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## ► To cite this version:

Ilyas Bendahmane, Quentin Garrigues, Emmanuelle Apper, Amélie Mugnier, Ljubica Svilar, et al.. Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies. 2024. hal-04777307

**HAL Id: hal-04777307**

**<https://ut3-toulouseinp.hal.science/hal-04777307v1>**

Preprint submitted on 12 Nov 2024

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1 **Maternal oral supplementation with *Saccharomyces***  
2 ***boulardii* I-1079 during gestation and early lactation**  
3 **impacts the early growth rate and metabolic profile of**  
4 **newborn puppies**

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6

7 **Keywords: Yeast; Nutritional Programming; Early growth; Proline; Metabolome; Newborn; Dog**

8

9 **Abstract:**

10 Nutritional programming is a manipulation of fetal and neonatal development through  
11 maternal feeding. In humans and pigs, maternal yeast supplementation was demonstrated as a  
12 promising approach to positively modulate newborns' health. This study aimed to investigate  
13 the effects of *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (SB) supplementation

14 in pregnant and lactating bitches on the newborns' early growth rate (EGR, between birth and  
15 two days of life), metabolic profiles, and the association between both of them. A total of 17  
16 female dogs and their 81 puppies were included. From day 28 of gestation until the end of the  
17 study, bitches were divided into two groups, one of which received orally  $1.3 \times 10^9$  colony  
18 forming units of live yeast per day. Puppies from mothers receiving the live yeast were  
19 defined as the SB group (n=40) and the others were defined as the placebo group (n=41). For  
20 each puppy, EGR was calculated, and blood and urine samples were collected at D2 for  
21 metabolome analysis using liquid chromatography-mass spectrometry (LCMS). Puppies from  
22 SB group presented higher EGR compared with the placebo group (12% vs 7%;  $p=0.049$ ).  
23 According to the Sparse Partial Least-Squares Discriminant Analysis (sPLS-DA), both urine  
24 and serum metabolome profiles were significantly different between the two groups with a  
25 total of 29 discriminating metabolites in urine and serum. Fourteen of them were implicated in  
26 the nitrogen metabolism pathway including, gamma-aminobutyrate, 3 methyl-l-histidine and  
27 xanthosine (less abundant in SB compared with placebo group, all  $p < 0.05$ ), adenine,  
28 aspartate and proline (more abundant in SB compared with placebo group, all  $p < 0.05$ ).  
29 Metabolic pathways pointed to proline synthesis, a crucial component in collagen synthesis  
30 and osteoarticular system development. Urinary proline abundance was positively correlated  
31 with EGR ( $r=0.45$ ;  $p < 0.001$ ). These findings highlight the potential benefits of maternal  
32 supplementation with SB promoting early neonatal growth, essential for the neonatal survival,  
33 through nitrogen metabolism orientation.

34

35

36 **1 Introduction:**

37

38 The maternal-fetal relationship plays a pivotal role in shaping development and health of  
39 offspring. According to the fetal programming theory initially proposed by Barker (1), factors  
40 and experiences during pregnancy may induce lasting modifications in the fetal anatomy,  
41 physiology, and metabolism, potentially contributing to the development of diseases in  
42 childhood and even in adulthood.

43 In the early stages of life, dogs encounter a spectrum of health challenges. One of the major  
44 concerns that puppies face during the first months of life are growth problems. For instance,  
45 the early growth rate (EGR) is strongly associated with neonatal mortality (2). Indeed, the first  
46 two days of life are crucial for the newborn dog, as they reflect the adaptation to the extra-  
47 uterine life but also the colostrum ingestion.

48 As all of the mentioned phenomena are strongly related to the dam, one could hypothesize  
49 that acting on the maternal organism could improve the newborn's health. Indeed, in a study  
50 in pigs demonstrated the possibility of modulating offspring growth through maternal  
51 nutrition during gestation and lactation (3). One of the strategies of maternal programming via  
52 nutrition aiming to improve neonatal outcomes is maternal probiotic supplementation during  
53 gestation.

54 Probiotics are live microorganisms that, when administered in adequate amounts, confer a  
55 health benefit on the host (4) - (5) - (6) - (7). Among probiotics, *Saccharomyces cerevisiae*  
56 var. *boulardii* (SB) is a yeast extracted from lychee and mangosteen fruit that has been shown  
57 to have beneficial effects on digestive health, general immunity in human (8) and even mental  
58 health in rats (9). In addition to effects observed on the host, its potential role as a tool for  
59 maternal nutritional programming on the offspring's health has been demonstrated recently.  
60 In pigs, supplementation of sows with SB during gestation and lactation modifies the  
61 intestinal microbiota of piglets, increasing specifically *Lactobacillus* species abundances

62 known to be beneficial for the host's health (10). The positive influence of SB  
63 supplementation in pregnant sows has been highlighted on the immunological status and  
64 response to stress of the newborn at birth, via a decreased blood cortisol concentration and  
65 increased white blood cell counts (11). To date, the effect of the maternal nutritional  
66 programming by yeast has never been investigated in the canine species.

67 The objectives of this study were thus 1) to evaluate the effect of live yeast SB  
68 supplementation of the pregnant and lactating bitches on EGR, urine and serum metabolome  
69 of their puppies at 2 days of life and 2) to study the relationship between these two elements.

70

## 71 **2 Materials and methods**

### 72 **2.1 Ethics statement**

73 This study was reviewed and approved by the local ethical committee (Comité d'Éthique en  
74 Expérimentation Animale, Science et Santé Animale n°115; reference number: SSA\_2020-  
75 004, Toulouse, France).

