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*Original article*

# Evaluation of in-feed larch sawdust anti-inflammatory effect in sows

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## Abstract

The study aimed to investigate the possible anti-inflammatory activity of larch sawdust as feed supplement in lactating sows' diet and its possible effect on the prevalence of Postpartum Dysgalactia Syndrome under field conditions. In a Greek farrow-to-finish pig farm, fifteen sows were randomly and equally allocated to a negative control group (NC group), a positive control group (PC group), and a treatment group (LT group). The animals of the first two groups received 99% basic diet and 1% corn starch, while LT group animals received 99% basic diet and 1% larch sawdust. The whole trial period lasted 35 days (7 days prior to farrow – day of weaning). At parturition day, animals of the PC group received 2 ml of an anti-inflammatory drug intramuscularly (meloxicam, Metacam<sup>®</sup>, Boehringer Ingelheim Vetmedica), while the animals of both other groups, received 2 ml of normal saline. Results showed insignificant differences among experimental groups for parameters such as post-partum rectal temperature and piglets performance. On the contrary, a significant increase of mean milk lactation index was observed in LT and PC groups on the 4th day of lactation period, when compared with NC group ( $p=0.014$ ). Additionally, mean IL-6 concentrations in blood in the LT group showed a tendency for reduction when compared with those found in NC, and insignificant difference ( $p>0.05$ ) when compared with those observed in PC group 24 hours postpartum. Moreover, the respective TNF $\alpha$  mean level in the LT group at 24 and 72 hours after parturition was similar to that found in PC group, respectively) and significantly lower than that determined in the NC group ( $p=0.003$ ,  $p=0.024$ ). The results suggest a possible anti-inflammatory effect of larch sawdust in sows.

**Key words:** larch sawdust, pigs, postpartum dysgalactia syndrome, anti-inflammatory

## Introduction

Postparturient disorders in sows are commonly categorized under the term mastitis-metritis-agalactia (MMA) complex and were recently termed postpartum dysgalactia syndrome (PPDS) (Klopfenstein et al. 2006, Maes et al. 2010, Martineau et al. 2012). The clinical signs are characterized mainly by disorders of lactation and health status of sows such as anorexia, pyrexia, constipation, abnormal postpartum vulval discharge, as well as decreased litter performance characterized by unsuccessful attempts for suckling, diarrhea, poor growth rates and increased pre-weaning mortality (Klopfenstein et al. 2006). Quite frequently, the first symptom of PPDS is fever exceeding 39.3°C to 39.5°C accompanied with greatly reduced milk production within 12 to 48 hours postpartum, rapidly leading to piglet starvation (Hoy 2003, Klopfenstein et al. 2006). Diet composition and conditions that contribute to the proliferation of bacteria and consequently rise the potential of endotoxemia (e.g. cystitis, metritis, vaginitis, mastitis) seem to play a significant role in the development of PPDS (Maes et al. 2010, Papadopoulos et al. 2010, Martineau et al. 2012).

European larch (*Larix Pinaceae L. decidua*) is a deciduous tree mainly native to higher regions of the Alps, the Sudetes and the Carpathian mountains (Martinez 1998). Larch sawdust is a by-product of wood industry that is mainly used for producing pellet fuels at present, but has been found to possess promising anti-inflammatory properties. Immunomodulatory and strong anti-inflammatory activity of a *L. decidua* sawdust hydroethanolic extract has been shown by Farinacci et al. (2008) in an *ex-vivo* study in phorbol 12-myristate 13-acetate (PMA) stimulated ovine neutrophils. Kim et al. (2002) supported that there might be an immunostimulating effect of larch arabinogalactan, since an increase in complement properdin was seen after administration of the test compound along with Echinacea species to humans. Similarly, stimulating properties of arabinogalactan on phagocytosis, particularly against *Escherichia coli* and *Klebsiella* species, have been suggested (Monograph 2000). Phenolic compounds such as lignans and flavonoids as well as diterpenes of larch sawdust have been shown to possess antioxidant and anti-inflammatory properties (Pietarinen et al. 2006, Pferschy-Wenzig et al. 2008).

