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**Rabbit dietary supplementation with pale purple coneflower (*Echinacea pallida*). 1. Effects on the reproductive performance and immune parameters of does**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1576968> since 2019-12-24T12:45:04Z

*Published version:*

DOI:10.1017/S1751731115002979

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(Article begins on next page)

1 **Effects of dietary supplementation with pale purple coneflower (*Echinacea***  
2 ***pallida*) on reproductive performance and immunity of rabbit does and on**  
3 **productive results of their kits**

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20

21 Running head: Pale purple coneflower in rabbit nutrition

22

23 **Abstract**

24 *Echinacea pallida* (EPAL), also known as pale purple coneflower, is an herbaceous  
25 flowering plant with immune-enhancement and antioxidative properties. EPAL effect  
26 was studied on rabbit does' reproductive performance, serum biochemistry and

27 haematological parameters as well as on their kits growth performance. One hundred  
28 21-weeks-old Grimaud rabbit does were randomly assigned to two groups. One  
29 group was fed a basal diet supplemented with 3 g EPAL /kg diet (*Echinacea* group,  
30 E) while the other was fed the basal diet without the supplementation (Control group,  
31 C). Reproductive performance of does was not affected by the treatment ( $P>0.05$ ).  
32 Haematological parameters of pregnant rabbits showed that any interaction between  
33 gestational day and treatment was observed except for neutrophils cells ( $P=0.033$ ).  
34 The control group was significant higher than the treatment group for basophils cells  
35 (0.55 and 0.29 %, respectively;  $P=0.049$ ). Gestational day significantly affected most  
36 haematological parameters ( $P<0.05$ ). No significant effect of gestational day or  
37 treatment was observed on blood serum chemistry. Regarding the immune  
38 parameters, no significant differences were observed between groups; while a  
39 significant effect of gestational day was observed for lysozymes (6.02 vs 7.99 vs  
40 1.91; for 0, 14 and 28 days respectively;  $P=0.014$ ). Eighty weaned kits (40 born from  
41 C does and 40 born from E does) were randomly assigned to four groups of 20  
42 animals each fed a growing commercial diet supplemented with or without 3 g EPAL  
43 /kg diet. The following experimental groups were formed: CC (rabbits fed the C diet  
44 and born from the C does), CE (rabbits fed the E diet and born from the C does), EC  
45 (rabbits fed the C diet and born from the E does) and EE (rabbits fed the E diet and  
46 born from the E does). Dietary EPAL treatment did not significantly ( $P>0.05$ ) affect  
47 the growth performance of weaned rabbits. In conclusion, a lack of effect of EPAL  
48 was reported. Indeed, its dietary supplementation did negatively influence the  
49 reproductive and haematological parameters of does nor the growing performance of  
50 fattening rabbits.

52 **Keywords:** pale purple coneflower, *Echinacea pallida*, rabbit does, haematology,  
53 fattening rabbits.

54

## 55 **Implications**

56 In recent years, after the ban on the use of antibiotics as growth promoters, phyto-  
57 additives have been proposed to improve rabbit health and reduce post-weaning  
58 mortality. The present study describes the effects of dietary supplementation with  
59 *Echinacea pallida* (known to possess immune-enhancement and antioxidative  
60 properties) on rabbit does reproductive performance and immunity and on their kits  
61 productive results. The EPAL dietary supplementation did not influence the  
62 reproductive and haematological parameters of rabbit does nor did promote the  
63 growth performance of their kits.

64

## 65 **Introduction**

66 Animal health is a critical issue in animal production strongly affecting the income  
67 generated from husbandry activity. Moreover, since the European Union has banned  
68 the use of antibiotics as feed additives, many researches in the animal nutrition area  
69 have been focused on gauging alternative feeding strategies preventing digestive  
70 diseases while enabling the achievement of a satisfactory growth performance. Given  
71 the advance in modern biotechnology, the application of naturally-occurring  
72 antimicrobial and antioxidant compounds has been preferably employed in animal  
73 nutrition due to its potential health benefits on the host physiology (Chrastinová *et al.*,  
74 2010). The immunomodulatory and anti-oxidative properties of officinal plants are  
75 well known, as well as their ability to promote positive outcomes on animal health and  
76 performance (Böhmer *et al.*, 2009; Arafa *et al.*, 2010).