### 76 **2.2 Animals, diet and experimental design**

#### 77 **2.2.1 Dams**

78 Of the thirty-six bitches included in the previous study by Quentin Guarigues et al. ([ref](#)), 17  
79 were selected for our study, to balance the cohort, ensure homogeneous groups, and healthy  
80 puppies (Table 2). These female dogs of medium-sized breed (Australian Shepherd, body  
81 weight between 15 kg and 25 kg) and large-sized breeds (Golden Retriever, Labrador  
82 Retriever, White Swiss Shepherd; body weight between 25 kg and 45 kg) were included  
83 within one breeding kennel and housed individually in the same conditions. No dam presented  
84 any health trouble at the time of inclusion.

85

86 Ovulation was identified using a progesterone assay, with progesterone levels ranging  
87 between 4 ng/mL and 10 ng/mL. Then two natural matings were conducted on days 1 and 3  
88 post-ovulation. Pregnancy diagnosis was carried out between days 25 and 30 post-ovulation  
89 using abdominal ultrasonography.

90 At the 28th day of gestation (**G28**), bitches were randomized according to their breed size,  
91 breed, age, parity, body condition score and fecal score and subsequently, they were allocated  
92 into two groups: placebo (n=7) or supplemented group (n=10; SB group).

### 93 **2.2.2 Diet and probiotic supplementation in dams**

94 Two extruded foods manufactured by CRUSTY FOOD SAS (Montardit, Verteuil d'Agenais,  
95 France) and formulated to meet NRC recommendations (2006) were provided during the  
96 study: The first diet (**Diet 1; Table 1**) was given from the date of ovulation until the G28 and  
97 the 2nd diet from G28 until the end of the study ((56 days *post-partum*; **Diet 2**). Vitamin A,  
98 vitamin D3 and vitamin E were added as nutritional feed additives (**Table 1**).  
99 (13)). All dogs had *ad libitum* access to water for drinking during the whole experiment.

100 The additive tested was *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (Levucell  
101 SB®, Lallemand SAS, Blagnac, France) given in 400-mg vegetal capsules  
102 (hydroxypropylmethylcellulose (HPMC) caps size 1 clear, Suheung Co, South Korea)  
103 containing 6.25% additive (YEAST, measured at  $6.4 \times 10^8$  CFU of additive/capsule), 92.75%  
104 potato starch and 1% E551a (silicic acid, precipitated and dried). Capsules were fed twice per  
105 day, in the morning and the evening, in a bullet of wet petfood to ensure full consumption.  
106 Each dog received  $1.3 \times 10^9$  CFU of additive/day. The control group was fed the same  
107 capsules but without the additive (Placebo).

### 108 **2.2.3 Puppies**

109 Among a total of 113 puppies born, 105 were born alive and only 81 puppies having all  
110 samples needed (urine and serum) were selected to this study. At birth, puppies were  
111 identified using a different colored collar, and their weight and sex were recorded. All  
112 included puppies were born naturally and remained healthy at the time of sampling (2 days  
113 *post-partum*). Puppies remained with their dams during the study period with unlimited access  
114 to maternal milk.

115 Included puppies underwent weight measurements within the first 24h following birth (**D0**)  
116 and Two days later (**D2**). One day after birth (**D1**), all puppies were examined and their rectal  
117 temperature and glycemia were measured to evaluate their health status.

118 At D2, samples were taken from each puppy (**Fig1**): about 1mL of urine was obtained by  
119 gentle swabbing of perineal area (to stimulate spontaneous miction) and blood was collected  
120 from the jugular vein (1mL), both on Eppendorf tubes. The blood was centrifugated (16000  
121 RCF for 10min) to isolate the serum, and aliquot was stored at -20°C for 2 weeks and then  
122 transferred to -80°C until analysis.

### 123 **2.3 Serum and urine metabolome analyses**

124 Urine and serum metabolome analyses and data pre-processing were conducted at the  
125 BIOMET platform attached to the cardiovascular and nutrition research center (C2VN)  
126 located in Marseille, France.

### 127 **2.4 Samples preparation**

#### 128 **Urine**

129 The tubes were thawed on ice, then vortexed for 1 minute. The samples were then centrifuged  
130 at 4°C for 15 minutes at 16000 RCF. Following this, 50µL of urine was drawn into 2mL  
131 Eppendorf tubes and 400µL of deionized water was added. After vortexing the mixture for 30  
132 seconds, another round of centrifugation was performed. A total of 350µl of the supernatant

133 was collected in a 0.45µm filter, then recentrifuged (4° C, 15 min 16000 RCF). Then, 50uL of  
134 the supernatant was collected in a glass vial with the insert then stored at -80°C before  
135 analysis by liquid chromatography–mass spectrometry (LC-MS).

### 136 **Serum**

137 The tubes were thawed on ice then vortexed for 1 minute. Then 50µL of the sample was taken  
138 and 200%L of cold methanol (MeOH) (-20°C) was added. The mixture was slowly agitated  
139 for 1 minute then incubated in the freezer (-20°C) for 1 hour, vortex mixed for 30 seconds  
140 then centrifuged (4°C, 15 min, 11000 RPM). A total of 200µl of the supernatant was collected  
141 in a 10kDa filter then recentrifuged (4°C, 45 min, 16000 RCF). Following this, the tubes were  
142 evaporated under a stream of nitrogen then the dry extract was taken up in 120µL  
143 Water/acetonitrile (90/10,v/v +0.1% formic acid) then vortexed for 1 minute. The mixture  
144 was then transferred to a 0.45 µm filter tube and centrifuged for 15 min (4°C, 16000 RCF).  
145 Then, 50µL of the supernatant was collected in a glass vial with the insert then stored at -80°C  
146 before analysis by LC-MS.