There are no previous studies investigating the anti-inflammatory or antimicrobial properties of larch sawdust as phytogetic feed additive in swine diets. There are reports only for the use of larch arabinogalactans as bioactive compounds for humans (Robinson et al. 2001) and dogs (Grieshop et al. 2002). In the present study, we aimed to investigate

potential anti-inflammatory properties of larch sawdust, used as feed supplement in sows' diet under field conditions.

## Materials and Methods

The experimental protocol of this efficacy study has been prepared under the license for experimenting on animals, issued by the local Veterinary Administration Office (County Pierias Veterinary State Authority). The clinical study was single blinded and performed according to the "code of practice for the conduct of clinical trials for veterinary medical products" (EMEA 2001). Animals were maintained in accordance with national and European animal welfare requirements (EMEA 1998, OECD 1998, FVE 2002).

### Animals and experimental design

The study was carried out in a commercial pig farm in northern Greece with a capacity of 900 sows under production. The farm had its own feed mill and artificial insemination laboratory. Fifteen (15) sows of the same genetic background (Topigs hybrids) were used. Trial animals were chosen in terms of achieving homogeneity of parity, body weight (BW), previous production parameters (litter size and litter average BW) and previous health status. The farm was selected due to recent history of PPDS occurrence. The farm suffered from reduced milk production associated with mastitis cases, pyrexia and decreased appetite in sows, mainly during the first week of lactation period. Subsequently, decreased growth rate of many suckling piglets, sporadic diarrhea and low mortality was observed in a number of litters (data not presented).

The trial sows were randomly allocated to a negative control group [NC group (n=5)] that received 99% basic diet and 1% corn starch, a positive control group [PC group (n=5)] that received the same feed as the NC group, and a treatment group [LT group (n=5)] that was given the basic diet and 1% larch sawdust. Trial animals received gestation feed during the last 7 days of gestation and lactation feed during the four weeks of the lactation period. Basic diets formulation is presented in Table 1. The feeding period lasted 35 days, beginning at 7 days prior to parturition (move of sows from gestation to farrowing unit) until the day of weaning i.e. 28th day post-partum. Before addition to the feed, larch sawdust was grounded and filtered, so that at least 95% of the material's particles had a size of approximately 1 mm in order to provide uniform distribution of the raw material in the feed,

Table 1. Feed ingredients of sows basic diets during the trial period (7 days prior to farrowing until 28 days postpartum).

Feed Ingredients			
Gestation diet (kg/ton of feed) <sup>1</sup>		Lactation diet (kg/ton of feed) <sup>2</sup>	
Corn	520	Corn	450
Soya 44%	15	Soya 44%	60
Salt	5	Salt	5
Wheat bran	260	Wheat bran	230
DDGS 27CP	170	DDGS 27CP	183
Lysine	2	Fish meal	10
Vitamin – Mineral premix	10	Fat	30
Limestone	15	Lysine	2
Dicalcium phosphate	3	Vitamin – Mineral premix	10
		Limestone	15
		Dicalcium phosphate	5

<sup>1</sup> Gestation diet was offered to trial animals during the last 7 days of gestation

<sup>2</sup> Lactation diet was offered to trial animals for 28 days postpartum during lactation period.

as well as good intestinal absorption of possible anti-inflammatory compounds present in the feed mixture. The material was well accepted at an inclusion level of 1% in the diet as shown by previous trials (Hagmuller et al. 2006, Jacela et al. 2010, Van Krimpen et al. 2010). Furthermore, the animals of the PC group received 2 ml (40 mg of meloxicam) of the anti-inflammatory drug meloxicam intramuscularly (Metacam®, Boehringer Ingelheim Vetmedica GmbH, Germany) at the day of parturition, while the animals of both other groups were intramuscularly injected with 2 ml of normal saline at the same day

### Plant material

Larch sawdust used as feed supplement was received from Jannach Ges.m.b.H., Thalheim, Austria. The powdered material (particle size approx. 1 mm) was phytochemically characterized by HPLC-analysis.

