years on *O. vicifolia* or *L. cuneata* have compared different forms of exploitation, namely direct grazing, conservation as hay or silage (Paolini et al, 2005, Heckendorn et al, 2006, Shaik et al., 2006, Werne et al, 2013) or more recently with dehydrated pellets (Terrill et al., 2007, Gujja et al., 2013, Girard et al., 2013, Kommuru et al., 2014).

4.2.2 Exploiting rangelands and their biodiversity of plant resources

In most tropical parts of the world, the ecosystems present a wide plant biodiversity and include several plant species with nutraceutical potential as natural forages. However, for many of those plant species, there is no information on their agronomy or on possible propagation methods. There are, however, a few exceptions, such as *Leucaena leucocephala, Arachis pintoi, Gliricidia sepium or Cratylia argentea (*von son de Ferneix et al., 2014*).* All are from the Fabaceae family and are exploited **i)** in plantations, **ii**) in silvo-pastoral systems that consist of rows of grass and trees, or **iii)** in so-called "fodder banks" that are tree plantations in a very dense system that are not allowed to grow beyond approximately 2 m in height. Thus, nutraceutical forages in tropical areas could offer a variety of situations for their production. However, most farmers currently are relying on the natural availability of nutraceutical plants. Meanwhile, the few people who can invest money in their farms may also be able to build silvo-pastoral systems or fodder banks. Irrespective of the production strategy, such plants may be used for direct browsing or in a cut-and-carry system.

At the moment, most farmers let their animals browse the natural vegetation. Under such conditions, it is not possible to determine whether animals have consumed enough nutraceutical material to obtain the desired AH effect. Cut-and-carry systems could instead be the answer to ensure that animals are exposed to sufficient nutraceutical material. However, these systems are constrained by time spent by farmers to harvest enough plant material for all animals, the need for a vehicle to move sufficient plant material, and difficulties with harvesting (e.g. thorns). In any case, such management strategies could eventually deplete the resource. Thus, the creation of low-cost fodder banks represents a more sustainable option. However, it poses considerable challenges in terms of investment and technical knowledge that is beyond the capability of most small farmers at present.

For the tropical areas, a considerable amount of data has been acquired in Mexico for tannin-containing leguminous trees, such as *Havardia albicans* and *Lysiloma latisiliquum*. These materials were tested first under *in vitro* conditions (Alonso-Díaz et al., 2008a, 2008b; Hernández-Orduño et al., 2008) and their promising results led to subsequent *in vivo* studies with sheep (Martínez-Ortíz de Montellano et al., 2010; Galicia-Aguilar et al., 2012; Mendez-Ortíz et al., 2012) (see section 3.1.2).

4.2.3 Exploring the value of agro industrial by-products

In different areas of the world, interest has been growing in exploring the potential of tannin-containing 'waste' or by-products from agro-industries. These represent an alternative option to "natural" nutraceuticals, in the sense that the PSM can be extracted from the by-products and then added to an existing feed (see Table 1). Transforming 'waste' tannin-rich materials into nutraceutical feeds with antiparasitic properties could have several advantages. First, this represents a viable alternative to add value to agro-industrial waste products; secondly, it may help to solve the problem of the inherent variability of PSM content (see below) in nutraceutical plants, as it would allow adjusting the bioactive PSM concentration(s) in feeds (Girard et al., 2013). Third and consequently, it may also help to avoid any negative consequences caused by an excess of PSM in the feed.

Some examples of tannin-containing plant by-products that have been under recent investigation for their AH activities are: 1/ by products from the nut industry in temperate areas (Desrues et al., 2012, Girard et al, 2013), 2/ carob pods (Manolaraki et al., 2010; Arroyo-Lopez et al., 2014) in the Mediterranean region, and 3/ coffee by-products and cocoa fruit husks and leaves in Yucatan, Mexico (Covarrubias-Cárdenas et al., 2013; Vargas-Magaña et al., 2014a, 2014b).