147 For both sample types (urine and serum), two controls for each were carried out: the pool  
148 (quality control samples) as a mixture of 20µL of each serum sample, urine sample and a  
149 blank samples, prepared the same way as the samples by taking 50µL of deionized water.

### 150 **2.5 LC-MS analysis**

151 The metabolic profile of the samples was acquired using a high-performance liquid  
152 chromatography (HPLC) system, a Thermo Fisher Scientific Ultimate 3000 Series, coupled  
153 with a Q-Extractive Plus, a high-resolution mass spectrometer (Thermo Fisher Scientific,).  
154 Chromatographic separation was achieved using hydrophilic and reverse phase  
155 chromatography in order to largely cover the metabolome identification. For reverse phase  
156 separation, a Hypersil Gold column (100 mm x 2.1 mm x 1.9 µm) from Thermo Fisher



157 Scientific was used together with a 0.1% formic acid in water (solvent A) or in acetonitrile  
158 (solvent B). Flow rate was kept at the 0.4 mL/min and column temperature was maintained at  
159 40°C. A molecules separation started with 0 % of A for 1 minute. Than the rising of B% to  
160 100% was achieved in 10 minutes, system wash was done for 2 minutes with 100% B, and the  
161 initial conditions were restored in 1 min. Equilibration of the column was keeping an 0% A  
162 for 2 minutes. Total run was 16 minutes. For a hydrophilic separation a Sequant zicHILIC 5u,  
163 200A, 150 × 2.1 (Merck, France) was used with a 0.25 mL/min flow rate at 25 °C of column  
164 temperature. Solvent A was 16 mM ammonium formiate in water, and solvent B a 0.1μ  
165 formic acid in acetonitrile. First 2 minutes a column was kept at 97% of B, then the gradient  
166 was run during 8 minutes decreasing B to 70% and for 5 minutes to 10%. 2 minutes solvent B  
167 was kept at 10%, then returned at 97% in 1 minute and held to 97% B for 9 minutes for the  
168 column conditioning. The total run was 27 minutes. Mass spectra were acquired using a  
169 switching polarity mode in the mass range 80-1000 *m/z*. A resolving power was set to 35 000  
170 full width at half maximum (FWHM). Electrospray voltage was kept at 3.5 kV, S-lens RF  
171 level at 55 V, capillary temperature at 320°C and sheath gas and auxiliary gas were kept at 30  
172 and 8 arbitrary units (a.u.), respectively. A tandem mass spectrometry spectra (MS/MS) was  
173 acquired using a Data Dependent Analysis experiment, at 17 500 FWHM resolving power. A  
174 Top 5 selection of ions were performed after each full scan acquisition in order to isolate and  
175 fragment a high number of ions.

176 Samples were analysed randomly in an analytical batch and in the LC-MS system. Five  
177 recovery solvent, then 5 samples of extraction blanks were injected in order to rinse and  
178 stabilize the analytical system, followed by the first series of quality control samples. The  
179 samples were then analysed randomly, interrupted every 5 experimental samples by a quality  
180 control sample. Finally, a MS/MS experiment was done on a pooled sample for the metabolite  
181 annotation and identification.

## 182        **2.6 Data pre-processing**

183        Full scan spectra were converted to a mzXML format using Proteo Wizard and pre-processed  
184        using XCMS library script under R language. The following parameters were applied: peak  
185        picking: CentWave, peak grouping: density, retention time correction: obiwarp. Analytical  
186        batch was corrected using Van-der-Kloet algorithm and further filtering was applied on the  
187        extracted signals, such as removal of signals with coefficient of variation higher than 30% in  
188        quality control samples, and removal of noise signal.

189        Signal annotation was achieved using “in-house” database with information about *m/z* and  
190        retention times in reverse and hydrophilic phase of nearly 800 metabolites. A match between  
191        the experimental and database information was done on the Galaxy Workflow4metabolomics  
192        platform using following conditions: *m/z* accuracy limit was set to 5 ppm, and retention time  
193        drift up to 0.7 minutes. After an automatic annotation, each annotated feature was visually  
194        verified and the annotation confirmed by fragmentation spectra comparison.

195        From this annotation, a table is obtained containing 205 identified/annotated metabolites with  
196        their intensities detected in the urine samples and 228 metabolites for serum. Subsequently,  
197        probabilistic quotient normalization (PQN) was carried out on each of the matrices.