### Determination of flavonoids

For determination of the main flavonoids dihydroquercetin and dihydrokaempferol, 1g was accurately weighed and exhaustively soxhlet-extracted with methanol for 8 h, and thereafter the solvent was removed in a rotary evaporator. The residue was dissolved in 10.0 ml MeOH, diluted 1:2 with MeOH and filtered through a cellulose acetate filter (pore size 0.45 µm). A quantity of 5 µl of this solution was analyzed. The HPLC analysis was performed on an Agilent 1100 series HPLC system, using a Zorbax® SB C-18 Narrowbore RR column (2.1x150 mm; particle size 3.5 µm) protected by a Zorbax® SB-C8 guard column (2.1x12.5 mm, particle size 5 µm) (Agilent, Waldbronn, Germany). Mobile phase consisted of

H<sub>2</sub>O/0.1% HCOOH (solvent A) and CH<sub>3</sub>CN/0.1% HCOOH (solvent B), and the gradient was as follows: 0-30 min, 10-25% B; 30-31 min, 25-100% B; 31-35 min, 100% B; 35-36 min, 100-10% B; 36-41 min, 10% B. Flow rate was set at 250 µl/min, oven temperature at 35°C, and DAD detection wavelength at 290 nm. For quantification of the two compounds, a calibration curve was prepared with 5 dilutions of a methanolic solution of dihydroquercetin (Roth, Karlsruhe, Germany) (1, 0.5, 0.1, 0.05 and 0.01 mg/ml), and 5 µl of each dilution were injected.

### Determination of larixylacetate (LXA) and dehydroabiatic acid (DHA)

In detail, 2.5 g were accurately weighed and extracted with n-hexane by means of Accelerated Solvent Extraction (ASE; Dionex), using the following parameters: preheat: 0, heat: 5', static: 6', flush: 100%, purge: 40 sec, cycles: 4, pressure: 68.9 bar, temperature: 40°C. Solvent was removed in a rotary evaporator, and the residue was dissolved in 5.0 ml ethanol, and filtered through a cellulose acetate filter (pore size 0.45 µm), and 5 µl of the solution were analysed by HPLC (apparatus and stationary and mobile phase; see flavonoid analysis), using the following gradient: 0-30 min, 65-85% B; 30-31 min, 85-100% B; 31-40 min, 100% B; 40-41 min 100-65% B; 41-50 min, 165% B. Flow rate was set at 250 µl/min, oven temperature at 20°C and DAD detection wavelength at 205 nm for LXA and at 228 nm for DHA.

For quantification, calibration curves were prepared with 7 dilutions of LXA and DHA (0.5, 0.25, 0.125, 0.05, 0.01 and 0.005 mg/ml) isolated as described in Pferschy-Wenzig et al. 2008, and 5 µl of each dilution were injected. Results were calculated

Table 2. Production parameters of sows (ADFI: Average daily feed intake, BWL: Body weight loss, BFL: Back fat loss) and litters (BW: Body weight, ADWG: Average daily weight gain) per trial group (mean  $\pm$  standard deviation, P value between groups for each parameter, SEM: Standard Error of the Mean).

Sows production parameters											
Group	ADFI – lactation period (kg/day)			BWL – lactation period (kg)			BFL – lactation period (mm)				
	Mean	P	SEM	Mean	P	SEM	Mean	P	SEM		
Negative Control (NC)	6.13 $\pm$ 0.527		0.24	41.4 $\pm$ 8.20		3.67	9.6 $\pm$ 1.14		0.51		
Larch 1% (LT)	6.18 $\pm$ 0.294	0.799	0.13	38.6 $\pm$ 10.48	0.908	4.69	9.4 $\pm$ 1.52	0.743	0.68		
Positive Control (PC)	6.30 $\pm$ 0.395		0.18	38.8 $\pm$ 14.10		6.30	9.0 $\pm$ 1.00		0.45		

  