4.2.4 Inherent variability of nutraceutical plant materials

Whatever the mode of exploitation (grown, browsed, or by-products), once a plant species has been identified through *in vitro* and *in vivo* studies as a potential candidate for use as a nutraceutical, it is still important to consider the inherent variability caused by several factors which influence the quantity and/or quality of PSMs and hence the antiparasitic effects of nutraceutical candidates.

Sainfoin (*O. viciifolia*) has been extensively studied to explore such factors that influence PSM contents and composition and the related AH activities (Manolaraki et al., 2011). Three main factors have been identified: environmental conditions (e.g. phenological stages, areas or soil conditions for growth, climate and seasons), genetic factors (cultivars or chemotypes) (Azuhnwi et al., 2013; Stringano et al., 2012) and also technological processes (e.g. fresh versus hay, silage and pellet samples).

It is possible that this issue of variability caused by the different factors is also important for growing sericea lespedeza (Muir et al., 2014), or for the leaves of *L. leucocephala* or *Manihot esculenta* that originate from plantations in tropical zones. Variation of PSM contents may be even more evident in plants that grow in the native vegetation. Under such conditions, variation of PSM content is significant between individual plants even during the same season in the same geographical region (Alonso-Díaz et al., 2010b).

Further factors, which are frequently not considered, include variation in harvesting methods by farmers, such as the leaf:stem ratio that results from pruning only young leaves (branch tip) or complete branches (often based on biomass feed requirement), and post-harvest practices, such as sun drying, wilting, which are often based on nutritional advice, but may not have considered the effects on the bioactive PSMs, such as their reduced content or activity in the feed. As the common and traditional view is that PSMs are anti-nutritional factors, this type of advice will need to be revised for nutraceuticals.

However, as far as we are aware, hardly any research has been conducted on selecting or breeding for bioactive plants with stable tannin or other PSM compositions. This seems to be an opportunity that would be worth exploring as we have shown that a few relatively ‘robust’ sainfoin and sorghum chemovars used earlier exist, where the PSM composition was much less dependent on the environment (Azuhnwi et al., 2013a; Mueller-Harvey and Dhanoa, 1991).

5. PERSPECTIVES

Based on the previous description of tannin-containing legumes against GIN in small ruminants and the development of methodological approaches for exploring the nutraceutical concept, several areas for future research have been identified that focus on on-farm applications.

5.1 What else? Novel parasitic “targets” for tannin-containing legumes

5.1.1 Against GINs in other host species.

Several recent *in vitro* results have shown that tanniniferous legumes can also regulate worm biology of the main genera of GINs in cattle (Sandoval-Castro et al., 2012). Significant results were obtained with extracts of sainfoin (*O. viciifolia*); *L. corniculatus* and *L. pedunculatus* against *Ostertagia ostertagii* and *Cooperia oncophora* (Novobilsky et al, 2011, 2013). These data are offering opportunities for *in vivo* studies and to promote the combined control of bloat and GINs in cattle production. Recently, a 50% reduction in worm burden was obtained in calves infected with *O. ostertagi* and fed sainfoin pellets (Desrues et al, 2015 WAAVP 2015 abstract).

Attempts have been made in South Africa to regularly include wattle extracts (*A. karroo*) as a supplementary feed for cattle that are infected with *Haemonchus* sp and *Oesophagostomum columbianum*. These studies reported a reduction in egg excretion (Xhomfulana et al., 2009).

Two recent studies, based on *in vitro* assays, have also underscored that different tanniniferous plant sources could also be used against GIN in monogastric livestock hosts given the positive assessment of AH effects against *Oesophagostomum dentatum* and *Ascaris suum* in pigs (Williams et al., 2014a and b). However, it remains to be seen whether these tannin resources will be used as herbal remedies or as nutraceuticals (Table 1). These *in vitro* data are supported by a field observation study that examined the effects of acorns (*Quercus robur*) fed to outdoor pigs which had been raised with natural nematode infections. The results indicated a dramatic reduction (> 90 %) in GIN faecal egg count (Salajpal et al., 2004).

