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How antimicrobial plant extracts can affect the *in vitro* cell-mediated response in free-range Mangalitsa pigs

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Summary

The Mangalitsa pig bread, becoming more and more popular, is raised in Romania, especially on low input farms, for its tasty and unique flavoured meat. In such systems, the survival of animals and the outcome of the impact of pathogens on health is highly depend on the pigs' immunity. Thus medicinal plants which are known for their biological activities, available on the pastures or in the feed provided, could act as immunity enhancers, strengthening the pigs' resistance to diseases. The research was carried out on extensively raised suckling, weaned piglets and sows of the Mangalitsa breed, divided by ages in groups of ten (n = 10). Blood samples were taken, for ethical and health concern reasons, during the campaign for brucellosis testing, official in Romania. The samples were then transported to the laboratory in less than four hours and immediately subjected to the leukocyte blast transformation test. For this, the blood was mixed 1 : 4 with RPMI1640 (Sigma Aldrich, USA), then divided in 200 µl aliquots/well, in 96 well-plates and supplemented with alcoholic plant extracts (*Calendula officinalis*, *Satureja hortensis*, *Allium sativum*, *Coriandrum sativum*, *Cucurbita pepo*), 1.5 µl/well, in duplicate. After a 48 h incubation period at 37°C, the cultures were tested for residual glucose in the supernatant by the orto-toluidin test, obtaining a green compound quantified by spectrophotometry (SUMAL PE2, Karl Zeiss, Jena, λ = 610 nm, d = 0.5 cm). The glucose consumption (%) was calculated versus the initial culture medium glucose content for each variant. The variant and the groups were then compared by Student's t test for statistical significance of the results. The results indicated statistically significant differences between the young age groups, suckling and weaned piglets (p = 0.017 to 0.000016) for all plants except marigold, for weaned piglets and sows (p = 0.0001-0.0359), for all plant extracts, while for suckling piglets – sows p = 0.0035 and p = 0.0461 were recorded for summer savory and garlic, respectively. The plant extracts used known for biological effects impacted based on the age of the pigs and plant family, proving their immune stimulating capacity.

Keywords: Mangalitsa, low input, adaptive immune response, plant extracts

In Romania, the prevalence of low input pig farms is still high, as a result of traditional value and preference of people to grow their own food. According to the National Veterinary Sanitary and Food Safety Authority in 2023 the ratio of pigs growing on low input farms was approximately 40% of the overall pig population (24). One of the local breeds, valued for its easy raising due to the efficient use of wild pasture and local product (potatoes, pumpkins) supplements, as well as low cholesterol and unique taste is the Mangalitsa pig (15).

Even though growing pigs in free-range farms could improve their meat qualities, various risks concerning their health and welfare could impede on these produc-

tions. One of the main factors that could influence welfare and health is the pathogenic microbiome, which could increase economic losses due to high morbidity and mortality, immune suppression and disease control and prevention costs (27).

Alternative therapies were suggested for both humans and animals, supported by traditional ethnomedicinal practices lasting for millennia. Consumption of medicinal plants or plant extracts, due to their active constituents, have been shown to possess biological activities including antimicrobial, antioxidant and healing properties.

Consequent to a continuously increasing antibiotic consumption and therefore progressively accumulating antimicrobial resistance, current pharmacology research shifted worldwide into studying the antibacterial effects of various medicinal plants, aiming at providing a reasonable alternative to allopathic treatment in infectious diseases, also enhancing the efficacy of the immune system as adjuvants (2, 33).

Certain, largely available plants, such as *Fabaceae*, *Oleaceae*, *Philadelphaceae*, *Asteraceae*, *Lamiaceeae*, as well as many others were subject to research in finding antimicrobial combinations or products.