Piglets production parameters												
Group	BW at birth (kg/piglet)			BW at weaning (kg)			ADWG lactation period (kg)			Litter size (piglets born alive)		
	Mean	P	SEM	Mean	P	SEM	Mean	P	SEM	Mean	P	SEM
Negative Control (NC)	1.17 $\pm$ 0.208		0.093	8.12 $\pm$ 0.848		0.379	0.234 $\pm$ 0.058		0.026	10.4 $\pm$ 2.07		0.927
Larch 1% (LT)	1.22 $\pm$ 0.115	0.883	0.051	8.25 $\pm$ 0.626	0.704	0.280	0.242 $\pm$ 0.032	0.890	0.014	10.6 $\pm$ 1.67	0.628	0.748
Positive Control (PC)	1.20 $\pm$ 0.137		0.061	8.48 $\pm$ 0.514		0.230	0.246 $\pm$ 0.028		0.013	9.2 $\pm$ 3.27		1.463

Values within a column without superscripts do not differ significantly at  $p \leq 0.05$

Table 3. Mean milk lactation index at the 1st, 2nd, 3rd and 4th day of lactation (mean  $\pm$  standard deviation, P value between groups for each parameter, SEM: Standard Error of the Mean).

Groups	Milk lactation index											
	1st day			2nd day			3rd day			4th day		
	Mean	P	SEM	Mean	P	SEM	Mean	P	SEM	Mean	P	SEM
Negative Control (NC)	290 $\pm$ 13.27		5.94	330 $\pm$ 20.31		0.08	350 $\pm$ 25.0		11.19	360 <sup>a</sup> $\pm$ 33.91		15.17
Larch 1% (LT)	310 $\pm$ 21.82	0.215	9.76	320 $\pm$ 19.27	0.667	8.62	330 $\pm$ 27.17	0.438	12.15	430 <sup>b</sup> $\pm$ 39.91	0.014	17.85
Positive Control (PC)	310 $\pm$ 22.14		9.90	320 $\pm$ 20.19		9.03	330 $\pm$ 27.57		12.33	420 <sup>b</sup> $\pm$ 26.48		11.84

<sup>a,b</sup> Values with different superscripts within a column differ significantly at  $p < 0.05$

as means  $\pm$  standard deviation of 3 extractions, each of them analysed in duplicate.

## Records

Several production [total feed intake, Average Daily Feed Intake-ADFI (kg/day), sows Body Weight (BW) and backfat at allocation and weaning, mean BW at birth (kg/piglet), Average Daily Weight Gain (ADWG), mean litter BW at weaning] and health parameters (rectal temperatures of sows 12 and 36 hours post farrowing, routine clinical examinations of sows and piglets for clinical signs of mastitis and reduction in milk production and newborn feeding disorders) of sows and their litters were recorded. The milk production index for all sows was calculated according to Daza et al. (2004). On days 1, 2, 3, and 4 of lactation, piglets were separated from their dams for 90 min. The functional pectoral and inguinal glands were milked manually after injecting each sow with 20 i.u. oxytocin. Milk samples were kept at  $-20^{\circ}\text{C}$  until analysis.

In addition, IL-6 and TNF $\alpha$  were analysed as markers of inflammatory stress. In detail, blood samples were collected from all trial animals at allocation day (i.e. day 0 of the study: 7 days before farrowing day) and at 24 and 72 hours post farrowing for serum TNF $\alpha$  and plasma IL-6 evaluation. A quantitative sandwich enzyme immunoassay technique was used for IL-6 and TNF $\alpha$  determination (Porcine IL-6 and TNF $\alpha$  immunoassays, Quantikine<sup>®</sup> R&D Systems Inc, Minneapolis, USA). The cytokines results were expressed as n-fold increase in mean values at each time point [i.e. mean value of each parameter at 24 or 72 hours postpartum/mean values of each parameter in the same group animals at 7 days pre-farrowing (day 0 of the study)].

## Statistical analysis

Data were analyzed with a model of Analysis of Variance using the GLM procedure of SAS Statistical Package (“The SAS<sup>®</sup> System” release 8.1 for WINDOWS – 2002/ SAS Institute Inc., Cary, NC 27513,

Table 4. Relative expression (n fold\*) of IL6 and TNF $\alpha$  in sows after 24 and 72 hours Post Partum (PP) in each trial group (mean  $\pm$  standard deviation, P value between groups for each parameter, SEM: Standard Error of the Mean).