77 *Echinacea* is a genus of herbaceous flowering plants belonging to the Asteraceae  
78 botanical family. It presents high levels of production and economic importance in the  
79 United States of America, Canada and European countries. The use of a mixture of  
80 *Echinacea purpurea*, *Echinacea angustifolia* and *Echinacea pallida* (EPAL) has been  
81 reported to have immune-enhancement properties and benefits, such as the  
82 prevention and treatment of upper respiratory tract infections (Barnes *et al.*, 2005).  
83 Active components from *Echinacea* extracts (mainly alkylamides, polysaccharides  
84 and proteoglycans) have been shown to exert immunomodulatory, anti-inflammatory  
85 and anti-viral activities (Barnes *et al.*, 2005). Extracts of EPAL have been proposed  
86 as phyto-immunostimulating agents and their activities are mainly directed towards  
87 the innate immune system. Most studies performed on the immunotropic properties  
88 of EPAL were related to its effect on nonspecific immunity (activation of macrophage  
89 functions, phagocytosis of granulocytes, NK cells cytotoxicity), while other studies  
90 have investigated the adaptive immune modulation of EPAL (Egger *et al.*, 2008).  
91 Improvement of immunity parameters and productive performance has been reported  
92 in various livestock species (poultry, quails and rabbits) fed diets supplemented with  
93 *Echinacea* spp. (Maass *et al.*, 2005; Ahmed *et al.*, 2008; Böhmer *et al.*, 2009; Arafa  
94 *et al.*, 2010; Nasir and Grashorn, 2010; Sahin *et al.*, 2012). Nevertheless, scarce and  
95 conflicting evidence is available concerning the use of *Echinacea* spp. products in  
96 rabbit does during pregnancy. Based on this evidence, the aim of this study was to  
97 evaluate the effects of EPAL dietary supplementation on reproductive performance,  
98 blood parameters and immune indices in rabbit does as well as on the productive  
99 performance of their kits.

100

## 101 **Material and methods**

102

103 *Animals, housing, diets and management of rabbit does*

104 One hundred nulliparous does (14 week old) of a strain of Grimaud rabbits, obtained  
105 from Grimaud Italy, were housed individually in a closed rabbitry, with flat-deck wire  
106 net cages (40×50 cm<sup>2</sup>, including nest boxes: 41×26 cm<sup>2</sup>), and under a constant  
107 photo-period of 16 h of light per day. The rabbitry temperature was kept within 18°-  
108 22°C. A relative humidity of 60-75% was maintained by a forced ventilation system.

109 The does were randomly assigned to two groups (50 does per group). The first group  
110 was fed *ad libitum* a commercial pelleted diet (control diet, C) while the second one  
111 was fed the same diet supplemented with 3 g of EPAL powder /kg diet (*Echinacea*  
112 diet, E).

113 The doe rabbit diets were provided by the Ferrero S.p.A. feed manufacturer  
114 (Farigliano CN, Italy). Dry ground EPAL roots, obtained from Biotrade Snc<sup>®</sup> (Via  
115 Pacinotti, 21, Mirandola, Italy), was included in the treated diets during the raw  
116 material mixing process. The feeding program consisted of a diet provided from  
117 insemination to 21 days after parturition and another diet provided from 21 days after  
118 parturition to kits weaning. The diets contained the following ingredients in  
119 decreasing order: alfalfa meal, sunflower meal, barley, wheat bran, dried beet pulp,  
120 maize germ, roasted soybean meal, cane molasses, soybean oil, calcium carbonate,  
121 sodium chloride. The diets were analyzed for dry matter (DM, AOAC 925.40), crude  
122 protein by total nitrogen contents (AOAC 984.13), ether extract (AOAC 945.16),  
123 crude fiber (AOAC 962.09) and ash by ignition to 550°C (AOAC 923.03) according to  
124 the Association of Official Analytical Chemists (AOAC, 2000). NDF, ADF and ADL  
125 were determined according to Van Soest et al. (1991). Starch was determined by  
126 means of Ewer's polarimetric method (European Economic Community, 1972). The

127 chemical composition of the different diets was reported in Table 1. Water was  
128 available *ad libitum* from nipple drinkers. The diets were completely exempt from  
129 medication (antibiotics or coccidiostat). All animals were reared under the same  
130 environmental and management conditions during the whole experimental period.  
131 Rabbit does were first artificially inseminated at 21 weeks of age (mean body weight:  
132  $3712 \pm 176\text{g}$ ). Then, artificial insemination was applied at 18 days post-partum (49  
133 day reproductive rhythm and single batch system). Cross-fostering was applied within  
134 the experimental groups with a maximum of 8, 9 and 10 kits per litter at first, second  
135 and following kindling, respectively. The kits were freely nursed by their doe and  
136 weaned at 35 days of age.

137

#### 138 *Does performance*

139 Data of the first five consecutive reproductive cycles were evaluated. Body weight of  
140 does at first and final kindling, does mortality and reproductive performance variables  
141 were studied. The following variables were calculated on the basis of IRRG's  
142 recommendations (International Rabbit Reproduction Group, 2005): total born; born  
143 alive; stillborn; litter size at 21 and 35 days of age; litter weight at 21 and 35 days of  
144 age; individual body weight of kits at 21 and 35 days of age; Kindling rate (%) =  
145 number of kindled does per number of inseminated does  $\times 100$ ; Prolificacy = number  
146 of born kits per number of does kindled; Numerical productivity at birth = number of  
147 born alive per inseminated doe; Overall productivity at birth = weight of born alive per  
148 inseminated doe; Perinatal mortality (%) = number of stillborn kits per number of total  
149 born  $\times 100$ ; mortality between 0-21 and 0-35 days of age.

