Effect of an Immunomodulatory Feed Additive in Mitigating the Stress Responses in Lactating Dairy Cows to a High Concentrate Diet Challenge

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Article

Dairy cows are often exposed to stressors during the lactation cycle. Nutritional stressors could be caused by rich-grain diet, leading to ruminal pH reduction and subsequent systemic inflammation. This metabolic pathology impacts animal health and productivity. Feed additives could provide beneficial effects on innate immune function in dairy cows, especially during stressing periods. The goal of this study was to determine the effect of OmniGen-AF on measures of immunity, inflammation, and liver function in lactating dairy cows fed a high-starch, low-fiber diet. Changes in rumination, pH, and volatile fatty acids were recorded. Treated cows resulted in better rumen volatile fatty acids profile and also showed shifts in hematological parameters compatible with a prompter regeneration of red blood cells, greater proportion of neutrophils, lower levels on GGT, PON, and BHB. These results show evidence of the nutritional stress induced by feeding a high-starch, low-fiber diet, and suggest that the fed additive tested modulates some of the metabolic and immunological responses to sub-acute ruminal acidosis.

Abstract: Dairy cows are often exposed to multiple stressors in a lactation cycle, with sub-acute ruminal acidosis (SARA) a frequent example of nutritional stress. SARA affects ruminal and intestinal equilibrium resulting in dysbiosis with localized and systemic inflammation impacting animal health and productivity. OmniGen-AF (OMN, Phibro Animal Health Corporation, Teaneck, NJ, USA) is a feed product recognized for modulating innate immune function, especially during periods of stress. The objective of this study was to determine the effects of OMN in lactating dairy cows fed a high-starch, low-fiber diet. Twenty-four blocked cows were assigned to control or treatment (55 g/d). After the additive adaptation (49 d) cows were fed the challenge diet (28 d). Milk, rumination and pH were continuously recorded; components, rumen fluid, and blood were taken in multiple time-point and analyzed. Results showed that the challenge decreased the rumination, shifted ruminal fluid composition, decreased milk production and the components, and slightly increased the time below pH 5.5, with no differences between groups. The treatment produced greater rumen butyrate and lower lactate, prompter regeneration of red blood cells, increase of neutrophils, lower paraoxonase, gamma-glutamyl-transferase, and β-hydroxybutyrate, with no differences on other tested inflammatory markers. Results show that OMN helps modulating some of the metabolic and immunological responses to SARA.

Keywords: dairy cows; stressors; immune modulation; rich grain TMR; SARA

1. Introduction

Dairy cows are exposed to multiple stressors during their life [1], especially during the periparturient period [2–4] and lactation [5]. Examples of stressors experienced by the high producing dairy cow include overcrowding and group changes, high environmental...
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温度，和喂养错误。这些应激源对生产力和繁殖性能产生负面影响[1]。此外，压力可能会影响代谢和免疫功能[6,7]。严重的或慢性压力会破坏稳态，改变生物功能并使动物易患多种疾病[8]。

消化紊乱由高粮饮食和缺乏从牧草的有效纤维导致的微纤维酸中毒（SARA）[9–11]，对性能产生负面影响，导致农民遭受重大经济损失[12]。

受损的瘤胃上皮不能阻止微生物和粘膜毒素同时进入全身循环[13,14]。SARA可能导致选择性瘤胃上皮屏障功能失效，从而允许粘膜免疫原物穿透血管和淋巴系统[15]。具体来说，各种粘膜毒素如内毒素和生物胺似乎通过改变上皮紧密连接功能来干扰上皮约束功能，从而破坏上皮细胞的完整性并使它们穿透改变细胞途径[14]。有越来越多的证据表明SARA导致对LPS的增强生长和释放大量LPS[16,17]。肠道LPS是一种潜在的促炎分子，已被广泛研究与免疫系统相关[16,18]。一旦肠道LPS进入血液循环，就会触发炎性级联反应，表现为血液急性期蛋白（APP）水平的适度升高，LPS结合蛋白、白介素和血清淀粉样蛋白A[19]是乳牛中LPS转移的主要标志。