As part of a broader study which was supported by the project PPILOW, which has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 816172 and by UEFISCDI Romania under grant agreement ERANET Core Organic Co-fund ROAM Free #249/2021, locally available plants such as *Calendula officinalis*, *Allium sativum*, *Coriandrum sativum*, *Satureja hortensis* and *Cucurbita pepo* were envisaged in the endeavour of seeking plants to combat parasites and pathogenic bacteria, while improving the immune status and welfare of pigs from local breeds in low input farms from North Western and Central Romania.

Calendula officinalis is a plant from the Asteraceae family, used in phytotherapy due to its richness in chemical compounds, such as carotenoids, flavonoids, terpenoids, coumarins, quinines, amino acids and others (23). The complex chemical composition of *Calendula* confers upon a variety of biological activities, such as immunostimulant, antigenotoxic, anti-inflammatory, antioxidant, promoting healing, *in vitro* antibacterial inhibitory effect against various Gram-positive and Gram-negative strains, gastroprotective, etc. (17). Studies conducted on pigs have shown that *Calendula* intake could be beneficial in combination with other plant extracts by enhancing neutrophil activity, leading to immune stimulant and antibacterial effect (19). In concentrations below 10% of the diet it increased feed intake and protected DNA from oxidative damage, therefore promoting growth (11, 14).

Satureja hortensis, popularly known as summer savory, is a medicinal and aromatic plant belonging to Lamiaceae family, widely distributed across the temperate zones of the globe and easily accessible (3). This plant is a rich source of phenolic compounds, flavonoids, terpens, minerals, vitamins (A and C) and essential oils. Due to its abundance in various chemical constituents, *Satureja hortensis* possess various biological activities, such as antibacterial, antiseptic, anti-inflammatory, antiviral, antioxidant (10). Swine diet supplementation with summer savory has shown beneficial effects, i.e., antiparasitic activity, improved gut health due to increased elimination of parasites, improving therefore the digestibility, heightening feed intake and increasing the weight gain (6, 9, 16, 18, 27).

Cucurbita pepo L. is a plant grown in a broad geographic area, belonging to Cucurbitaceae family. *Cucurbita* possess therapeutic effects, being an effective antibacterial, antioxidant, antitumor, hypoglycemic and hypolipidemic plant (1). In spite of the paucity of studies that discuss the beneficial effects of *Cucurbita* in swine health, the existing researches demonstrate that a diet supplemented with *Cucurbita pepo* has determined weight gain, anthelmintic effect and increased prebiotic activity (5, 20).

Allium sativum L. (garlic), a member of the Amaryllidaceae (formerly Alliaceae) family, has been used since ancient times for its medicinal properties, as a therapeutic or prophylactic ailment in cardiovascular diseases, infectious diseases, parasitic infections or cancer (21). Dietary changes, with supplementation of garlic or garlic extracts, in pig feed, have shown notable positive effects due to microbiota changes. Thus, lower concentration of allicin, one of the main active components in garlic, displays an inhibiting effect on pathogenic *Enterobacteriaceae*, while higher concentrations inhibit *Lactobacillus* spp. (27, 28, 31). Added to fodder, it also induces an increased food intake, a higher average daily gain, but also heightened intestinal villi and thus, a better absorption of nutrients (8, 27, 34).

Coriandrum sativum is an herb/spice/medicinal plant component of the Apiaceae family, rich in polyphenols and essential oils. Renowned biological activities, prominently antidiabetic, cardiovascular and antimicrobial effects, are the result of its chemical composition (31, 33). Therein, the literature does not provide adequate information of *Coriandrum sativum* effects or its health benefits in pigs, neither *in vivo* nor *in vitro*.

The purpose of this research, as a first step of a broader study envisaging antibacterial, antiparasitic and immunological effects of the selected plant panel, was to quantify the *in vitro* immune stimulating effect of *Calendula officinalis*, *Allium sativum*, *Coriandrum sativum*, *Satureja hortensis* and *Cucurbita pepo* for designing further tailored therapies in administering these plants to pigs as efficient pest control and immune stimulation means.