Group	IL6			IL6			TNF $\alpha$			TNF $\alpha$		
	24 hours PP	P	SEM	72 hours PP	P	SEM	24 hours PP	P	SEM	72 hours PP	P	SEM
Negative Control (NC)	4.05 <sup>a</sup> $\pm$ 1.07		0.48	2.48 $\pm$ 1.71		0.76	3.69 $\pm$ 1.62 <sup>a</sup>		0.72	2.68 <sup>a</sup> $\pm$ 1.32		0.59
Larch 1% (LT)	2.96 <sup>ab</sup> $\pm$ 1.02	0.016	0.46	1.59 $\pm$ 0.86	0.219	0.38	2.11 $\pm$ 0.71 <sup>b</sup>	0.003	0.32	1.07 <sup>b</sup> $\pm$ 0.55	0.024	0.24
Positive Control (PC)	1.86 <sup>b</sup> $\pm$ 0.92		0.41	1.12 $\pm$ 0.72		0.32	0.86 $\pm$ 0.29 <sup>b</sup>		0.13	0.98 <sup>b</sup> $\pm$ 0.77		0.34

<sup>a,b</sup> Values with different superscripts within a column differ significantly at  $p \leq 0.05$

\* Fold increase value: mean value of each parameter at 24 or 72 hours postpartum/mean values of each parameter in the same group of animals at 7 days pre-farrowing (day 0 of the study)

USA), with Site Code No. 0084912001. Duncan's-test was also used for testing differences in variations. The level of significance was set at 0.05.

## Results

The larch sawdust used as feed supplement was phytochemically characterized by analysing both the methanol and the *n*-hexane extracts, which allows the detection of both medium-polar and non-polar constituents. Results of HPLC determination of constituents (with known anti-inflammatory activity) in larch sawdust were (% of raw material  $\pm$  standard deviation): 0.699 $\pm$ 0.022 for dihydroquercetin, 0.695 $\pm$ 0.031 for dihydrokaempferol, 0.056 $\pm$ 0.002 for LXA and 0.010 $\pm$ 0.000 for DHA.

The mean postpartum rectal temperatures (PPRT) of sows at 12 and 36 hours post farrowing were between 38.94 to 39.76°C and insignificant differences ( $p > 0.05$ ) were observed between the trial groups. Results on production parameters of sows and piglets' performance (total feed intake, ADFI, mean BW at birth and at 21 days of age, ADWG) showed insignificant differences ( $p > 0.05$ ) between the experimental groups as shown in Table 2. Table 3 demonstrates the mean milk lactation index at the 1st, 2nd, 3rd and 4th day of lactation for all sows per group. Significantly increased values were observed in the LT and PC groups, in comparison with those found in the NC group on the 4th day ( $p = 0.014$ ).

In Table 4, the relative expression (n fold) of IL6 and TNF $\alpha$  in sows, 24 and 72 hours postpartum per experimental group are presented. Levels of IL-6, 24 hours postpartum, were significantly increased in the PC group compared with those determined in the NC group ( $p = 0.016$ ), while respective level in the LT group did not alter significantly in comparison with those observed in both other groups, although a tendency for reduction was observed. In contrast, TNF $\alpha$  concentrations at 24 and 72 hours after parturition in the LT and PC groups were significantly decreased when compared with those found in the NC group

( $p = 0.024$ ). The TNF $\alpha$  levels in PC group did not show significant difference when compared with LT group in both measurements.