150

#### 151 *Haematological, serum biochemistry and serum electrophoresis of rabbit does*

152 Blood samples were collected from 8 rabbits per group at different time points during  
153 the second gestation. Considering the day of artificial insemination as starting day  
154 (T0), blood samples were collected at: day 0, day 14 and day 28, respectively. The  
155 samples were collected from the lateral saphenous vein with a heparinized syringe to  
156 prevent the blood clot. At each sampling time point, one ml of blood was collected  
157 into sterile tubes containing ethylenediaminetetraacetic acid -2K (SB-41: Sysmex  
158 Corporation) for the evaluation of haematological parameters. Meanwhile, serum  
159 obtained by collecting four ml blood samples in a sterile serum plain tube, after  
160 incubation at room temperature (22°C) for two hours and centrifugation at 2500 g for  
161 10 minutes, was used for serum biochemistry and serum electrophoresis. Serum was  
162 stored at -80° C until analysis. Full blood count was performed using an automated  
163 laser cell counter calibrated for rabbits (MS4-S Hematology Analyzer, Melet  
164 Schloesing, Osny - France) to assess the following parameters: red blood cells (RBC,  
165 M/mm<sup>3</sup>), haemoglobin (Hb, g/dl), haematocrit (HCT, %), mean corpuscular volume  
166 (MCV, fl), mean corpuscular haemoglobin (MCH, pg), mean corpuscular  
167 haemoglobin concentration (MCHC, g/dl), red cell distribution width (RDW, %),  
168 platelets (PLT, m/mm<sup>3</sup>), relative volume of thrombocytes (PCT, %), mean platelet  
169 volume (MPV, fl), platelet distribution width (PDW, %), white blood cell count (WBC,  
170 m/mm<sup>3</sup>), lymphocytes (LYM, %), monocytes (MON, %), neutrophils (NEUT,%),  
171 eosinophils (Eos, %), basophils (Bas, %). For the serum blood chemistry, the  
172 concentrations of total protein (TP, g/dl), glutamate oxaloacetate transaminase (GOT,  
173 UI/L), blood urea nitrogen (BUN, mg/dl), albumin (g/dl), urea (mg/dl) and cholesterol  
174 (mg/dl) were measured using an automated system photometer (Screen Master  
175 Touch, Hospitex Diagnostics, Sesto Fiorentino, FI, Italy).



176 For immune indices, the serum electrophoretic patterns were obtained using a semi-  
177 automated agarose gel electrophoresis system (Sebia Hydrasys, EVRY, France) to  
178 determine serum protein. Serum lysozyme was measured with a lysoplate assay,  
179 carried out in a moist incubator at 37°C for 18 min. The method is based on the lyses  
180 of *Micrococcus lysodeikticus* in 1% agarose. The diameter of the lysed zones was  
181 measured with a ruler and compared with the lysed zones of a standard lysozyme  
182 preparation (Sigma Aldrich, Milan, Italy). The value was expressed as µg/ml  
183 (Osserman and Lawlor, 1996). The haemolytic complement assay was carried out in  
184 microtitre plates. The complement titre is the reciprocal of the serum dilution causing  
185 50% lysis of red blood cells of rams. Its concentration was expressed as CH<sub>50%</sub>  
186 (Moscati *et al.*, 2008).

187

#### 188 *Performance of fattening rabbits*

189 At the second parturition, forty weaned kits were randomly chosen from both C and E  
190 does. Rabbits were allocated into individual wire cages (0.41 m long × 0.30 m wide ×  
191 0.28 m high) and randomly assigned to four equal-size experimental groups (n=20).  
192 Two groups of rabbits were fed a growing commercial basal diet (C) while the  
193 remaining two groups were fed the same diet supplemented with 3 g of EPAL powder  
194 / kg diet (E). According to the maternal diet, the following experimental groups were  
195 formed: CC group (rabbits fed the C diet and born from the C does), CE group  
196 (rabbits fed the E diet and born from the C does), EC (rabbits fed the C diet and born  
197 from the E does) and EE group (rabbits fed the E diet and born from the E does). The  
198 chemical composition of the different diets is reported in Table 2. The diets were  
199 completely exempt from medication (antibiotics or coccidiostat). Feed and water were  
200 provided *ad libitum*. During the whole trial, temperature was maintained at 22±2°C

201 and a 16L: 8D photoperiod was applied. Health status was monitored daily from  
202 weaning to 77 days of age.

203 Rabbits were weighed at 35, 49 and 77 day of age and the following performance  
204 parameters were calculated: daily feed intake, daily weight gain and feed conversion  
205 ratio at different periods of age.

206

### 207 *Chromatographic identification of Echinacea ingredients*

#### 208 *Chemicals*

209 Echinacoside (purity 98%), chlorogenic acid (purity  $\geq$  95%), HPLC-MS and analytical  
210 grade solvents were purchased from Sigma-Aldrich (Milan, Italy).

#### 211 *Extraction procedure*

212 500 mg of dry ground EPAL roots, were sonicated for 10 min with 10 ml of a mixture  
213 of MeOH/H<sub>2</sub>O (70/30) three times. The resulting total extract (30 ml) was filtered and  
214 analyzed by UHPLC-PDA-MS/MS system.