Material and methods

Biological material. The experiment was carried out *in vitro*, using blood sampled from extensively raised Man-

galitsa pigs (n = 30) from a farm of 240 animals in total. The farm was located in a temperate continental climate region, where all the animals had permanent access to the outdoor environment and benefits of enrichments (mud bath, roughage, straw and toys) (5, 6).

The animals were divided into three groups, based on their age: suckling piglets (n = 10), weaned piglets (n = 10) and sows (n = 10), for defining the influence of age on the medicinal plants' extracts *in vitro* immune enhancing effects.

The blood was sampled in commercial tubes containing heparin (Vacutest Kima, Na Heparin, 6 ml) from the jugular confluent in piglets and auricular vein from weaners and sows: a single sampling from each individual. To avoid additional stress for the animals as well as for ethical and health concerns, blood samples were taken during the campaign for official brucellosis testing, within the Romanian animal disease surveillance program, approved by the National Veterinary Sanitary and Food Safety Authority. The samples were then transported to the laboratory in less than four hours, in an isothermal box with the addition of a Deltaphase® Isothermal Pad (Braintree Scientific Inc., US) to keep the blood at 37°C. Upon arrival to the laboratory the samples were immediately subjected to the leukocyte blast transformation test.

Obtainment of the plants extracts for *in vitro* use. Five plants were selected to be tested, namely *Alium sativum* L., *Coriandrum sativum* L., *Cucurbita pepo* L., *Calendula officinalis* L. and *Satureja hortensis* L., due to their local availability and their traditional use in the study area. The plants were provided by licensed companies, specialized in cultivating medicinal and aromatic plants and commercializing them for human use in dedicated stores (i.e., SN Plafar SA, Romania). The plant parts (*C. officinalis*, *S. hortensis – herba*, *C. sativum*, *C. pepo – semen*, *A. sativum – bulbus*) were extracted and analyzed for their chemical composition at the "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca (4). For this, 10 g of each plant were mixed with 100 ml of 70° ethyl alcohol and macerated for 72 h. Subsequently, the mixture was transferred into a turbo extractor, for 3 minutes at 4000 rpm. The final extract is filtered and submitted to analysis via High performance liquid chromatography/mass spectrometry (HPLC-MS), which were coupled to identify and quantify the main components of the alcoholic plant extracts (HPLC: Agilent 1100 HPLC Series, Agilent Technologies, Santa Clara, CA, USA, MS: Brucker Ion Trap SL, Brucker Daltonics GmbH, Leipzig, Germany). The data were processed by using ChemStation and DataAnalysis software from Agilent (Agilent Technologies, Santa Clara, CA, USA) (4). The content of polyphenols, tocopherols and sterols were quantified from each sample.

The leukocyte blast transformation test. The implemented protocol assessed the *in vitro* proliferative reaction of mononuclear leukocytes to mitogens, which non-specifically stimulate these cells, along with plant extracts, by gauging glucose consumption as an indicator of cellular reactivity (34).

For this, the blood was diluted 1 : 4 with RPMI 1640 medium, and afterwards distributed in 200 µl aliquots in 96-well plates. Phytohemagglutinin obtained from *Phaseolus vulgaris* (PHA, 5 µg/mL), which induces the transformation of T lymphocytes, served as a stimulation control, while the alcohol (7.5 µg/mL) served as solvent control for the plant extract, treated experimental variants. The experimental variants of each sample were added with 7.5 µg/ml of alcoholic extracts from *Calendula officinalis*, *Satureja hortensis*, *Allium sativum*, *Coriandrum sativum* and *Cucurbita pepo*). All samples were performed in duplicate. After 48 h, glucose concentrations were measured in the initial RPMI 1640 and in all plant treated variants, using a glucose solution with a standard concentration (100 mg%), by an orto-toluidine colorimetric test. For this, 50· µL of the cultural supernatant were mixed with 2·ml of orto-toluidine reagent, boiled for 8·min, cooled suddenly in cold water and read at 610·nm wavelength, d = 0.5 mm, in a spectrophotometer (SUMAL PE2, Karl Zeiss, Jena, Germany), versus the o-toluidine solution. The blast transformation index (BTI) was calculated as follows: $BTI\% = [(MG - SG)/MG] \times 100$, where MG was the glucose concentration in the initial culture medium and SG the glucose concentration in the sample after incubation (32).