未受控制的急性或慢性炎性反应对LPS可能不仅会对组织造成损害，而且会以能量消耗的形式损失，改变营养素优先级，影响能量平衡，降低动物生长和生产力[1]。第一个免疫屏障是由胃肠道上皮细胞（14）和第二免疫系统[21]组成的，它在调节炎性反应中起着关键作用。一个有效的免疫系统提供的恢复力对于限制疾病的使用抗菌药物在食品生产动物[22]，符合欧盟立法的建议[23]。

不同营养素通过调节免疫系统响应而被证明对肠道具有益处，如OmniGen-AF（OMN；Phibro Animal Health Corporation，Teaneck，NJ，USA）。这种混合物由几种成分组成，其中一种是酵母细胞壁材料。酵母细胞壁含有能够刺激或激活免疫反应的几类成分。实际显示，OMN对受损奶牛具有支持免疫功能的作用，尽管其具体机制尚未完全阐明。Brandao et al. [26]通过喂OMN证明了LPS对泌乳牛的影响，发现灌胃LPS组和对照组血浆结合蛋白水平更高。Ortiz-Marty et al. [27]和Mezzetti et al. [29]分别在受应激牛和受应激牛中观测到OMN对免疫功能和代谢的影响。Mammi et al. [30]发现OMN通过促进干乳期泌乳牛的上皮细胞再生和产奶量，降低应激水平和减少脂肪动员。

尽管OMN对动物具有积极影响，但到目前为止，尚未有研究分析OMN对乳牛的影响。我们的研究目的是观察OMN对乳牛的营养和免疫系统的影响。
2. Materials and Methods

2.1. Experimental Design, Housing and Diets

This study was conducted at the University of Bologna dairy research farm. The experimental design is outlined in Figure 1.

![Experimental design diagram]

According to the capability of the University of Bologna dairy and research barn, twenty-four lactating multiparous Italian Holstein-Friesian cows were distributed in two treatment groups balanced by parity, DIM, milk yield and components (Table 1), and the groups were randomly assigned to treatment. Cows in the OmniGen AF treatment (OMN) were fed 55 g/d of OmniGen AF (Phibro Animal Health Corporation, Teaneck, NJ, USA) and cows in the control treatment (CON) received no supplement.

Table 1. Characteristics of the cows assigned to CON and OMN treatment groups at the beginning of the experiment (Covariate) and before the challenge period (T0) (mean ± SD).

| Cows’ Characteristics | Beginning Experiment | Beginning Challenge |
|-----------------------|----------------------|---------------------|
|                       | CON                  | OMN ¹                | CON                  | OMN ¹                |
| Age, y                | 2.65 ± 0.66          | 2.62 ± 0.52          | 2.78 ± 0.79          | 2.75 ± 0.65          |
| Lactation, n          | 1.64 ± 0.65          | 1.67 ± 0.65          | 1.64 ± 0.65          | 1.67 ± 0.65          |
| DIM ²                 | 51.5 ± 28.9          | 52.3 ± 30.5          | 100.5 ± 28.9         | 101.3 ± 30.5         |
| BW ³, kg              | 630 ± 58.3           | 633 ± 64.1           | 644 ± 73.2           | 634 ± 70.4           |
| Milk yield, kg/d      | 40.0 ± 7.74          | 40.4 ± 7.78          | 41.77 ± 6.85         | 43.0 ± 9.06          |
| Fat, %                | 3.91 ± 0.74          | 3.89 ± 0.54          | 3.59 ± 0.18          | 3.53 ± 0.15          |
| Total protein, %      | 3.30 ± 0.24          | 3.28 ± 0.23          | 3.24 ± 0.18          | 3.26 ± 0.20          |
| Lactose, %            | 4.95 ± 0.17          | 5.02 ± 0.10          | 4.96 ± 0.17          | 5.03 ± 0.08          |
| MUN ⁴, mg/dL          | 8.27 ± 2.90          | 8.35 ± 3.26          | 9.79 ± 1.99          | 9.15 ± 1.99          |
| SCC ⁵, log₁₀/mL       | 1.45 ± 0.32          | 1.63 ± 0.42          | 1.78 ± 0.29          | 2.20 ± 0.32          |

¹ OmniGen-AF supplemented at 55 g/d. ² Days in milk. ³ Body weight. ⁴ Milk urea nitrogen. ⁵ Somatic cell count.