#### 215 *HPLC Analysis*

216 EPAL extract analyses were carried out on a Shimadzu Nexera X2 system equipped  
217 with a photodiode detector SPD-M20A in series to a triple quadrupole Shimadzu  
218 LCMS-8040 system provided with electrospray ionization (ESI) source (Shimadzu,  
219 Dusseldorf Germany). An Ascentis® Express C18 column (150 mm x 2.1 mm i.d., 2.7  
220  $\mu$ m particle size), (Supelco, Bellefonte, PA) was used (operated at 30°C). The mobile  
221 phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile  
222 (B), at a flow rate of 0.4 ml min<sup>-1</sup>. Polyphenols elution was achieved using the  
223 following linear gradient: starting condition, 95% A, 5% B; 3 min, from 5 to 15% B; 17  
224 min, from 15 to 100% B; 5 min and 100% B for 2 min. The injection volume was 5  $\mu$ l.  
225 UV spectra were acquired in the 210-450 nm wavelength range. The identification of

226 the components was based on the co-injection of pure standards and on their UV  
227 spectra and mass spectral information in both positive and negative ionization mode  
228 (respectively, ESI+ and ESI-).

229 Quantification of Echinacoside: A standard stock solution (1mg/ml) of Echinacoside  
230 was prepared in methanol and stored at -18°C. Suitable dilutions of the standard  
231 stock solution in methanol/water (1/10) were prepared to obtain final concentrations  
232 from 10 to 100 mg/ml. Calibration curve was built by analysing the resulting standard  
233 dilutions three times by HPLC-PDA.

234

### 235 **Statistical analysis**

236 Statistical analyses were performed using SPSS software package (IBM SPSS,  
237 2012). Data concerning the reproductive parameters from the first to the fifth  
238 reproductive cycles were combined and analyzed in a single dataset. Statistical  
239 analyses for significant differences in reproductive performance between the control  
240 and Echinacea groups were performed using a Student's t-test. Mortality, kindling  
241 rate and prolificacy were analyzed using Chi-square test. The effect of dietary  
242 treatments on blood indices and immune parameters across three gestational periods  
243 (day 0, day 14, day 28) was statistically analyzed with a mixed between-within  
244 subjects model (GLM Repeated Measures). Performance of the fattening rabbits was  
245 analyzed using a one-way ANOVA with group as fixed factor. Duncan's New Multiple  
246 Range test was used for post-hoc comparisons. The significance was declared at  
247  $P < 0.05$ .

248

### 249 **Results**

250 *HPLC profile of EPAL*

251 The HPLC profiles of EPAL root extract are shown in Figure 1. The analysis identified  
252 the presence of caftaric acid, cichoric acid, chlorogenic acid and Echinacoside which  
253 specifically characterized EPAL species (Hu and Kitts, 2000; Speroni *et al.*, 2002;  
254 Barrett, 2003). Chromatographic analysis was reported to find 0.37 % Echinacoside.  
255 Echinacoside was found to be the main caffeic acid derivative in EPAL extract,  
256 responsible for the immunostimulatory action of Echinacea extracts (Hu and Kitts,  
257 2000; Pellati *et al.*, 2005; Dalby-Brown *et al.*, 2005). Echinacoside has been studied  
258 for its antioxidant, anti-inflammatory and cicatrizing activities (Speroni *et al.*, 2002).  
259 However, a purified phytochemical does not imitate the immunological effects of  
260 whole plant extracts. It appears that the immunopharmacological activities of  
261 Echinacea depend on a combination of several active compounds (Randolph *et al.*,  
262 2003).

263

#### 264 *Reproductive performance*

265 Reproductive performances of the first five reproductive cycles are reported in Table  
266 3. There were no significant differences between groups for any of the studied  
267 parameters. Numerical and overall productivities calculated during the five cycles  
268 were: born alive, 1438 and 1471 kits; number of kits at day 35, 1229 and 1260 for  
269 control and E groups, respectively.

270

#### 271 *Haematological findings*

272 The haematological parameters of pregnant rabbits are reported in Table 4. The  
273 results indicated a significant ( $P<0.05$ ) effect of treatment and gestational day on  
274 some haematological parameters. The control group was significant higher than the  
275 treatment group for basophils cells (0.55 and 0.29 %, respectively;  $P=0.049$ ).

276 Gestational day significantly affected RBC, Hb, HCT, MCV, MCH, MCHC, RDW,  
277 MPV, PDW, WBC, LYM, NEUT and Eos ( $P<0.05$ ). For any studied variables, no  
278 significant interaction between treatment and gestational period was reported except  
279 for NEUT ( $P=0.033$ ).

280 No significant effect of gestational day or treatment was observed on blood serum  
281 chemistry. Regarding the immune parameters, no significant differences were  
282 observed between groups; while a significant effect of gestational day was observed  
283 for lysozymes ( $P=0.014$ ). The higher concentration of lysozymes was observed in  
284 day 14 of gestation in comparison with days 0 (+32.7%) and 28 (+318.3%) (6.02 vs  
285 7.99 vs 1.91; for 0, 14 and 28 days respectively).