5.1.2. Against other digestive parasites

Recent investigations have also explored the effects of tannin-containing plants against *Eimeria* infections in small ruminants. Although early results were disappointing from an *in vitro* assays that examined a wide range of sainfoin extracts for their capacity to inhibit oocyst sporulation (Saratsis et al., 2012), the results of *in vivo* studies on natural infection were much more encouraging. These studies evaluated sainfoin fed to lambs (Saratsis et al., 2012), and sericea lespedeza (*Lespedeza cuneata*) fed to either lambs (Burke et al., 2013) fed before and at weaning or kids (Kommuru et al., 2014). Moreover, some early results are also available that examined the effect of *Pistacia lentiscus* in young goats (Markovics et al., 2012).

In most of these *in vivo* trials, significant reductions in oocyst excretion were measured when young animals were fed with legumes. In particular, kids fed with *L. cuneata* showed reductions greater than 90 %. In addition, there was some evidence that sericea lespedeza had a positive effect on host resilience, as it led to a lower requirement for anti-coccidian treatments. These promising results suggest that tannin containing legumes, in particular pellets of sericea lespedeza, represent an option for a plant-based control of coccidiosis in small ruminants around weaning time.

The ability of lambs and kids to consume sufficient nutraceutical amounts to prevent coccidiosis around weaning could be a limiting factor that will need to be considered. However, results of a recent experiment with calves, which were under 15-day old, are promising: supplementation of milk with concentrated pomegranate extract may, depending on the concentration, reduce faecal oocyst count and diarrhoea intensity and duration because of *Cryptosporidium parvum* infection (Weyl-Feinstein et al., 2014) [it should be noted, however, that pomegranate contains mostly ellagitannins – and not CTs].

5.2. Exploring plant resources as nutraceuticals: example of other plant families, other bioactive PSMs.

The logical and successive steps described in section 3 have helped to identify other botanical resources, which could be exploited as nutraceuticals. Two examples that meet the general criteria of nutraceuticals as defined in Table 1 will be mentioned here to illustrate these issues.

An impressive range of studies has been performed in Spain that investigated the AH effects of different heather species that belong to three *Erica spp* and one *Caluna sp* (Ericeacae) and are browsed by Cashmere goats (Moreno-Gonzalo et al., 2012). This is a first example for which a whole series of consistent results were obtained that covered *in vitro* assays (e.g. Moreno-Gonzalo et al., 2013), controlled *in vivo* studies with experimental infections in confined conditions (Frutos et al., 2008; Moreno-Gonzalo et al., 2014) and systemic studies with natural infections (Osoro et al., 2009).

Chicory (*Cichorium intybus*) (Asteraceae), when used as a forage for ruminants, represents another example of potential nutraceutical with AH properties, which has illustrated the “robustness” of the methodological approach described in section 3. Chicory has been shown to possess AH properties based on experiments that ranged from *in vitro* assays (Molan et al., 2003b; Foster et al., 2011 a,b), simple *in vivo* studies (Peña-Espinoza et al., 2015 WAAVP2015 abstract), to systemic studies (Athanasiadou et al., 2007; Tzamaloukas et al., 2006 a,b; Nielsen et al., 2009).

Two other points are worth mentioning in comparison of chicory and the legume models and illustrate which additional studies are required to expand the nutraceutical concept for tackling digestive parasites in infected livestock.

- Based on several *in vitro* results, the nature of the AH PSMs of chicory seems to stem from sesquiterpene lactones and not from CTs and related polyphenols (Molan et al., 2003b, Foster et al., 2006, 2011a and b).
- Despite the different PSMs, results acquired with chicory forages also illustrated that environmental and genetic factors affected the anti-parasitic activity and were linked to variations in PSM quantity or composition. This is evidenced by marked differences between cultivars (Miguel Peña-Espinoza, personal communication, 2015). Therefore, these factors require addressing before to seeking on-farm implementations for such nutraceuticals (Foster et al., 2006, 2011a).