Statistical analysis. Average values and standard deviations were calculated by use of Microsoft Excel program. The results for each group were compared using Student's t-test (method used in testing of hypothesis for comparison of means between groups), to establish their statistical significance ($p < 0.05$) (22).

Results and discussion

The microbial aggression's effect is reliant on pigs' management and, implicitly, their immune status. The rise of microbial infections, especially in low input farms, is due to epidemiological pressure, lack of prevention, excessive consumption of antibiotics or biased immune response. To reduce the microbial aggression it is important to enhance the pig's immune response and to strengthen their resistance to diseases, by administering immunity enhancers, as medicinal plants.

In low input farming, such plants can be found on pastures or administered in the fodder pigs are being fed with (13). The relatively low cost and broad availability of the plants subjected to the present experiment could provide limited resource farmers with a readily available tool to provide a triple, antiparasitic, antimicrobial and immune enhancing effect (5, 6).

Plant extract analysis. The results of the plant extracts' analysis indicated that each medicinal plant, tested via HPLC-MS, possessed a unique chemical composition, including different compounds or compound ratios. The content in active components is substantially dependent on the geographical area, the soil structure, the harvesting season and the climatic conditions during the growing and harvesting periods (1, 2, 8, 9). Although a generic composition table could be available for each tested plant, before evaluating their *in vitro* effects, the concentrations of the bioactive components needed to be evaluated. Even though tocopherols (radical-chain breakers with antioxidant activity) (3) have been found in different concen-

Tab. 1. Chemical composition and compounds concentration of tested plant extracts (adapted from 4)

Bioactive constituents		Plant extracts submitted to HPLC-MS* analysis and their constituents concentration				
		Callendula officinalis	*Allium sativum*	*Coriandrum sativum*	*Satureja hortensis*	*Cucurbita pepo*
Polyphenols (µg/ml)	chlorogenic acid	220.767	–	4.177	–	–
	isoquercitrin	38.877	–	–	6.515	–
	rutoside	18.819	–	–	–	–
	syringic acid	1.51	–	0.09	–	–
	protocatechuic acid	0.67	–	–	2.28	–
	vanillic acid	0.44	–	0.94	0.95	–
	caffeic acid	–	1.221	–	0.65	–
	ferulic acid	–	0.456	0.759	0.557	–
	sinapic acid	–	0.228	–		–
	p-coumaric acid	–	–	0.501	1.464	–
	quercitrin	–	–	–	0.365	–
	quercetol	–	–	–	0.394	–
	luteolin	–	–	–	6.621	–
	apigenin	–	–	–	2.442	–
Tocoferols (ng/ml)	α-tocoferol	61.6	36.1	–	86.8	–
	γ-tocoferol	248.9	–	–	89	446
	δ-tocoferol	9.3	–	–	13.2	23.2
Sterols (ng/ml)	ergosterol	500	–	0.584	–	–
	stigmasterol	72888	–	9.675	–	22.024
	β-sitosterol	241997	–	34.548	–	5.355
	campesterol	1635	–	1.78	–	0.358
Sulfoxide (µg/ml)	aliin	–	14.726	–	–	–
Methoxylated flavones (ng/ml)	acacetin	–	–	–	12691.97	–
	jaceosidin	–	–	–	8820.76	–
	hispidulin	–	–	–	2483	–

Explanations: *HPLC-MS – high performance liquid chromatography-mass spectrometry; "–" – compound not found

trations in almost all plant samples, other bioactive constituents were identified, such as polyphenols and sterols in *Calendula officinalis*, polyphenols and alliin in *Allium sativum*, sterols in *Cucurbita pepo*, sterols and polyphenols in *Coriandrum sativum*, which had no tocopherol in its content, polyphenols and flavones in *Satureja hortensis* (Tab. 1) (4). The components and their concentrations identified for the purpose of this experiment were in the range suggested by other authors (11, 17).