The experiment consisted of three phases listed as covariate, pre-challenge, and challenge phase. In the pre-trial and pre-challenge phase cows were housed in free-stall pens (OMN or CON) and group fed a TMR (Table 2); during the covariate phase, cows were sampled and data recorded in order to balance groups. After that, OMN was supplied since the beginning of the trial (pre and challenge periods) and mixed into the TMR of the OMN group. The ration was formulated to mimic a standard Parmigiano Reggiano ration, based on dry forages and approved concentrates, and it was balanced using a software based on the CNCPS model (DinaMilk5; Fabermatica, Ostiano, Italy).
Table 2. Composition of experimental diets.

| Diets’ Composition | Pre-Challenge Diet | SARA Challenge Diet |
|---------------------|---------------------|---------------------|
| Ingredients 1, kg/cow/d, as fed |                     |                     |
| Grass hay, finely chopped | 9.5 | 6.0 |
| Wheat straw, finely chopped | 1.0 | 1.0 |
| Corn flakes | 6.0 | 13.0 |
| Concentrate 2 | 7.5 | 8.0 |
| Liquid feed 3 | 1.0 | 1.0 |
| Grass hay, long | Ad libitum | - |
| Forage:Concentrate  | 45.4:54.6 | 24.8:75.2 |

| Chemical composition, %DM |                    |                     |
|---------------------------|-------------------|-------------------|
| DM                        | 87.22 ± 3.00 | 88.11 ± 0.74 |
| Ash                       | 7.50 ± 1.28 | 6.25 ± 0.43 |
| Ether extract              | 3.21 ± 0.47 | 2.78 ± 0.65 |
| aNDFom 4                  | 35.94 ± 4.16 | 29.00 ± 2.80 |
| ADF 5                     | 24.55 ± 2.56 | 18.86 ± 1.64 |
| ADL 6                     | 5.27 ± 1.09 | 4.65 ± 0.19 |
| uNDF240h 7                | 9.93 ± 3.32 | 8.05 ± 0.96 |
| Starch                    | 22.95 ± 2.62 | 33.62 ± 2.45 |
| pNDF<sub>1.18mm</sub> 8   | 17.56 ± 1.35 | 13.80 ± 0.98 |

1 Additionally, 55 g/d of OmniGen-AF was added to the diet of cows in the OMN treatment. 2 Concentrate: 29.6% wheat bran, 29.4% sorghum grain, 21.6% canola meal, 14.7% flaked fullfat soybean, 2.2% calcium carbonate, 1% sodium chloride, 0.4% magnesium oxide, 0.9% sodium bentonite, and 0.3% vitamin and mineral premix (providing 40,000 IU/kg of vitamin A, 4000 IU/kg of vitamin D3, 30 mg/kg of vitamin E 92% α-tocopherol, 5 mg/kg of vitamin B1, 3 mg/kg of vitamin B2, 1.5 mg/kg of vitamin B6, 0.06 mg/kg of vitamin B12, 5 mg/kg of vitamin K, 5 mg/kg of vitamin H1 (para-aminobenzoic acid), 150 mg/kg of vitamin PP (niacin), 50 mg/kg of choline chloride, 100 mg/kg of Fe, 1 mg/kg of Co, 5 mg/kg of I, 120 mg/kg of Mn, 10 mg/kg of Cu, and 130 mg/kg of Zn). 3 Cane and beet pulp molasses blend fully characterized for composition, sugars and digestibility [31,32]. 4 Amylase- and sodium sulfite-treated NDF with ash correction. 5 Unavailable NDF estimated via 240 h in vitro fermentation. 6 Physically effective NDF (aNDFom*pef), calculated using the Ro-Tap system.