286

#### 287 *Fattening rabbit performance*

288 The results of fattening rabbits performance are illustrated in Table 5. For all studied  
289 variables, no statistically significant differences were reported amongst the  
290 experimental groups ( $P>0.05$ ). In addition, regarding the health status, no illness and  
291 death were observed during the fattening period.

292

## 293 **Discussion**

#### 294 *Reproductive performance*

295 Body weight of does at kindling, kindling rate, litter size at birth, at days 21st and 35th  
296 of age, and the mortality of kits did not differ between the two groups. This indicates  
297 that *Echinacea* supplements in does' diets did not exert a promoting effect on  
298 reproductive function when administered at 3 g EPAL/kg of diet. Our results differ  
299 from those obtained in mice by Barcz *et al.* (2007) who found that two *Echinacea*  
300 drugs (*Esberitox* and *Echinapur*) lowered the number of embryos in one litter, even if

301 the results were on the edge of statistical significance. During murine pregnancy,  
302 *Echinacea purpurea* reduced the number of viable foetus (Chow *et al.*, 2006). A  
303 prospective study suggested that the use of *Echinacea* in pregnancy during  
304 organogenesis is not associated with an increased risk of major malformations (Gallo  
305 *et al.*, 2000). Further theoretical evidence via an expert panel on botanical medicine  
306 reported that oral consumption of *Echinacea* in recommended doses appeared safe  
307 and effective to use during pregnancy (Perri *et al.*, 2006).

308

### 309 *Haematological findings*

310 Blood parameters in rabbits are used as an aid for the clinical diagnosis of metabolic,  
311 infectious and parasitic diseases and to assess animal condition. A variety of factors  
312 can affect animal haematological and biochemical parameters, including breed,  
313 gender, diet, age, reproductive status and seasonal variations (Ozegbe, 2001; Wells  
314 *et al.*, 1999). The haematological and biochemical parameters of this study were  
315 within normal ranges for rabbit species (Archetti *et al.*, 2008; Özkan *et al.*, 2012). The  
316 application of *Echinacea* extract should booster immunological reactivity and should  
317 contribute to improve health status (Böhmer *et al.*, 2009). In the present trial, EPAL  
318 had no influence the heamatological and health status of rabbit does. The change in  
319 blood coagulation-related parameters during the later stage of gestation is a common  
320 physiological response for the protection against excessive haemorrhage or for the  
321 preservation of the homeostasis at parturition (Mizoguchi *et al.*, 2010). In our study,  
322 the modulation of RBC and HCT may be related to physiological anemia resulting  
323 from haemodilution (Ozegbe, 2001). Watery supplementation with *Echinacea*  
324 *purpurea* extract induced higher results of Hb, PCV and RBC in growing rabbits  
325 (Ahmed *et al.*, 2008). Likewise, a study by Chow *et al.* (2006) found an increase in

326 RBC in pregnant mice when fed *Echinacea purpurea*. In addition, the increment of  
327 erythropoietin level (glycoprotein hormone which controls erythropoiesis) has been  
328 reported in *Echinacea purpurea*-treated men. This should support the RBC increment  
329 deriving from the supply of phyto-additives (Whitehead *et al.*, 2007). On the other  
330 hand, Maass *et al.* (2005) did not find any significant difference for these parameters  
331 in sows, piglets and grower/finisher pigs that received dried *Echinacea purpurea* herb  
332 as feed additive in their diets. Differences concerning plant species tested (EPAL vs  
333 *Echinacea purpurea*), preparation methods (raw material vs extraction), physiological  
334 status (pregnant vs non-pregnant) and species (rabbit vs swine, mice and human  
335 beings) could explain these contrasting results. An author showed that WBC  
336 parameters increased during the whole period of gestation in pregnant women  
337 (Cincotta *et al.*, 1995), in rabbit does (Haneda *et al.*, 2010) and also in rats (DeRijk *et*  
338 *al.*, 2002). Cundell *et al.* (2003) found a significant increase of lymphocytes after one  
339 week in rats fed with dried Echinacea preparations. A higher proliferation rate of  
340 spleen lymphocytes in EPAL supplemented mice has been reported in an in vitro  
341 study, but the haematology indices were not influenced (Zhai *et al.*, 2007). The  
342 increase in WBC generally is a good indicator of immunity efficiency increase  
343 (Wieslaw *et al.*, 2006). In our study, the effect of EPAL was observed only for Bas.  
344 According to other studies, this effect may be related to its phytochemically active  
345 constituents of EPAL (Hu and Kitts, 2000; Pellati *et al.*, 2005; Dalby-Brown *et al.*,  
346 2005).

347 With respect to blood serum chemistry, no significant difference was observed in total  
348 protein. In contrast, Wells *et al.* (1999) reported a decrease in total protein and  
349 albumin in pregnant rabbits and this is thought to reflect the increased blood volume.