Blast transformation test. The innate component of the immune system in pigs reaches maturity at birth, but piglets are deprived of adaptive immune cells and antibodies due to the peculiarities of the placenta in this species (35). Through colostrum, and later milk, the piglets are provided with immune cells and antibodies, which allow them to cope with the environmental antigens, appearing with the stimulated T and B cells and corresponding memory cells. This will fulfill the adaptive immune functions as the animals grows (30). Weaning is a moment in the piglets' life when changes in feeding and increase in the digestive intake of microbial load leads to a decrease in the adaptive cell-mediated response (30). Due to the lack of data on changes in the age-related development of the adaptive immune response in Mangalitsa pigs, the general trend of development of the immune response in pigs was considered in interpreting the experimental data. The environment factors could bias the interpretation of the results, since the adaptive response is highly variable with age and limit the value of the study, therefore on the farm selected for the testing the environment was highly consistent, the animals sharing the same shelter and caretakers.

Samples collected from suckling piglets (Fig. 1) have shown increased response to classic mitogens PHA when compared to the untreated control culture, suggesting that the mononuclear cells have the ability to respond to stimuli *in vitro*. Nevertheless, the differences between the results recorded for various plants, either inhibition or stimulation, were obvious. In this category, although there are no statistically significant differences between the variants, it can be seen that the alcohol induces an inhibiting effect when compared

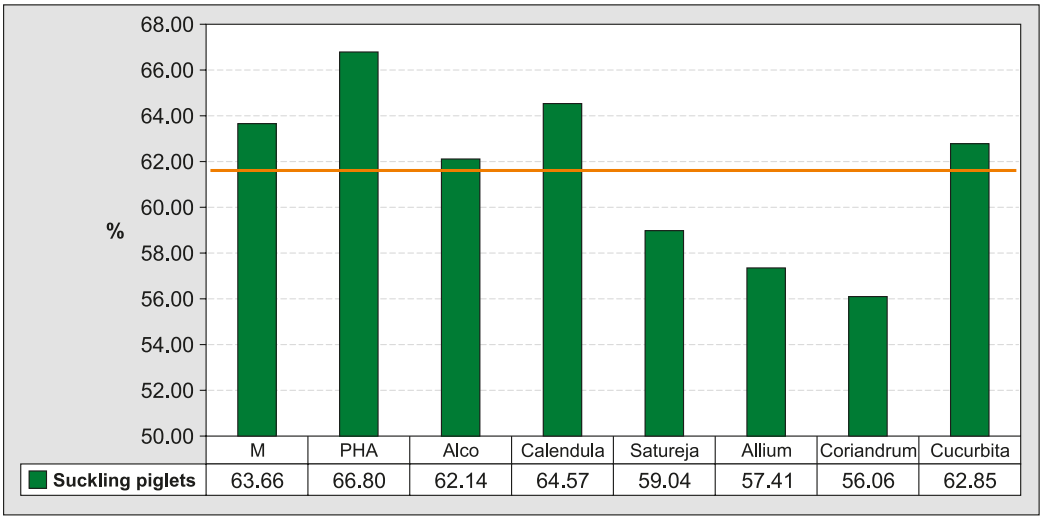

Fig. 1. Blast transformation indices (BTI, %) recorded for the blood samples collected from suckling piglets. Although there are no statistically significant differences between the variants, it can be seen that the alcohol induces an inhibiting effect when compared to the untreated control, while all the plant extracts, with the exception of *Calendula officinalis* exert an inhibiting effect as well. The inhibition caused by the coriander extract is the most pronounced while the garlic and summer savory extract are less inhibiting, in that order