Diets were mixed and fed once daily at 0900 h and offered ad libitum (approx. *1.1 expected intake). Additionally, grass hay was available ad libitum during the covariate and pre-challenge phase and not available during the SARA challenge phase. Cows were milked twice daily in a 2 × 5 herringbone parlor. Milk yield and BW were recorded at every milking (Kibbutz Afikim, Israel). The covariate phase lasted 14 d. The pre-challenge phase lasted for 49 d, which is the time that previous research [33] has shown is needed to demonstrate differences in immune function to feeding OMN; in Wu et al. [15], this time was effective in increasing the gene neutrophil expression of the adhesion molecule SELL and the cytokine CXCL8. The pre-challenge phase was followed by a 28 d SARA challenge phase. Cows were exposed to the challenge in three consecutive time blocks of eight cows each (four cows per treatment). During the challenge, cows were housed in tie-stalls bedded with sawdust, and they had free access to individual feed bunks and water dispensers. The diet consisted of a TMR made with the same ingredients fed during the pre-challenge phase, but the inclusion rates of some ingredients were modified to increase starch, while decreasing aNDFom, pNDF, and uNDF240 (Table 2). The starch raised from 22.95 to 33.62% DM thanks to the increase in corn flakes content (from 6 to 13 kg/cow/day as fed). Fibrous fractions decreased from 35.94, 17.56, and 9.93 to 29.00, 13.80, and 8.05% DM of aNDFom, pNDF, and uNDF240, respectively, thanks to the reduction of grass hay (from 9.5 to 6 kg/cow/day as fed). OMN (55 g/d) was top dressed to the corresponding cows immediately after the ration delivery. DMI was measured by weighing feed offered and orts, and water intake was automatically recorded by flow meters. For milking, cows were moved to the same milking parlor previously described; each milking lasted for approx. 45 min.
2.2. Feed and Milk Sampling

Samples of feedstuffs, diets, and orts were collected twice weekly throughout the experiment (Mondays and Thursdays), dried in a forced-air oven at 65 °C. Samples were firstly checked by NIR techniques (TANGO FT-NIR Spectrometer, Bruker Optics GmbH, Ettlingen, Germany, [34]) and analyzed for DM, CP, aNDFom, ADF, peNDF, and starch as previously described [35,36]. In vitro aNDFom digestibility (24 h and 240 h) was determined in buffer media containing ruminal fluid [37]. Digestibility was performed on forages and TMR according to the procedure described by Palmonari et al. [38]. In vitro aNDFom digestibility at 240 h was performed using the Tilley and Terry modified technique [39]. Milk samples from two consecutive milkings from each cow were collected on d −14 and −3 prior to start of the experiment, on d 0, 7, 14, 21, and 28 of the SARA challenge (Figure 1) and analyzed by a certified laboratory (Associazione Provinciale Allevatori Bologna) for fat, total protein, lactose, and SCC. ECM was then calculated.

2.3. Rumen Sampling and Measurements

Cows were monitored for reticular pH with an indwelling wireless transmitting unit (SmaXtec Animal Care Sales GmbH, Graz, Austria), a system previously validated in rumen-cannulated dairy cows [40]. These devices (3.5 cm i.d., 12 cm long, and weighing 210 g) were calibrated following the manufacturer instructions and manually inserted into the rumen via the esophagus one week before the start of the pre-challenge period. Previous research has showed that these devices tend to sit in the ventral reticulum area [40]. pH and temperature were recorded every 10 min and data transmitted real-time to a base station using the ISM band (433 MHz). Data were then collected using an analog-to-digital converter and stored in an external memory chip. Reticular pH data were aggregated as daily means, and a pH threshold of 5.5 was used to calculate time and dispersion below that threshold [41,42]. Rumen fluid was collected via esophageal tube at 0845 h on d 0, 14, and 28 of the SARA challenge. The first 500 mL of rumen fluid collected were discarded before taking samples. Rumen fluid was analyzed for volatile fatty acid (VFA) concentrations by gas chromatography [43]; ammonia was assessed using a commercial kit (urea/BUN—color, BioSystems S.A. Barcelona, Spain); and L-lactic and D-lactic acids were determined with a commercial kit (K-DLATE, Megazyme Co., Wicklow, Ireland). Commercial standards were used for the calibration of the kits. Rumination time was continuously monitored during the entire experiment using the Hi-Tag rumination monitoring system (SCR Engineers Ltd., Netanya, Israel).