5.3. Self-medication and nutraceuticals: a novel field for basic researches on the host-gastro intestinal nematode interactions.

Given that the antiparasitic effects of nutraceuticals depend on the PSM concentration in a feed and on the length of consumption by the infected host, the tannin-containing legumes provide a valuable model to explore the host-parasite (GIN) interactions in regard of regulation of infection related to the host feeding behaviour and /or to assess the balance between host immunity and nutritional behaviour (Hoste et al., 2010). Interesting studies can and have yet been performed that examine 1/ the ability of the host to select a feed with AH properties when hosts are infected or not with GINs (=self-medication) (Lisonbee et al, 2009; Villalba et al, 2013, Junkhe et al, 2012); **2/** the influence of various host or parasite factors on the self-medication behaviour (Amit et al, 2013); and **3**/ the trade-offs between negative nutritional effects and beneficial health effects that accrue from the consumption of nutraceuticals (Frutos et al, 2008).

It is important to indicate, however, that the influence of GIN on the ingestion of nutraceutical plant materials is not easy to evaluate in regard of methodological issues. Attempts to evaluate this phenomenon using cafeteria studies, where animals are exposed to different types of plant materials, or direct observation methods in the field, that compare animals with and without parasites, will be influenced by the quantity of parasites present in the animals (light to heavy burdens), the time that animals had been naturally infected with GIN before the study began (naïve or immune competent animals), the existing feeding experience, physiological adaptations (e.g. tannin binding saliva) of the animals investigated, amongst several other aspects, many of them being difficult to control (Alonso-Díaz et al., 2010a; Vargas-Magaña et al., 2013). Because self-medication has been the subject of 2 recent review (Villalba and Provenza, 2007; Villalba et al., 2014), this issue will not be developed further.

6. CONCLUSIONS.

- Because of the constant, worldwide, rapid development of resistance to synthetic chemical AHs, and also because of the increasing societal demand, there is nowadays a clear and urgent need to explore and validate alternative options for specific livestock systems (e.g. organic farming systems, small dairy ruminant systems in EU or in caprine breeding).
- For these reasons, in regard of the control of GINs in livestock, as previously evoked (Thamsborg et al., 1999) with the 21st century, we are now probably entering the post-anthelmintic era and are moving from relatively simple solutions for on-farm applications (use of synthetic AHs) towards much more complex options.
- The development of nutraceutical products with real potential for the control of GIN in ruminants is a possibility that is well underway of becoming a reality in different parts of the world for different livestock breeding systems and relying on different plant materials.

- The complexity of the scientific questions which need to be addressed are intrinsic to nutraceuticals. Therefore, the possibility of developing on-farm applications against digestive parasites requires a multidisciplinary approach between scientists with expertise in parasitology, as well as phytochemistry animal production, digestive physiology, ethology and other.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 1: The criteria defining the main concepts of xenobiotics and plant secondary metabolites for controlling gastrointestinal nematodes of livestock (VFI = voluntary feed intake).

Nature of the bioactive compounds	**Synthetic chemical compounds**		**Natural chemical compounds (Plant secondary metabolites)**	
Formulations	**Therapeutic drugs**	**Chemical additives (supplements)**	**Herbal drugs**	**Nutraceuticals**
Mode of administration	Forced Administration	Added to the feed	Forced administration	PSMs Included in and/or added to the feed
	Independent of VFI	Dependent of VFI	Independent of VFI	Dependent of Voluntary Feed Intake (VFI)
	Short term Well defined posology	Long term Posology defined by a range within the feed	Short term	Long term
Objective	**Curative /** (preventive)	**Preventive**	**Curative/** (preventive)	**Preventive/** (curative)
Quality of the active compounds	Standardised Identified	Standardised Identified	Variable Usually non identified	Variable Identification of the family of phytochemical compounds
Mode of action	Usually, well identified	Usually, well identified	Unknown	Hypotheses
Development of resistance	High	High	Unknown	Suspected