198

## 199        **2.7 Statistical analysis:**

200        All statistical analysis was performed using R software, version 4.2.1. (14).

### 201        **2.7.1 EGR**

202        The early growth rate was calculated for each individual as follows: (weight D2 – weight D0)  
203        × 100 / weight D0. An analysis of variance (ANOVA) was then performed to evaluate the  
204        effect of treatment, breed and litter on growth rate, using the model:

$$205 \qquad \text{EGR} = \mu + \text{treatment} + \text{breed} + \text{litter} + \epsilon$$

## 206 2.7.2 Metabolome

207 As metabolomes are compositional data (15), we firstly applied the Geometric Bayesian-  
208 multiplicative method (GBM) to handle the zero values, assuming that the probability of  
209 detecting the metabolite in the samples is not zero. The `cmultRepl` function from the  
210 `zComposition` package (16) was employed to perform this method. Then, using the abundance  
211 table generated from the GBM output, the `composition` package was utilized to transform the  
212 data into the Centred Log Ratio (CLR). Subsequently, after obtaining the CLR output, a linear  
213 model was applied to identify the impact of each fixed effect on each metabolite, using the  
214 following formula:

$$215 \quad \text{Formula1: } y_1 = \mu_1 + \text{mother} + \text{breed} + \text{litter size} + \epsilon_1$$

216 P-values were obtained and used to calculate the percentage of metabolites affected by each  
217 confusion effect. The results revealed that the breed effect impacts 50% of the metabolites,  
218 while the maternal effect and litter size affect approximately 20%. Notably, the correction for  
219 the breed effect eliminates the influence of the maternal and litter size effects. Consequently,  
220 we opted to correct for the breed effect and proceeded to retrieve the residual data table for  
221 subsequent multivariate analyses:

$$222 \quad \text{Formula2: } y_2 \sim \mu_2 + (1 | \text{breed}) + \epsilon_2$$

223 The residuals derived from the final model were employed for the following analyses.  
224 Utilizing the corrected data matrix obtained from Formula2, we employed the `"tune.splsda"`  
225 function to determine the optimal number of principal components (`ncomp`) and the number  
226 of variables (`KeepX`) per component for achieving the most effective discrimination between  
227 the two groups (Placebo and SB). Subsequently, a sparse Partial Least Squares Discriminant  
228 Analysis (PLS-DA) was performed using the `"splsda"` function from the `MixOmics` package  
229 (17), with the parameters (`ncom` and `KeepX`) recommended by `"tune.splsda"`. Evaluation of

230 error rate was conducted through the utilization of balanced error rate (BER) analysis and area  
231 under the receiver operating characteristic curve (AUROC) was performed to assess the  
232 classification performance. Subsequently, PlotLoadings and variable importance in projection  
233 (vip) functions were employed on the principal components selected (as determined by  
234 “tune.splsda” function), in order to extract the relevant variables that discriminate our groups.

235 Metabolite set enrichment analysis was performed using Metaboanalyst 5.0 (ref: ) . website in  
236 order to identify significant urine and serum metabolic pathways. Correlations between the  
237 variables of different matrices were calculated based on multiblock sPLS-DA using  
238 “network” function of the MixOmics package. A cut-off score of 0.50 was set in order to only  
239 represent variables whose scores are higher than the latter.

### 240 **2.7.3 Link between metabolome and EGR**

241 Pearson's correlation with a threshold of 0.4 was used to highlight the links between  
242 individual metabolites and growth rate. Then, scatterPlot was produced using the ggplot2  
243 package (18). The final diagram was produced using the BioRender website. Data are  
244 presented as mean  $\pm$  standard deviation (SD).

245

## 246 **3 Results:**

### 247 **Characteristics of the included puppies**

248 A total of 81 puppies alive at D2 (49 males and 32 females, 7 per litter on average) from 17 selected  
249 bitches were included in the study. Birth weights ranged from 214g to 576g with an average of 387g.  
250 At D1, the mean rectal temperature was  $36.4 \pm 0.8^\circ\text{C}$  (min:  $33.8^\circ\text{C}$  – max:  $38.3^\circ\text{C}$ ) and the glycemia  
251 was  $120 \pm 30.6$  mg/dl (min: 64 mg/dl – max: 207 mg/dl) with no sign of diseases.

252 Among the included puppies, 49% (40 puppies) were born from 10 mothers supplemented with SB  
253 during gestation and lactation, while 51% (41 puppies) were born from 7 mothers supplemented with a

254 placebo. Both groups of puppies were balanced according to the sex ratio, litter size, breeds, and birth  
255 weight (**Table 2**).

256 Out of all puppies, EGR varied between -14.2% and 37.7%. According to the ANOVA test, the EGR  
257 was significantly higher in puppies from the SB group compared to those from the placebo group  
258 (+57%; p-val= 0.049) despite a high variability in the SB group (**Fig2**).

259

### 260 **Urinary and serum metabolome in newborn puppies**

261 A total of 228 serum and 206 urine metabolites were detected in puppies at D2 according to the LC-  
262 MS analysis (**Table 1-2supp**). The metabolites found in the urine and serum of neonates were for the  
263 vast majority part of the nitrogen metabolism pathway (**Fig3**).

264 After the enrichment analysis and p-value correction, only the ammonia recycling and glycine-serine  
265 metabolism pathways were significantly overrepresented in urine samples (respectively, p-adj<0.01, p-  
266 adj=0.016; Fig3), while no pathway was significantly overrepresented in serum samples.

267

### 268 **Impact of maternal SB supplementation on the urinary metabolic profiles of newborn** 269 **puppies**

270 The sPLSDA analyses performed on urinary metabolites allowed us to discriminate puppies  
271 of the SB group from those in the placebo group with a high accuracy of the model  
272 (AUROC $\geq$ 0.98 for urine metabolome). The two principal components explained 12% of the  
273 variance for urine metabolome and discriminated groups with an error rate (BER) of 0.27  
274 (**Fig4a**). On these principal components, VIP analyses allowed the selection of 20 out of the  
275 initial 206 urinary metabolites (Loading score comprised between -0.31 and 0.57),  
276 discriminating puppies of the SB from the placebo group (**Fig5a; Table 3**). All these  
277 metabolites presented a VIP score greater than 1.0.