Explanations for Fig. 1-4: BTI% – glucose consumption, as calculated at the end of the culture incubation period; M – untreated control; PHA – samples treated with *Phaseolus vulgaris* phytohemagglutinin; Alco – samples treated with 70° alcohol; Calendula – samples treated with *Calendula officinalis* alcoholic extract; Sat – samples treated with *Satureja hortensis* alcoholic extract; Allium – samples treated with *Allium sativum* alcoholic extract; Coriandrum – samples treated with *Coriandrum sativum* alcoholic extract; Cucurbita – samples treated with *Cucurbita pepo* alcoholic extract

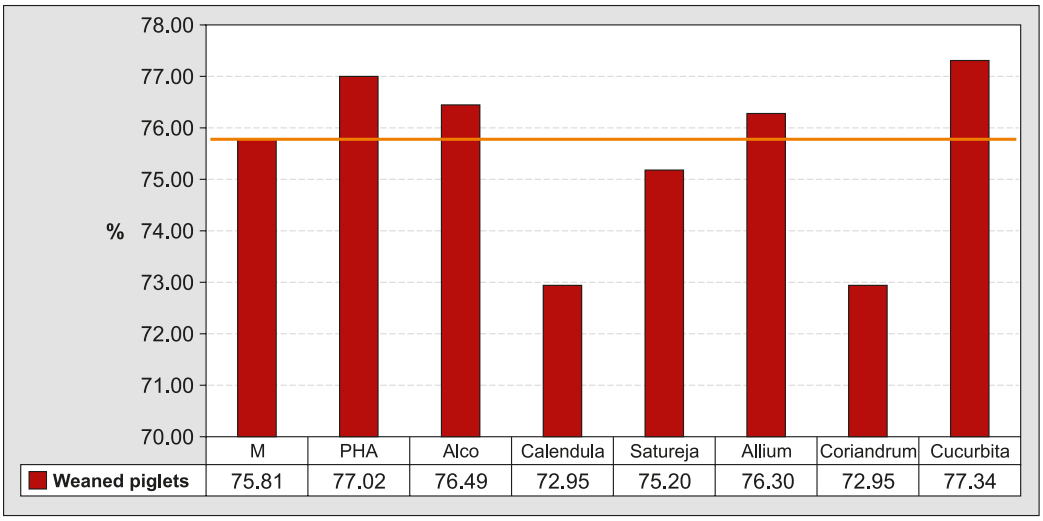

Fig. 2. Blast transformation indices (BTI, %) recorded for the blood samples collected from weaned piglets. This age category reacted in a different manner to the solvent, which induced stimulation while compared with the unstimulated control culture. Both *Calendula officinalis* and *Coriandrum sativum* exerted equally an inhibiting effect versus untreated and alcohol treated controls. Summer savory was still inhibiting, but at a lesser extent than marigold and coriander, while for this category the garlic stimulated cell growth when compared to the untreated control but not with the solvent control

to the untreated control, while all the plant extracts, with the exception of *Calendula officinalis*, exert an inhibiting effect as well. The inhibition caused by the coriander extract is the most pronounced, while the garlic and summer savory extract are less inhibiting, in that order.

In weaned piglets, the overall picture indicated increased BTI when compared to the suckling piglets for all tested variants (Fig. 2, Fig. 4). This age category reacted in a different manner to the solvent, which induced stimulation while compared with the unstimulated control culture. Both *Calendula officinalis* and *Coriandrum sativum* equally exerted an inhibiting effect, as opposed to untreated and alcohol treated controls. Summer savory was still inhibiting, but at a lesser extent than marigold and coriander, while for this category the garlic stimulated cell growth, when compared to the untreated control but not with the solvent control.

The BTI values recorded for adult female animals were for all plant extracts below the values of the untreated control. The alcohol solvent did not influence the cell growth; therefore, most probably the inhibition occurred due to the active components of the plants (Fig. 3, 4).