350 Innate immunity has an important role to prevent the infection as first-line defence  
351 and also contributes antigen-presenting cells that activate the adaptive immune  
352 response, which is specific and powerful (Tizard, 2013). Dietary supplementation with  
353 *Echinacea* can stimulate the innate immunity by increasing cytokine production  
354 (Hwang *et al.*, 2004) and phagocyte-stimulation (Böhmer *et al.*, 2009). Lysozymes  
355 and the complement system are interesting indicators to study the innate immune  
356 function. In our experiment, only lysozyme results showed a time related change. It  
357 must be highlighted that our work was performed in a standard environment without  
358 infection, stress or other factors influencing immune responses. Therefore, the  
359 experimentation in normal conditions may hardly result in a significant effect on  
360 immunity despite the supplementation with an immunomodulating agent.

361

#### 362 *Fattening rabbit performance*

363 Growth performance of *Echinacea* supplemented groups did not showed significant  
364 differences. Our results differ from Arafa *et al.* (2010) who found, in a similar study  
365 using *Echinacea purpurea* at 130 mg/kg body weight, a significant decrease in  
366 mortality rate and an increase of live weight in 6-week-old growing rabbits fed E diets  
367 ( $P<0.05$ ). Usually, dietary herb supplementation leads to an improvement of the  
368 flavour, which accounts for an increase of feed ingestion and better performance  
369 (Franz *et al.*, 2010; Christaki *et al.*, 2012). Ahmed *et al.* (2008) highlighted a  
370 significant improvement of final body weight, daily weight gain and feed conversion  
371 ratio in growing rabbits to which were orally given in liquid 7.5 mg of *Echinacea*  
372 *purpurea* extracts/kg body weight and day. However, the outcomes of above reported  
373 references are not fully comparable with our trial due to some dissimilarities in  
374 experimental plans concerning: tested *Echinacea* species, concentration of the



375 supplement, administration route (oral by liquid mixture), supplement preparation  
376 (extraction) and supplemented periods in doe's diet  
377 Generally, mixtures of *Echinacea purpurea*, *Echinacea angustifolia* and EPAL are  
378 used in human medicine and animal production. To this regard, positive outcomes on  
379 productive performance were reported in rabbits with *Echinacea purpurea* addition  
380 (Ahmed *et al.*, 2008; Arafa *et al.*, 2010), whereas studies conducted with other  
381 livestock species did not find any improvement (Hermann *et al.*, 2003; Maass *et al.*,  
382 2005; Böhmer *et al.*, 2009; Sahin *et al.*, 2012).  
383 In conclusion, there is no evidence that diets supplemented with EPAL cause any  
384 beneficial effects in normal management condition. Nonetheless, further studies are  
385 suggested in order to evaluate the effect of *Echinacea pallida* on animal performance  
386 and to study the relation between its active components and physiological functions.

387

### 388 **Acknowledgements**

389 This research was supported by a “University of Torino (ex 60%)” grant (Es. fin.  
390 2013). The authors thank Dr. Claudio Malavasi (Biotrade Snc®, Modena, Italy) for  
391 providing the dry ground EPAL samples, Dr. Cerrina (Ferrero S.p.A Cuneo, Italy) for  
392 his support on feed formulation, Dr. Luca Operti (*Department of Drug Science and*  
393 *Technology, University of Torino*) for EPAL analysis, Mrs. Vanda Malfatto for her  
394 technical support, Mr. Dario Sola, Dr. Paolo Montersino and Mr. Mario Colombano for  
395 rabbit care.

396

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532 **Table 1** *Rabbit does diets composition*

	Does diet (from artificial insemination to 21 days after parturition)		Does diet (from 21 days after parturition to 35 days after parturition)	
	Control	Treatment	Control	Treatment
Chemical composition <sup>1</sup>				
Dry matter (DM)	89.3	90.2	89.9	89.9
Crude protein (% DM)	18.7	18.8	17.5	17.2
Ether extract (% DM)	2.6	2.9	4.5	4.6
NDF (% DM)	35.0	33.7	32.4	32.2
ADF (% DM)	22.4	22.2	17.5	17.9
ADL (% DM)	5.5	5.7	5.4	5.4
Ash (% MS)	9.5	9.5	7.5	7.9
Starch (% DM)	26.2	27.2	17	17.4
<i>Echinacea pallida</i> (g/kg)	0	3	0	3
Minerals and vitamins <sup>2</sup>				
Calcium (% DM)	0.9	0.9	1	1
Lysine (% DM)	0.8	0.8	0.7	0.7
Phosphorus (% DM)	0.5	0.5	0.4	0.4
Methionine (% DM)	0.3	0.3	0.4	0.4
Sodium (% DM)	0.3	0.3	0.3	0.3
Vitamin A (UI/kg)	12.5	12.5	12.5	12.5
Vitamin D3	1.2	1.2	1.2	1.2
Vitamin E	100	100	100	100
Ferrous carbonate (mg/kg)	662	662	704	704
Manganese oxide (mg/kg)	195	195	209	209
Zinc oxide (mg/kg)	186	186	186	186
Copper sulfate (mg/kg)	98	98	98	98
Potassium iodide (mg/kg)	2.4	2.4	2.5	2.5
Sodium selenite (mg/kg)	0.6	0.6	0.6	0.6

533 <sup>1</sup>The experimental diets were analyzed by the laboratory of the Department of Agricultural,  
534 Forest and Food Sciences, Turin, Italy. <sup>2</sup>These data were provided by the Ferrero Mangimi  
535 S.p.A, (Farigliano CN, Italy), which formulated and prepared the experimental diets.