2.4. Blood Sampling

Blood was collected from the coccygeal vein at 0845 h on d −14, −7, and −3 prior the start of the experiment, on d 0, 1, 2, 3, 7, 14, 21, and 28 of the SARA challenge. Samples were taken into vacuum tubes containing either EDTA (for complete blood counts), clot-activator (silicate, for serum assays), or Li-heparin (for plasma assays) (Vacutest, Kima, Padova, Italy). EDTA tubes were kept at 4 °C after collection and blood counts were performed within 4 h. Clot-activator and Li-heparin tubes were centrifuged at 2000 × g for 20 min and 3000 × g for 10 min to obtain serum and plasma, respectively (Centrifugeetté 4203, ALC International Srl, Cologno Monzese, Italy). Serum and plasma samples were stored at −80 °C until analysis. Complete blood counts (CBC) were performed at the Clinical Pathology Laboratory University of Bologna Veterinary Hospital using an automated hematology system (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) according to previous studies [44,45]. The CBC listed several parameters: hemoglobin (HG), haematocrit (HTC), erythrocytes (ERT), reticulocytes (RET), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), red cell distribution width (RDW), leukocytes (LEU), lymphocytes (LYM), neutrophils (NEU), and eosinophils (EOS). Plasma samples were analyzed at the Università Cattolica del Sacro Cuore (Piacenza, Italy): a clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington, MA) was used to determine the concentration of beta hydroxybutyrate (BHB), gamma-glutamyl transferase...
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(GGT), haptoglobin (HAPT), ceruloplasmin (CRP), albumin (ALB), and cholesterol (CHOL) following Calamari et al. [46]. Reactive oxygen metabolites (ROM) and ferric reducing antioxidant power (FRAP) were determined according to Jacometo et al. [47]; and paraoxonase (PON) was determined according to Bionaz et al. [48]. Calibrations were performed through commercial standards for CRP, ALB, BHB, ROM and FRAP, and through internal standards for the rest. Four different quality controls were used to test the repeatability and precision for each parameter. Furthermore, plasma samples were used to determine IL-1ß and serum amyloid A (SAA) using a multi-detection microplate reader (BioTek Synergy 2, Winooski, VT, USA) and commercial ELISA kits specific for the bovine species (Pierce, Thermo Scientific, Rockford, IL, USA) for IL-1ß, or TP-802 (Tridelta D.L., Ireland) for SAA. Serum samples were analyzed at the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna (Brescia, Italy). Commercial kits were used on these samples to measure IL-6 (DuoSet ELISA, cat. no. DY8190, R & D Systems, Minneapolis, MN, USA) and γIFN (BovigamTM TB Kit, cat. no. 63,320, Thermo Scientific Prionics AG, Schlieren, Zurich, Switzerland). In both cases, the calibrations were performed through standard solutions according to the manufacturer’s instructions. Full methodologies including the coefficient of variation are reported in Calamari et al. [46].

2.5. Data Analysis

Data were analyzed using the software JMP v15.1 (SAS Institute Inc., Cary, NC, USA). Linear mixed effects models were used. Model main fixed effects were treatment, diet and interaction. Data related to reticular pH and temperature, rumination time, milk yield, and BW were analyzed considering only the last 28 d of the pre-challenge phase in order to balance the model. Data related to rumen fluid parameters, milk components, and blood parameters were analyzed as repeated measurement (first-order autoregressive AR1) considering the sampling day as the time effect, and using the baseline established prior to the start of the experiment (d −14, −7, and −3) as covariate depending on scheduled sampled (Figure 1). Data of DMI and WI were recorded only during the tie stall period; thus, only the treatment effect was considered. A preliminary analysis including blocks as fixed effect was conducted and resulted in no significance; thus, this factor was included as nested effect into the random factors. Each cow within block and treatment was considered as experimental unit and used as random variable for all analyses. Normal distribution of the data was checked for the residuals resulted from an initial mixed model, and normalized, when necessary, by BoxCox transformation. Means are reported as least square mean and pairwise multiple comparisons were performed using Student t-test as post hoc test when a p-value ≤ 0.10 was detected. A p-value ≤ 0.10 was considered a tendency; a p-value ≤ 0.05 was considered statistically significant; and a p-value ≤ 0.01 was considered highly significant.