278 Significant differences were noted between the SB and placebo groups in the abundance of all  
279 discriminating urine metabolites. Specifically, within the purine metabolism pathway, the SB  
280 group exhibited a significant increase in the abundance of urine adenine and xanthine,  
281 accompanied by a significant decrease in urine allantoin compared to the placebo group.  
282 Regarding amino acid metabolism, the SB group showed a significant increase in urinary  
283 abundance of phenethylamine, aspartate, and proline. In contrast, urinary abundances of  
284 cystathionine and butyryl-L-carnitine and L-asparagine were decreased compared to the  
285 placebo group.

#### 286 **Impact of Maternal SB Supplementation on the Serum Metabolic Profiles of Newborn** 287 **Puppies**

288 The sPLSDA analyses performed on serum metabolites also achieved high accuracy in  
289 discriminating puppies of the SB group from those in the placebo group (AUROC $\geq$ 0.98 for  
290 serum metabolome). Samely, the two principal components explained 12% of the variance of  
291 serum metabolome and distinguished the groups with an error rate (BER) of 0.24 (Fig4b).  
292 From these components, VIP analyses identified 9 serum metabolites out of the initial 228  
293 (Loading score between 0.07 and 0.59), which differentiated the SB group from the placebo  
294 group (**Fig5b; Table 4**). Each of these metabolites had a VIP score greater than 1.0.

295 Significant differences were observed between the SB and placebo groups in the abundance of  
296 these discriminating serum metabolites. Notably, within the purine metabolism pathway, the  
297 SB group showed a significant decrease in serum xanthosine levels compared to the placebo  
298 group. Additionally, the SB group had decreased levels of serum gamma-aminobutyrate and  
299 3-methyl-L-histidine compared to the placebo group.

300

#### 301 **Correlations between urinary and serum metabolites and early growth of puppies.**

302 A network based on multiblock sPLS was used to investigate the correlation between urinary  
303 and serum metabolites (**Fig6**). Correlation analysis was restricted to metabolites that exhibited  
304 significant differences between SB and placebo groups. The network exhibited a considerable  
305 degree of connectivity with each node being connected to several nodes. In line with the  
306 correlation score, two distinct correlation network groups were identified. Serum metabolites,  
307 particularly gamma-aminobutyrate and hydroxyphenyl lactate (both with a connectivity of 4),  
308 along with xanthosine (with a connectivity of 3), emerged as the most central, while urinary  
309 metabolites were peripheral. Xanthine presented the strongest (negative) correlations, in  
310 particular with hydroxyphenyl lactate ( $r=-0.67$ ) and gamma-aminobutyrate ( $r=-0.71$ ),  
311 followed by cystathionine positively correlated with indole-3-carbaldehyde ( $r=0.60$ ) gamma-  
312 aminobutyrate ( $r=0.60$ ) and hydroxyphenyl lactate ( $r=0.53$ ).

313 According to Pearson's correlation analysis, the early growth rate was positively correlated  
314 with proline ( $r=0.45$ ) and mevalolactone ( $r=0.56$ ) in urine, while it was negatively correlated  
315 with alpha-methyl-dl-histidine dihydrochloride ( $r=-0.64$ ) in urine and 3-methyl-l-histidine (-  
316 0.46) in serum. (**Fig7a-b**).

#### 317 **4 Discussion:**

318 Although the interest in research on the neonatal period (i.e., the first three weeks of life) in  
319 the dog is continuously increasing, the canine neonatal morbidity and mortality rates remain  
320 relatively high (11%) (19). Several strategies are proposed to canine breeders and their vets  
321 (20) to improve the newborn's health, mostly focused on the management of whelping and  
322 suckling periods. The modulation of offspring vitality and vulnerability through the mother  
323 during the gestation and lactation period is increasingly documented in humans and pigs (7),  
324 but very few studies focused on canine developmental programming through maternal  
325 nutrition in dogs. Our study demonstrated for the first time the interest of maternal

326 supplementation with yeast during gestation and early lactation on the neonatal growth and  
327 metabolic profile, proposing a new strategy to breeders and veterinary practitioners.

328 The median growth rate during the first two days of life in the dog was estimated at about 3%  
329 in the study by Mila et al (2). However, in our study, the growth rate in the placebo group was  
330 found to be twice as high at 7%. Despite this notable result, the SB group presented even a 1.5  
331 higher growth rate over the first two days of life than puppies from the placebo group;  
332 however, there was a large variability among individuals (Fig2).

333 The early growth rate can be impacted by maternal milk availability, its quality, and/or the  
334 capacity of its ingestion by the offspring. In pigs, maternal supplementation with SB increased  
335 milk production by 9%, improving the daily weight gain of the piglets (up to +27 g/day)  
336 compared to the control group (21). Maternal milk production and its ingestion by puppies  
337 was not measured in our study but a higher energy content was recently demonstrated in the  
338 colostrum of bitches supplemented with SB over gestation (22).