The strong inhibiting effect of the coriander extract, in spite of its quite high content in sitosterol, a compound indicated as T cell enhancer and growth promoter (7). The coriander extract is also scarce in components when compared to the other plant extracts, except the pumpkin extract, where the tocopherols and sterols represent the only components.

Comparing the activities of the other plant extracts between the age groups, there is a relevant influence of the age of the animals on the effects of the plants *in vitro*. Although the aim of the experiment was to monitor the *in vitro* whole alcoholic plant extracts' effects on mononuclear cells' proliferation, lacking investigations on the separate compounds, it can be stated that not only the compound category (tocopherols, sterols, polyphenols) but also the individual

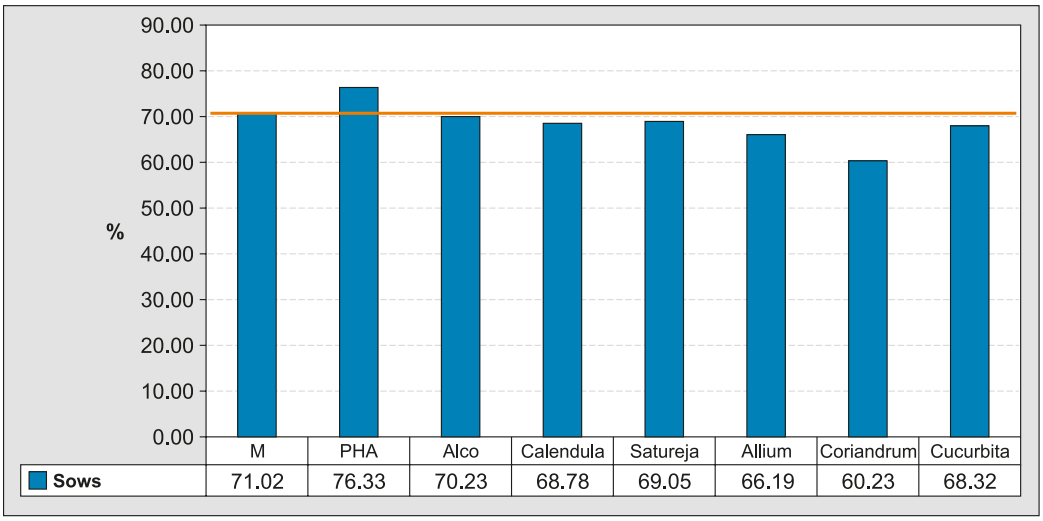

Fig. 3. Blast transformation indices (BTI,%) recorded for the blood samples collected from sows. The BTI values recorded for adult animals were for all plant extracts below the values of the untreated control. The alcohol solvent did not influence the cell growth, therefore, most probably the inhibition occurred due to the active components of the plants