3. Results

3.1. Intake and Milk Production

As formulated, the diet fed during the SARA challenge contained, vs. the standard diet, considerably more starch (34% vs. 23%), and less aNDFom (29% vs. 36%), peNDF (14% vs. 18%), and uNDF240 (8.1% vs. 9.9%) (Table 2). The results of intakes, BW and production are reported in Table 3. No differences were detected between the treatment groups during the SARA challenge neither on DMI (25.8 vs. 25.7 kg/d in CON and OMN, respectively, p = 0.99) nor water intake (144 vs. 147 L/d in CON and OMN, respectively, p = 0.70). Milk yield was similar between treatment groups (41.7 and 42.0 kg/d for CON and OMN, respectively, p = 0.94) but decreased equally in both groups in the SARA challenge period (−2 kg/d of milk, p < 0.01). A drop in milk fat and total protein yields were recorded during the SARA challenge (−287 and −93.4 g of fat and total protein yields, respectively, p < 0.01) with no differences between treatments (1310 vs. 1223 and 1332 vs. 1367 g of fat and total protein yields in CON and OMN, respectively, p = 0.35 and 0.73). Somatic cell count and MUN did not vary between treatments but the somatic cell count
increased (+0.59 log10 cells × 1000/mL, p < 0.01) and MUN decreased (−3.9 mg/dL, p < 0.01) when the SARA challenge diet was fed. BW did not change throughout the experiment or between treatment groups (644 and 628 kg, in CON and OMN, respectively, p = 0.61).

Table 3. Effect of the dietary challenge on intakes, rumination time, reticular pH and temperature, milk yield and components in CON 1 or OMN 2 cows when fed the pre-challenge or SARA challenge diets.

| Item                              | Pre-Challenge Diet | SARA Challenge Diet | SEM  | p-Values |
|-----------------------------------|-------------------|---------------------|------|----------|
|                                  | CON 1             | OMN 2               | CON  | OMN      |
| DMI, kg/d                         | -                 | -                   | 25.8 | 25.7     | 1.25     | 0.99  | -     | -     |
| WI, L/d                           | -                 | -                   | 144  | 147      | 10.4     | 0.70  | -     | -     |
| BW, kg                            | 646               | 617                 | 641  | 639      | 17.2     | 0.61  | 0.31  | 0.11  |
| Rumen time, min/d                 | 502               | 488                 | 434  | 405      | 13.8     | 0.23  | <0.01 | 0.27  |
| Reticular pH                      | 6.05              | 6.05                | 57.2 | 57.1     | 12.5     | 0.65  | <0.01 | 0.64  |
| Reticular pH < 5.5, min/d         | 35.6              | 40.2                | 37.9 | 38.9     | 0.11     | 0.52  | <0.01 | 0.91  |
| Reticular temperature, °C         | 37.8              | 38.8                | 37.9 | 38.9     | 0.1       | 0.72  | <0.01 | 0.11  |
| Milk yield, kg/d                  | 42.3              | 43.4                | 41.1 | 40.6     | 3.2       | 0.94  | <0.01 | 0.12  |
| Fat yield, g/d                    | 1455              | 1365                | 1165 | 1081     | 9.4       | 0.35  | <0.01 | 0.90  |
| Protein yield 3, g/d              | 1351              | 1441                | 1312 | 1293     | 10.1      | 0.73  | <0.01 | 0.11  |
| ECM, kg/d                         | 38.2              | 39.5                | 34.3 | 34.9     | 2.5       | 0.72  | <0.01 | 0.35  |
| MUN, mg/dL                        | 10.13             | 9.16                | 6.04 | 5.46     | 0.72      | 0.23  | <0.01 | 0.28  |
| SCC, log10 cells/mL               | 1.78              | 2.20                | 2.45 | 2.71     | 0.29      | 0.52  | <0.01 | 0.34  |