339 In order to understand the mechanism explaining the increased growth in puppies born from  
340 SB mothers in our study, we decided to explore their urinary and serum metabolomes. Firstly,  
341 we investigated the different metabolic pathways found in the urine and serum of newborn  
342 puppies at two days of age. Our results showed that whether in urine or serum, the identified  
343 metabolites are mostly involved in nitrogen metabolism (amino acids and purines metabolic  
344 pathways, urea cycle, or even ammonia recycling cycle). Nitrogen metabolism is essential for  
345 cell division (23), which is important for the development of tissues and organs in puppies at  
346 this age. The need for nitrogen is the highest right after birth due to the intensive growth  
347 process and then decreases with age. In humans during the first 6 months, the daily protein  
348 requirement of infants is 2.2 g/kg, then this requirement gradually decreases to reach 0.75  
349 g/kg/day in adulthood (24). The same pattern could be observed in puppies, supporting the  
350 overrepresentation of the nitrogen metabolism in puppies at two days of life in our study.



351 Secondly, we aimed to explore the effect of maternal SB supplementation during gestation  
352 and lactation on the metabolome of puppies. We have demonstrated that maternal yeast  
353 supplementation modifies the metabolic profile of her offspring as observed in piglets (11). A  
354 total of 29 metabolites from serum or urine were impacted by the supplementation, 14 were  
355 amino acids, precursors or products of their degradation, nucleotides, and energy metabolites.  
356 The metabolic pathways in which these metabolites are involved as well as their relationship  
357 were compiled in Fig8. This figure shows that two major metabolic pathways are modified  
358 thanks to the addition of SB: amino acids and purine metabolic pathways. The main changes  
359 are observed in reactions involving amination and transamination, in particular those using the  
360 glutamine/glutamate system. The glutamine/glutamate system is essential for protein  
361 anabolism and nucleotide synthesis, because of its ability to donate or take up an amine group  
362 (25) - (26). The transformation of glutamine by deamination and the conversion of aspartate,  
363 histidine, and tyrosine by transamination are crucial processes for the biosynthesis of  
364 glutamate, an essential precursor of gamma-aminobutyrate and proline (27) (Fig9). One of the  
365 most significant findings in our study is the increased urinary levels of proline and reduced  
366 levels of gamma-aminobutyrate observed in the SB group.

367 Proline is a crucial component in collagen synthesis and osteoarticular system development  
368 (28). This finding is particularly significant in light of the observed increased growth rate of  
369 puppies from mothers supplemented by SB. Interestingly, we also found a significant  
370 correlation between urinary proline abundance and growth rate in puppies. A similar positive  
371 correlation between blood proline concentration at 12 months and growth between 6 and 12  
372 months was observed in babies, growth being evaluated using weight-for-height ratio and  
373 length velocity (29).

374 In parallel to this change in amino acid metabolism after SB maternal supplementation, we  
375 hypothesized a modification of the purine metabolism pathway in puppies at 2 days of age

376 (Fig 8). In the same way as proline, we have noted an increase in urine adenine abundance.  
377 This could reflect an increase of purine metabolism (30), aligning with the needs of rapid  
378 growth. Imaging studies must be conducted to confirm if these changes in nitrogen  
379 metabolism induced by maternal SB supplementation are linked to the bone development of  
380 puppies at this age.

381 This effect of maternal SB supplementation on changes in metabolites involved in the  
382 nitrogen metabolism pathway in the offspring can be elucidated through various mechanisms.  
383 First, maternal SB supplementation could modify the fetal environment during gestation with  
384 its effect observed postnatally. Indeed, maternal amino acids have the potential to modify fetal  
385 epigenetics, promoting growth and development (31). In a parallel study, we noticed that SB  
386 supplementation in late gestation led to a change in maternal metabolism, thus reinforcing the  
387 idea of fetal epigenetic modulation through the transplacental pathway (12). In the previous  
388 study mothers supplemented with SB had a higher blood level of metabolites involved in the  
389 metabolism of amino acids such as proline, tryptophan, beta-alanine, glycine, serine and  
390 threonine during gestation compared to placebo group. We cannot conclude at this stage  
391 whether the changes in the nitrogen metabolism of the mothers have an influence on the  
392 nitrogen metabolism of the young, or whether these two changes are the result of the same  
393 cause.

394 Secondly, nitrogen metabolism modification in puppies can also come from maternal milk  
395 quality. In the study of Garrigues et al. (22), no significant protein modification was observed  
396 in the colostrum after SB supplementation of the bitches. However, breast milk contains also  
397 a fraction of non-protein nitrogen in the form of free amino acids, peptides, which could be  
398 ingested and used by newborns, playing an important role in their growth and development  
399 (32). A detailed analysis of the colostrum should be carried out to reach a conclusion.

400 Yeasts can act in different ways on the body: indirectly (via an action on the intestinal  
401 microbiota) or directly (via a supply of nutrients). Of all these possibilities, the one that could  
402 explain at least part of our observations is its richness in vitamins B, including B6 (33,34).  
403 Vitamin B6 acts as an essential cofactor for more than 100 enzymes playing a crucial role in  
404 catalytic reactions, including decarboxylations and transaminations (35). Colostrum is rich in  
405 vitamins, whom vitamin B6 (36), which can also be transmitted transplacentally to the fetus.  
406 Intake of vitamin B6 during pregnancy or via colostrum may play an important role in  
407 modulating nitrogen metabolism in newborns (37) - (38). In future studies, it would be  
408 interesting to evaluate the effect of maternal SB supplementation on the abundance of vitamin  
409 B6 in the plasma and colostrum of female dogs and its link with offspring metabolome  
410 changes.