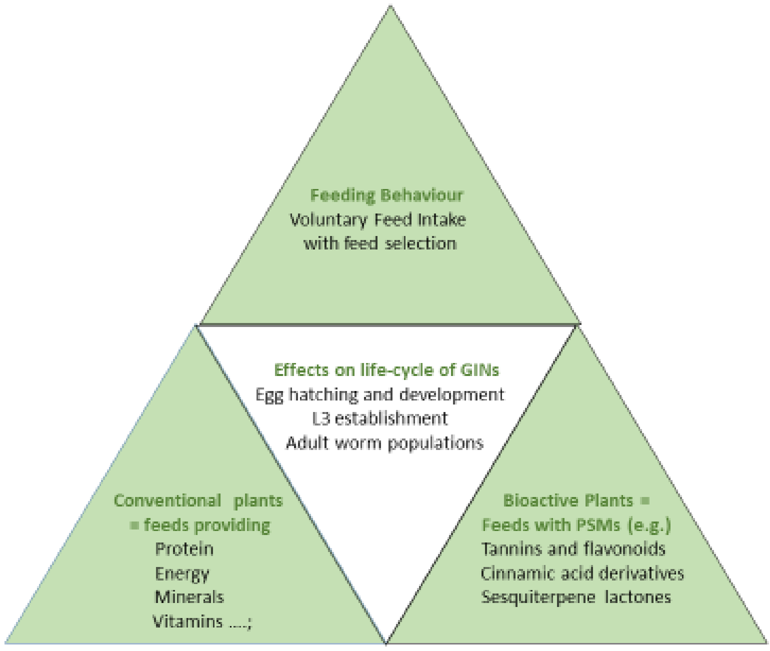
Figure

FIGURE 1: A proposed model of the components defining the concept of nutraceuticals against gastrointestinal parasites in livestock

FIGURE 2: The different *in vitro* assays available to screen the AH effects of potential nutraceuticals (see Jackson and Hoste, 2010), **EHA:** Egg Hatching Assay; **LFIA**: Larval Feeding Inhibition Assay; **LDIA**: Larval Development Inhibition Assay; **LMIA**: Larval Migration Inhibition Assay; **LEAI** : Larval Exsheathment Inhibition Assay; **AMIA**: Adult Motility Inhibition Assay; ***C. elegans***: Assays developed using the *C. elegans* model of free living nematodes.

FIGURE 3: Three key stages of the GIN life cycle have been identified as possible targets when tanniniferous plants are consumed by infected small ruminants: 1/ a reduced excretion of nematode eggs by the adult worms (maximum values up to 80 % reduction) (Shaik et al., 2006); 2/ a reduced establishment of the infective third-stage larvae in the host (up to 70 %) (Brunet et al, 2008); and 3/ a reduced development of eggs to third-stage larvae (maximum values of reduction up to 90 %) (Niezen et al., 2002).

FIGURE 1



Feeding Behaviour
Voluntary Feed Intake
with feed selection
Effects on life-cycle of GINs
Egg hatching and development
L3 establishment
Adult worm populations
Conventional plants
= feeds providing
Protein
Energy
Minerals
Vitamins ….;
Bioactive Plants =
Feeds with PSMs (e.g.)
Tannins and flavonoids
Cinnamic acid derivatives
Sesquiterpene lactones