Table 4. Evolution of rumen VFAs (acetic, propionic, isobutyric, nor-butyric) and lactic acid concentration in CON 1 or OMN 2 cows when fed the pre-challenge or SARA challenge diets.

| Item            | D 0  | D 14 | D 28 | SEM  | p-Values |
|-----------------|------|------|------|------|----------|
|                 | CON 1| OMN 2| CON 1| OMN 1| TRT      | Time | TRT x T |
| Total VFA, mmol/L | 89.4 | 89.3 | 110.2 | 103.0 | 103.4 | 105.5 | 5.16 | 0.71 | <0.01 | 0.62 |
| Acetic, %mmol    | 56.6 | 56.0 | 52.5 | 55.5 | 50.5 | 53.5 | 1.34 | 0.13 | 0.03 | 0.30 |
| Propionic, %mmol | 27.8 | 28.4 | 36.0 a | 29.7 b | 37.1 | 32.0 | 1.76 | 0.04 | <0.01 | 0.08 |
| Iso-butyric, %mmol | 0.50 | 0.36 | 0.26 b | 0.47 A | 0.42 | 0.47 | 0.06 | 0.12 | 0.69 | <0.01 |
| Nor-butyric, %mmol | 13.1 | 12.3 | 9.8 b | 11.5 a | 10.6 | 11.2 | 0.55 | 0.33 | <0.01 | 0.08 |
| L-lactic, mg/dL  | 151  | 119  | 187  | 155  | 154  | 121  | 25.1 | 0.09 | 0.35 | 0.99 |
| D-lactic, mg/dL  | 172  | 125  | 241  | 147  | 174  | 121  | 37.2 | 0.02 | 0.41 | 0.81 |

1 CON is control cows. 2 OMN is cows receiving OmniGen-AF (55g/d). 3 Total protein.

3.2. Ruminal Parameters

Results of rumination time and reticular pH and temperature are reported in Table 3. Rumination time decreased during the SARA challenge (−76 min/d, p < 0.01) in both treatment groups with no differences between treatments. No significant differences were observed in daily average reticular pH neither between diets nor treatment groups. However, time below pH 5.5 increased in both treatment groups going from the pre- to the challenge diet (+19.26 min/d, p < 0.01). Even if very slightly, reticular temperature increased during the SARA challenge diet (+0.01 °C, p < 0.01). Additionally, both L- and D-lactic acid were lower in OMN animals since the beginning of the SARA challenge period (−32 mg/dL and −65 mg/dL, p = 0.09 and p = 0.02, respectively).

Table 3. Effect of the dietary challenge on intakes, rumination time, reticular pH and temperature, milk yield and components in CON 1 or OMN 2 cows when fed the pre-challenge or SARA challenge diets.
3.3. Hematological, Metabolic, and Immunological Parameters

The SARA challenge had strong effects on metabolic, health, and immunological parameters in all cows (Figures 2–6). Most of RBC, WBC, inflammatory, metabolic, and oxidative status parameters were affected by the SARA challenge. In particular, the levels of HG, HTC, ERT, MCHC, LEU, NEU, CHOL, ALB, BHB, and FRAP declined progressively along the SARA challenge period, while MCV, RWI, EOS, CER, SAA, ILs, GGT, PON, and ROM increased. Among CBC parameters, OMN increased RET (0.061 vs. 0.045%, in OMN and CON, respectively, p = 0.09, Figure 2d) and NEU (44.88 vs. 40.26%, in OMN and CON, p = 0.02, Figure 3b), while LYM resulted decreased (46.32 vs. 50.58%, in OMN and CON, p = 0.02, Figure 3c). A treatment effect was also shown on inflammatory and metabolic parameters: CORT increased (11,537 vs. 9319 pg/mL, in OMN and CON, respectively, p = 0.01, Figure 6a) and GGT (−2.16, U/L, in OMN, p = 0.01, Figure 5d) resulted lower in OMN cows compared to CON cows. Finally, BHB, compared to CON cows, was lower in OMN cows during the last days of the challenge (0.49 vs. 0.40 mmol/L d21 and 0.48 vs. 0.41 mmol/L d 28, in CON and OMN, respectively, p = 0.05, Figure 5c).