411 Gamma-aminobutyrate is crucial for brain development (39). During the first days of life it is  
412 known that the tissues of the body grow at different rates, with bone tissue development being  
413 prioritized over other tissues, including the nervous system (40). Overall, according to here  
414 presented results, we could hypothesize that yeast favors the use of glutamate towards the  
415 synthesis of proline and to the detriment of gamma-aminobutyrate, which is most probably  
416 expressed by accelerated osteoarticular growth of newborns in our study.

417 These findings highlight the potential benefits of maternal supplementation with SB in  
418 promoting offspring growth through metabolic nitrogen metabolism regulation. However,  
419 further research is needed to explore the long-term implications of these modifications on the  
420 overall health and development of the offspring.

421

#### 422 **Conflict of Interest**

423 AM<sup>2</sup> and EA were employees of Lallemand SAS, Blagnac, France.

424 The other authors declare that they have no competing interests.

425

#### 426 **Author contributions**

427 The authors contributed to the project as follows: Conceptualisation – HM, SC, QG and EA;

428 Sampling – QG; Data curation – IB, GQ, LS; Data Analysis – IB, AM<sup>3</sup>, HM; Original writing

429 – IB, AM<sup>3</sup>, HM, LS; Review and editing – IB, GQ, EA, AM<sup>2</sup>, LS, JCM, SC, AM<sup>3</sup>, HM.

430 All authors read and approved the final manuscript.

#### 431 **Funding**

432 This research project was financially supported by the Occitanie Pyrénées-Méditerranée

433 Region and Lallemand SAS (Blagnac, France).

#### 434 **Acknowledgments**

435 The authors would like to thank the breeder for enabling the feasibility of this project, and

436 likewise, all veterinary students participating in the project (Pauline David, Elodie Mansalier,

437 Fanny Mercier, Nicolette Rover) for their aid in conducting the samplings.

438

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557 **Table 1.** Nutrient composition of diets used in the study.

		<b>Feed</b>	
		<b>Diet 1</b>	<b>Diet 2</b>
<b>Nutrient content</b>	Moisture, %	5.7	6.3
<b>(on raw mater basis)</b>	Crude ash, %	8.7	9.3
	Crude protein, %	30.8	28.4
	Ether fat, %	15.1	16.8
	Crude fibre, %	2.7	<2
	Carbohydrates <sup>1</sup> , %	37.0	37.7
<b>Feed additives</b>	Vitamin E, mg/kg	124	114
	Vitamin A, IU/kg	7 670	7 330
	Vitamin D, IU/kg	1 080	1 000
	Metabolizable energy <sup>3</sup> , kcal/100g	385.8	408.6

558 <sup>1</sup>Carbohydrate was calculated from equation: %Dry Matter-(%Ether extract+%Crude Protein+%Crude  
559 Ash+%Crude fibre); <sup>3</sup>Metabolizable energy was calculated from equation (NRC, 2006 (12) and FEDIAF, 2019.

560 Diet 1 : from ovulation until 27 day of gestation

561 Diet 2 : from the 28 day of gestation until the end of the study

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568 **Table 2.** Characteristics of puppies selected and included for metabolome analysis

	<b>Placebo group</b>	<b>SB group</b>
<b>Puppies (n)</b>	41 (51%)	40 (49%)
<b>Sexe Males (n)</b>	27 (33%)	22 (27%)
<b>Females (n)</b>	14 (18%)	18 (22%)
<b>Litters (n)</b>	7	10
<b>Puppies fed by each lacting bitch (mean±SD)</b>	7±2	8±2
<b>Puppies included per litter (mean±SD)</b>	6±2	4±2
<b>Breeds (n)</b>	4	4
<b>Birth weight (mean±SD)</b>	395±75g	379±68g

569 Placebo group includes the following breeds : Golden Retriever (30 % of puppies), Australian  
570 shepherd (46 % of puppies), Labrador (10 % of puppies) and Bernese Mountain dog (14% of puppies. ;  
571 The Saccharomyces boulardii group includes the following breeds : Golden Retriever (52 % of  
572 puppies), Australian shepherd (35 % of puppies), Labrador (5 % of puppies) and Swiss White  
573 Shepherd (7% of puppies)

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583 **Table 3.** Uniquely distinctive urinary metabolites between *Saccharomyces boulardii* and  
 584 Placebo groups, based on Variable Importance in Projection (VIP).

<b>Urine metabolites</b>	<b>VIP</b>	<b>Values in SB</b> <b>compared to</b> <b>placebo</b>	<b>P-adj</b>
<b>Hydroxyphenyllactate</b>	7.12	↓	p=0.000
<b>Xantine</b>	4.15	↑	p=0.001
<b>Indoxyl sulfate</b>	3.92	↑	p=0.001
<b>Aspartate</b>	3.59	↑	p=0.002
<b>L-Asparagine</b>	3.54	↓	p=0.002
<b>Adenine</b>	3.22	↑	p=0.004
<b>4-Hydroxybenzoate</b>	3.19	↑	p=0.004
<b>Fucose</b>	3.03	↓	p=0.005
<b>3-Hydroxybutanoate</b>	2.93	↓	p=0.005
<b>Butyryl-l-carnitine</b>	2.78	↓	p=0.006
<b>Allantoin</b>	2.34	↓	p=0.010
<b>Glucosamine 6-Phosphate</b>	2.12	↑	p=0.013
<b>Alpha-Hydroxyisobutyrate</b>	2.09	↓	p=0.014
<b>Mandelate</b>	2.03	↑	p=0.014
<b>Phenethylamine</b>	1.89	↑	p=0.017
<b>Proline</b>	1.85	↑	p=0.017
<b>2-Hydroxy-4-(Methylthio)butanoate</b>	1.84	↓	p=0.018
<b>Cystathionine</b>	1.75	↓	p=0.019

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<b>Mevalolactone</b>	1.71	↑	p=0.020
<b>Alpha-methyl -DL-histidine dihydrochloride</b>	1.68	↓	p=0.021

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585 Arrow (↓) means a lower abundance of metabolites and the arrow (↑) a higher relative  
586 abundance in *Saccharomyces boulardii* compared to the placebo group. P-adj was obtained  
587 using Anova and post-hoc Bonferoni test.