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538 **Table 2** *Growing rabbits diets composition*

	Diets <sup>2</sup>	
	Control	Treatment
Chemical composition <sup>1</sup>		
Dry matter (DM)	89.8	89.8
Crude protein (% DM)	17.1	17.3
Ether extract (% DM)	3	3
NDF (% DM)	39.4	39.6
ADF (% DM)	23.7	24
ADL (% DM)	6.6	6.6
Ash (% DM)	9.7	10.4
Starch (% DM)	12	12.3
<i>Echinacea pallida</i> (g/kg)	0	3
Minerals and vitamins <sup>2</sup>		
Calcium (% DM)	1	1
Lysine (% DM)	0.7	0.7
Methionin (% DM)	0.4	0.4
Phosphorus (% DM)	0.4	0.4
Sodium (% DM)	0.3	0.3
Vitamin A (UI/kg)	12.5	12.5
Vitamin D3	1.2	1.2
Vitamin E	100	100
Ferrous carbonate (mg/kg)	662	662
Manganese oxide (mg/kg)	195	195
Zinc oxide (mg/kg)	186	186
Copper sulfate (mg/kg)	98	98
Potassium iodide (mg/kg)	2.5	2.5
Sodium selenite (mg/kg)	0.57	0.57

539 <sup>1</sup>The experimental diets were analyzed by the laboratory of the Department of Agricultural,  
540 Forest and Food Sciences, Turin, Italy. <sup>2</sup>These data were provided by the Ferrero Mangimi  
541 S.p.A, (Farigliano CN, Italy), which formulated and prepared the experimental diets.

542 **Table 3** Effects of pale purple coneflower (*Echinacea pallida*) dietary supplementation on reproductive performance of rabbit does

	Control group	<i>Echinacea</i> group	Standard error of mean difference	P-value
No. of does at first kindling	50	50	-	-
No. of does at fifth kindling	37	38	-	-
Mortality of does (%)	26	24	-	0.817 <sup>1</sup>
Body weight (LW), g				
at first kindling	3868	3869	-	0.982
at fifth kindling	4782	4770	-	0.929
No. of kindled does/artificial insemination	148 / 221	151 / 221	-	-
Kindling rate,%	67	68	-	0.760 <sup>1</sup>
Prolificacy	8.78	8.88	-	0.852 <sup>1</sup>
Total born	10.5	10.5	0.36	0.978 <sup>2</sup>
Born alive	9.72	9.74	0.37	0.945 <sup>2</sup>
Stillborn	0.78	0.76	0.18	0.907 <sup>2</sup>
Litter size				
at 21d	8.36	8.42	0.25	0.816 <sup>2</sup>
at 35d	8.30	8.34	0.26	0.877 <sup>2</sup>
Litter weight (g)				
at 21d	2750	2747	101.22	0.981 <sup>2</sup>
at 35d	7023	7038	229.82	0.946 <sup>2</sup>
Individual body weight (g)				
at 21d	329	326	3.80	0.495 <sup>2</sup>
at 35d	846	844	4.05	0.585 <sup>2</sup>
Perinatal mortality (%)	7.40	7.25	-	0.868 <sup>1</sup>
Mortality (%)				
0-21d	14	13.6	-	0.788 <sup>1</sup>
21-35d	0.65	0.86	-	0.528 <sup>1</sup>

543 <sup>1</sup>: parameter analyzed by Chi-square test; <sup>2</sup>: parameter analyzed by Student's t-test

545 **Table 4** Effects of pale purple coneflower (*Echinacea pallida*) dietary supplementation on blood and immune parameters of  
 546 pregnant rabbit does (n=8 per group)