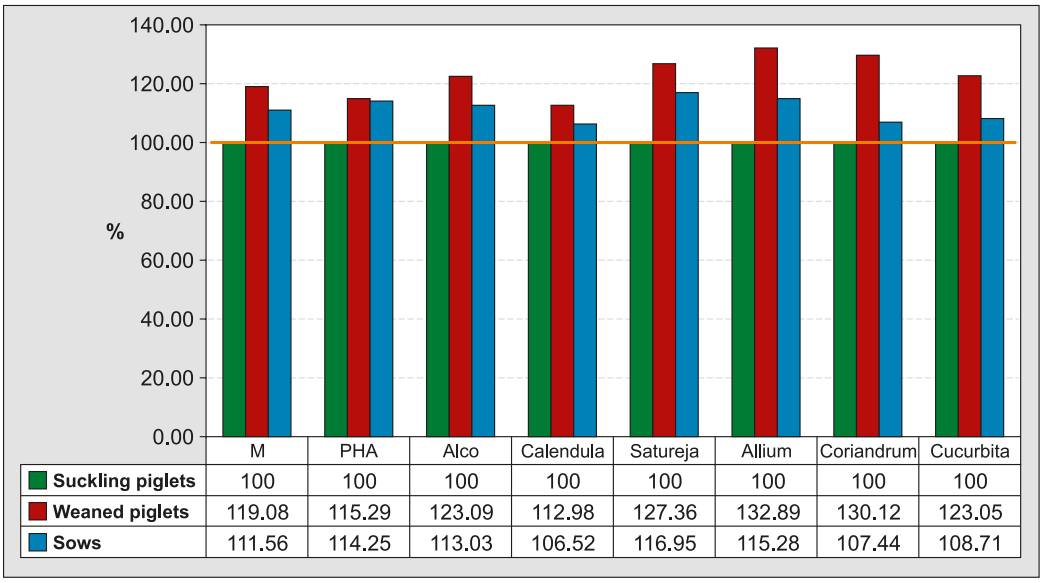

Fig. 4. The percentages of increase in BTI in weaned piglets and sows, when compared to the suckling piglets as reference. As a starting point, it could be observed that the cellular responses were weaker in the untreated control in suckling piglets, due to their underdeveloped immune system. All plants acted in an age category dependant manner, suggesting a better response in both weaned pigs and sows, when compared to the suckling piglets, with the most pronounced effects for *Allium* and *Satureja* for weaned piglets and sows respectively

compound type (i.e., chlorogenic acid, caffeic acid, α-tocopherol, δ-tocopherol, β-sitosterol, alliin, etc.) influence the immune cells' response. On average, for weaners and sows, the garlic and the summer savory increased the *in vitro* cellular response the most (Fig. 4), sharing caffeic acid, ferulic acid and α-tocopherol in higher amounts, all those compounds being known for their immune enhancing activities (3, 7).

When comparing the results obtained for weaned pigs and sows with those of suckling piglets, lesser responders due to their less reactive immune system, all plants acted in an age category dependant manner, suggesting an increase in response in both weaned pigs and sows, but higher in the weaned pigs (Fig. 4), with the most pronounced effects for *Allium* and *Satureja* for weaned piglets and sows respectively.

Statistical analysis results. The results indicated statistically significant differences between the young age groups, suckling and weaned piglets showing very low p values (p = 0.017 to 0.000016) for all plants except marigold, for weaned piglets and sows (p = 0.0001-0.0359) for all plant extracts, while for suckling piglets sows p = 0.0035 and p = 0.0461 were recorded for summer savory and garlic, respectively (Tab. 2). These results supported the observation that the response to the extracted plants was dependent on age category at least as much as on the chemical composition of the plants.

Conclusions. The plant extracts used for biological effects impacted on the basis of age of the pigs and plant family, proving their immune stimulating capacity. The immune stimulating activity of the plant alcoholic extracts also depended on their taxonomy, i.e., *Calendula* and *Cucurbita* were active in suckling

Tab. 2. P values for the statistical significance of the differences between the experimental categories. The differences are statistically significant at very low p values for most of the plant extracts, based on age category

	M	PHA	Alco	*Calendula*	*Satureja*	*Allium*	*Coriandrum*	*Cucurbita*
Suckling piglets – weaned pigs	0.00016	0.017	0.000016	0.18 NS	0.000023	0.000016	0.0002	0.0022
Weaned pigs-sows	0.1982 NS	0.8285 NS	0.0066	0.0359	0.0071	0.0013	0.0003	0.0001
Suckling piglets – sows	0.065725 NS	0.0254	0.023799	0.140936	0.003471	0.046091	0.284752 NS	0.106031 NS

piglets, *Cucurbita* in weaned piglets, none were immune enhancing in sows. The response in sows could also be because pigs are iteroparous organisms, in which the reproduction/immunity trade-off might cover the reproductive success. Further studies are needed to clarify the underlying mechanisms, i.e., by comparing different dosages of the alcoholic plant extracts in each age category and monitoring the dynamics in the same individual over a longer time period.

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