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603 **Table 4.** Uniquely distinctive serum metabolites between *Saccharomyces boulardii* and  
 604 Placebo groups, based on Variable Importance in Projection (VIP).

Serum metabolites	VIP	Values in SB compared to placebo	P-adj
Hydroxyphenyllactate	8.91	↓	p=0.000
Gamma-aminobutyrate	8.19	↓	p=0.000
Mono-Methyl adipate	5.38	↓	p=0.001
Xanthosine	4.70	↓	p=0.001
Indole-3-carbaldehyde	3.68	↓	p=0.002
6-Hydroxymelatonin	2.75	↓	p=0.004
3,5-Dihydroxybenzoic acid	2.49	↓	p=0.004
N-methyl-2-pyridone-5-carboxamide	1.14	↓	p=0.007
3-Methyl-L-histidine	1.02	↓	p=0.008

605 Arrow (↓) means a lower relative abundance of metabolites in *Saccharomyces boulardii*  
 606 compared to the placebo group. p-adj was obtained using Anova and post-hoc Bonferoni test.

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## Figure Legend

612 **Figure 1:** Experimental design of the study.

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614 **Figure 2:** Box and whisker plot of early growth rate in puppies from mothers supplemented  
615 with *Saccharomyces boulardii* (n=40; in yellow) during gestation and lactation and puppies  
616 from mothers supplemented with placebo (n=41; in blue). Early growth rate (%) = (weight at  
617 2 days - weight at birth) / weight at birth \* 100. An ANOVA was carried out. One asterisk (\*)  
618 indicates p value smaller than 0.05.

619

620 **Figure 3:** Summary of the enrichment analysis with the top 25 metabolic pathways obtained  
621 on 81 puppies at two days of age by Metaboanalyst 5.0. The figure (A) represents 206 urinary  
622 metabolites and figure (B) 228 serum metabolites. The enrichment rate was calculated by  
623 dividing the number of metabolites found by the number of metabolites expected for each  
624 pathway. The P value (P-adj) was calculated by hypergeometric test then a post-hoc Holm test  
625 was performed. In red are the metabolic pathways found significantly enriched after p-value  
626 correction. One asterisk (\*) indicates p value smaller than 0.05, two asterisks (\*\*) indicate p  
627 value smaller than 0.01.

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629 **Figure 4:** Sparse partial least-squares-discriminant analysis (sPLS-DA) of urine (model with  
630 2 principal components and KeepX (30, 20)) (A) and serum (model with 2 principal  
631 components and KeepX (10, 5)) (B) metabolome in newborn puppies at two days of age  
632 coming from mothers supplemented with *Saccharomyces boulardii* (40 puppies; yellow  
633 triangles) or placebo (41 puppies; blue dots).

634

635 **Figure 5:** PlotLoadings on urine (model with 2 principal components and KeepX (30, 20))  
636 (A) and serum (model with 2 principal components and KeepX (10, 5)) (B) metabolomes and  
637 their scores responsible for the discrimination between puppies from mothers supplemented  
638 with *Saccharomyces boulardii* (40 puppies; yellow bars) and those from the placebo group  
639 (41 puppies; blue bars).

640

641 **Figure 6:** Correlation matrix of urine and serum metabolites selected by multiblock sPLS-  
642 DA. Network map of urine (in green) and serum (in grey) metabolites highly correlated  
643 ( $\geq 0.5$ ). The number indicated on the line between nodules represents the value of the  
644 coefficient of correlation between two metabolites (only coefficients greater than 0.5 were  
645 retained in the network analysis) (n=81).

646

647 **Figure 7:** ScatterPlot of Pearson correlation between (A) Proline (B) Mevalolactone (C)  
648 alpha-methyl-dl-histidine dihydrochloride (D) 3-methyl-l-histidine relative abundances at 2  
649 days of age and early growth rate (%) (n=81).

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651 **Figure 8:** Diagram of the central pathways impacted in the serum and urine of puppies (at 2  
652 days) by maternal supplementation with *Saccharomyces boulardii*. The arrow ( $\downarrow$ ) means a  
653 lower abundance of metabolites and the arrow ( $\uparrow$ ) a higher relative abundance in  
654 *Saccharomyces boulardii* compared to the placebo group. Urine metabolites are written in  
655 red, urine metabolites in green and metabolites found modified in both in orange  
656 (Hydroxyphenyl lactate).



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658 **Figure 9:** Schematic overview of the metabolic pathway influenced by maternal  
659 *Saccharomyces boulardii* supplementation during gestation and lactation that could explain  
660 enhanced growth in offspring. The metabolites involved in the synthesis of glutamate by  
661 transaminations and deaminations are represented in gray and the glutamate-proline-growth  
662 axis in the blue rectangle.

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