FIGURE 2

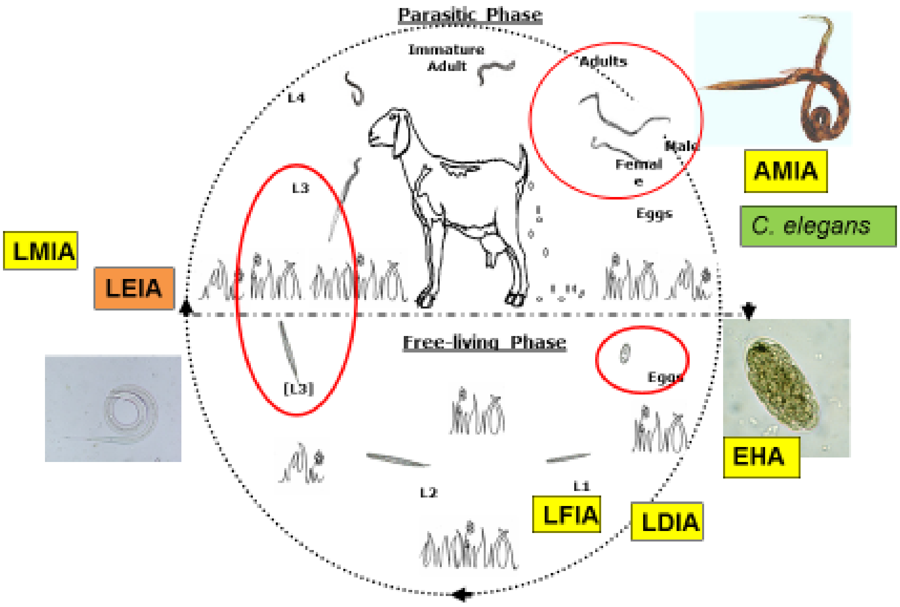
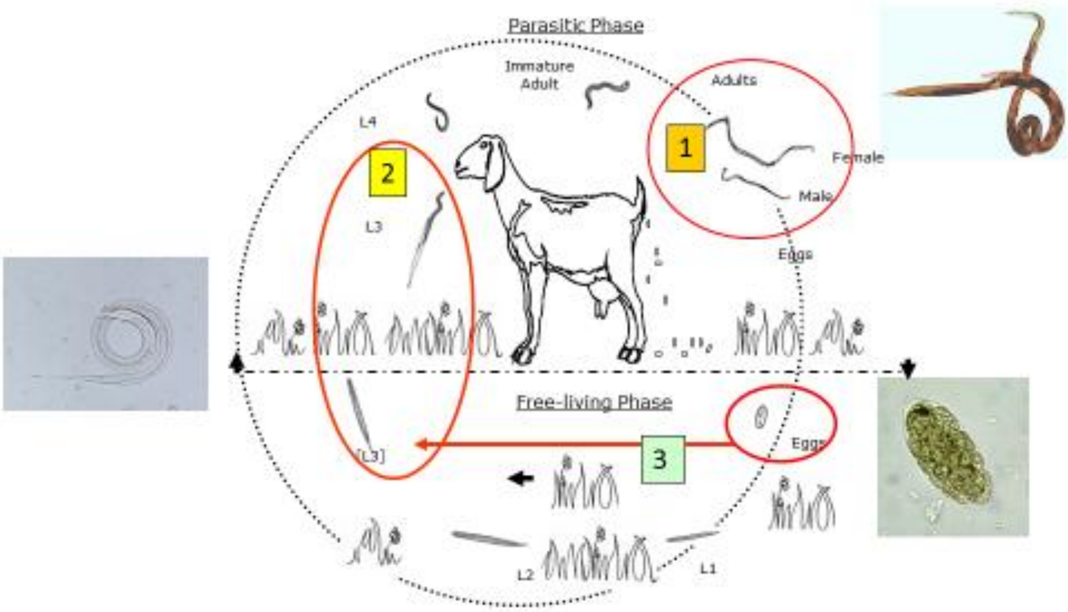


FIGURE 3



Parasitic Phase
Immature Adult
Adults
L4
1
2
Female
Male
L3
Eggs
Free-living Phase
3
Eggs
[L3]
L2
L1