	Treatment		Gestational day			Within subjects effects			Between subjects effects	
	Control group	<i>Echinacea</i> group	0	14	28	Gestational day P-value	Gestational day × Treatment P-value	Root Mean Square Error	Treatment P-value	Root Mean Square Error
No. of animals	8	8	8	8	8					
<i>Haematology</i>										
RBC (M/mm <sup>3</sup> )	5.80	5.50	5.38	5.96	5.62	0.025	0.963	0.422	0.145	0.265
Hb (g/dl)	11.99	11.40	10.96	12.27	11.86	0.013	0.992	0.885	0.274	1.912
HCT (%)	38.02	36.18	35.65	39.46	36.19	0.014	0.907	86.827	0.271	18.164
MCV (fl)	65.58	65.87	66.27	66.32	64.59	0.003	0.383	33.808	0.870	22.463
MCH (pg)	20.61	20.70	20.29	20.57	21.10	0.048	0.763	0.677	0.894	2.870
MCHC (g/dl)	31.53	31.46	30.69	31.06	32.74	<0.001	0.553	0.932	0.770	0.439
RDW (%)	10.81	11.65	10.14	11.53	12.03	<0.001	0.339	0.702	0.343	5.216
PLT (m/mm <sup>3</sup> )	137.07	168.00	146.20	154.50	156.90	0.897	0.441	53.741	0.223	64.048
PCT (%)	0.09	0.11	0.09	0.10	0.11	0.432	0.304	0.032	0.158	0.045
MPV (fl)	6.74	6.83	6.51	6.55	7.29	<0.001	0.380	0.253	0.620	0.460
PDW (%)	6.77	6.78	6.66	6.29	7.38	0.009	0.729	0.692	0.974	0.538
WBC (m/mm <sup>3</sup> )	9.59	9.38	11.14	11.11	6.22	<0.001	0.507	2.070	0.829	2.588
LYM (%)	14.97	14.57	15.41	12.56	16.34	0.011	0.803	2.541	0.850	5.688
MON (%)	6.53	5.91	6.62	5.49	6.54	0.052	0.663	1.055	0.533	2.606
NEUT (%)	76.87	78.17	76.87	80.49	75.21	0.009	0.033	3.396	0.657	7.717
Eos (%)	1.08	1.04	0.56	0.99	1.63	<0.001	0.626	0.475	0.851	0.565
Bas (%)	0.55	0.29	0.54	0.44	0.28	0.092	0.671	0.249	0.049	0.300
<i>Blood serum chemistry</i>										
BUN (mg/dl)	20.87	16.82	14.95	15.34	26.25	0.183	0.471	9.305	0.413	8.127
GOT (UI/L)	29.06	32.22	26.79	35.58	29.55	0.395	0.790	14.315	0.401	9.763
Total Protein (g/dl)	4.60	4.34	4.48	4.22	4.72	0.325	0.641	0.711	0.296	0.643
Albumin (g/dl)	2.91	2.91	2.68	2.87	3.18	0.109	0.191	0.494	0.971	0.391
Urea (mg/dl)	29.28	36.10	32.09	32.92	33.06	0.973	0.143	6.929	0.319	10.851
Cholesterol (mg/dl)	48.66	39.41	33.85	63.21	35.04	0.352	0.219	49,781	0.658	55.169

*Immune parameters*

Lysozymes (µg/ml)	5.64	4.98	6.02	7.99	1.91	0.014	0.590	4.122	0.862	10.067
Complement	36.72	29.44	34.31	34.06	30.87	0.826	0.267	12.959	0.174	12.438
Alfa1 (g/dl)	0.14	0.16	0.20	0.09	0.18	0.234	0.117	0.155	0.746	0.182
Alfa 2 (g/dl)	0.28	0.19	0.32	0.19	0.19	0.176	0.304	0.170	0.164	0,158
Beta 1 (g/dl)	0.28	0.28	0.33	0.28	0.23	0.175	0.198	0.114	0.828	0.100
Beta 2 (g/dl)	0.39	0.38	0.38	0.36	0.42	0.557	0.687	0.118	0.801	0.134
Gamma (g/dl)	0.60	0.42	0.56	0.43	0.54	0.697	0.515	0.355	0.115	0.281

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548 RBC: Red Blood Cells; Hb: Haemoglobin concentration; HCT: Haematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC:  
549 Mean Corpuscular Haemoglobin Concentration; RDW: Red cell distribution width; PLT: Platelets; PCT: Relative volume of thrombocytes; MPV: Mean Platelet  
550 Volume; PDW: Platelet distribution width; WBC: White Blood Cells; LYM: Lymphocytes; MON: Monocytes; NEUT: Neutrophils; Eos: Eosinophils; Bas:  
551 Basophils; BUN: blood urea nitrogen; GOT: glutamate oxaloacetate transaminase.

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560 **Table 5** Effect of pre and postnatal dietary supplementation with pale purple coneflower (*Echinacea pallida*) on growth performance  
 561 of fattening rabbits (n=20 per group)

	Groups				RSD	P-value
	CC	CE	EC	EE		
Live weight (g)						
At 35 day	885	889	889	882	53.8	0.976
At 49 day	1713	1711	1745	1717	79.7	0.513
At 77 day	3031	2998	3107	3041	160	0.190
Growth performance in 35-49 days						
Daily feed intake (g per day)	134	138	140	139	10.6	0.323
Daily weight gain (g per day)	59.2	58.7	61.2	59.6	3.58	0.160
Feed conversion ratio	2.28	2.36	2.29	2.35	0.15	0.200
Growth performance in 49-77 days						
Daily feed intake (g per day)	176	178	181	181	10.8	0.478
Daily weight gain (g per day)	45.4	44.4	46.9	45.7	4.05	0.254
Feed conversion ratio	3.87	4.03	3.88	3.98	0.30	0.282

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Growth performance in 35-77 days

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Daily feed intake (g per day)	162	165	168	168	11.5	0.368
Daily weight gain (g per day)	49.9	49.0	51.6	50.2	3.11	0.082
Feed conversion ratio	3.25	3.37	3.26	3.34	0.19	0.122

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562 CC: rabbits fed the C diet and born from the C does, CE: rabbits fed the E diet and born from the C does, EC: rabbits fed the C diet and born  
563 from the E does, EE: rabbits fed the E diet and born from the E does.

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576 **Figure 1**

577 *LC-PDA profile at 325 nm of Echinacea pallida (Nutt.) Nutt root extract at 325 nm.*

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