## Discussion

Major pathophysiological factor of PPDS are bacterial endotoxins, resulting in fever and hyperthermia (Reiner et al. 2009, Martineau et al. 2013). Postpartum rectal temperature (PPRT) higher than 39.3 or 39.5°C categorize sows as PPDS-affected (Persson et al. 1989, Madec and Leon 1992). Some authors support that PPRT of a clinical healthy lactating sow is 39.5°C (Persson et al. 1989), while others consider that PPRT higher or equal to 39.3°C is a characteristic clinical sign in PPDS-affected sows (Hoy 2003, Klopfenstein et al. 2006). In our study, there was no significant difference ( $p < 0.05$ ) of the mean PPRT between the various groups, at first 12 and 36 hours post farrowing. However, it must be pointed out that PPRT in the LT group remained below 39.5°C and 39.3°C at 12 and 36 hours postpartum, respectively, thus it could be hypothesized that the addition of larch sawdust in sows feed under the specific dosage scheme used in this study, could have an effect on the reduction of PPRT. However, further studies with a larger sample size could confirm this hypothesis.

Besides abnormal PPRT, diagnosis of PPDS must be based on a combination of criteria, such as clinical mammary gland changes, as well as decreased milk production and appetite (Mirko and Bilkei 2004). In our study, there were insignificant differences ( $p > 0.05$ ) in mean milk lactation index on the 1st, 2nd and 3rd day of lactation between trial groups. However, a clear and statistically significant increase was observed in the LT and PC groups on the 4th day of lactation. Therefore, a beneficial effect of larch sawdust inclusion in sows feed, in milk production and mammary gland function could be also suggested as possible.

The TNF $\alpha$  is mainly produced by endotoxin-activated macrophages and is part of the activation processes of other cytokines, such as IL-6 (Riollet et al.

2000). As previously reported (Wang et al. 2006, Zhu et al. 2007, 2008), there is greater local production of TNF $\alpha$  and proinflammatory cytokines in the mammary glands in PPDS-affected sows, because of cellular inflammation processes. The TNF $\alpha$  and IL-6 are involved in the pathogenesis of PPDS and their measurement is considered as a useful index of bacterial infections in lactating sows, since it provides information about the severity of the inflammatory process in the reproductive tract and/or mammary gland, as well as the monitoring of the course of disease (Wang et al. 2006, Szczubiał and Urban-Chmiec 2008). Our findings indicate that the inclusion of larch sawdust at a concentration of 1% in sows diets resulted in TNF $\alpha$  expression levels similar to those found in sows that received anti-inflammatory drug injection (Table 2).

The phytochemical analysis of the methanolic extract of the used larch sawdust revealed the presence of dihydroquercetin and dihydrokaempferol as the main flavonoids, which is consistent with the literature (Martinez 1998). These compounds are known to possess anti-inflammatory and anti-oxidative activity (Pietarinen et al. 2006). On the other hand, the *n*-hexane extract was also analyzed, and the content of two diterpenes in the used material was determined. It has been previously reported that some diterpenes (from a lipophilic larch sawdust extract), can influence arachidonate metabolism *in vitro* and inhibit cyclooxygenases to a lower extent (Pferschy-Wenzig et al. 2008).

In addition, larch wood also contains considerable amounts of arabinogalactans (polysaccharides with molecular weights of 10.000 to 100.000 Da). The lower molecular weight arabinogalactan fraction typically exhibits anti-inflammatory, anticomplement and anti-allergy effects (Monograph 2000). Arabinogalactans have been suggested as immune modulating factors since they could inhibit superoxide production and functional activation of neutrophils (Farinacci et al. 2008). Other studies have previously reported similar anti-inflammatory or anti-microbial effects. Stefanon et al. (2009), investigated the effects of *L. decidua* sawdust dietary administration in sheep under ACTH challenge and suggested that it can counteract ACTH effects on the inflammatory processes. Taken together, we could suggest that constituents of larch sawdust such as arabinogalactans, flavonoids and diterpenes, possibly along with yet unidentified compounds, might had contributed to anti-inflammatory effects observed in our study.

In conclusion, feeding sows with larch sawdust at the level of 1% of feed for 7 days prior and during a four week lactation period, resulted in a decrease in plasma TNF $\alpha$  concentrations 24 and 72 hours post-

partum, along with a significant increase in mean milk lactation index on the 4th day of lactation and a tendency for IL-6 reduction 24 hours postpartum. The above-mentioned main findings suggest that in-feed inclusion of larch sawdust could induce an anti-inflammatory activity and has positive effect on mammary glands function in sows.

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