![Figure 2. Cont.](image-url)
Figure 2. Effect of the dietary challenge on red blood cells parameters: hemoglobin (HG, g%, (a)), hematocrit (HTC, %, (b)), erythrocytes (ERT, n°/µm (c)), reticulocytes (RET, %, (d)), mean corpuscular volume (MCV, fL, (e)), mean corpuscular hemoglobin (MCHC, g%, (f)) and red cell distribution width (RWI, %, (g)) in cows in the CON ¹ (○) or OMN ² (+) treatments during the SARA challenge. ¹ Control group. ² Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect.

Figure 3. Effect of the dietary challenge on white blood cells parameters: leukocytes (LEU, n°/µmc, (a)) neutrophils (NEU, %, (b)), lymphocytes (LYM, %, (c)) and eosinophils (EOS, %, (d)) in cows in the CON ¹ (○) or OMN ² (+) treatments during the SARA challenge. ¹ Control group. ² Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect.
Figure 4. Effect of the dietary challenge on inflammatory markers: cortisol (CORT, pg/mL, (a)), ceruloplasmin (CER, µmol/L, (b)), Serum Amyloid A (SAA, µg/mL, (c)), Interleukin 1 beta (IL1β, pg/mL, (d)) and Interleukin 6 (IL6, pg/mL, (e)) in cows in the CON 1 (○) or OMN 2 (+) treatments during the SARA challenge. 1 Control group. 2 Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect.

Figure 5. Cont.
The present trial deals with the mitigation of the stress responses to a high concentrate diet challenge in lactating dairy cows by the supplementation of an immunomodulatory feed additive. 1 Control group. 2 Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect. ** p-value ≤ 0.05 between TRT within time point.

Figure 5. Effect of the dietary challenge on metabolic status markers: albumins (ALB, g/L, (a)), cholesterol (CHOL, mmol/L, (b)), beta hydroxybutyrate (BHB, mmol/L, (c)) and gamma glutamyl transferase (GGT, U/L, (d)) in cows in the CON 1 (○) or OMN 2 (†) treatments during the SARA challenge. 1 Control group. 2 Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect.

Figure 6. Effect of the dietary challenge on oxidative status markers: paraoxonase (PON, U/mL, (a)), Ferric reducing antioxidant power (FRAP, µmol/L, (b)), and reactive oxygen metabolites (ROM, H2O2/100mL, (c)) in cows in the CON 1 (○) or OMN 2 (†) treatments during the SARA challenge. 1 Control group. 2 Treated group (cows receiving OmniGen-AF, 55g/d). TRT: treatment p-value effect; T: time p-value effect; I: interaction TRT x T p-value effect.
4. Discussion

The present trial deals with the mitigation of the stress responses to a high concentrate diet challenge in lactating dairy cows by the supplementation of an immunomodulatory feed additive.

Regarding the results showing the effect of the challenge in the enrolled animals the rumination time, rumen pH, production, and blood markers changed consistently. The decrease in rumination time in response to the dietary challenge may be related to the levels of starch and aNDFom in the diet (34% and 29% of DM, respectively), rather than the content of peNDF (13.8% of DM). The latter was lower than min. levels recommended by other authors [49], but previous experience with feeding diets similar to the one fed in this trial, a diet based on hay and straw as required in the Parmigiano Reggiano production area [50,51] has shown it is possible to decrease the level peNDF to 11.2% of DM without compromising rumen health [52–54]. In all those examples the starch content of the ration (avg. 23.2% DM) was lower than in the SARA challenge diet, and comparable to the pre-challenge diet, fed in this trial. At the same time, a min. safe level of 9% uNDF of DM has been recommended [54,55], while this was 8% in our SARA challenge diet. Therefore, a high starch content, combined with a low uNDF content, were likely the reasons for the drop observed in rumination time, which has been identified as a marker for SARA [56,57].

Daily mean reticular pH was not as low as expected and did not change because of the dietary challenge, but time below reticular pH 5.5 slightly increased (Table 3). Rumen pH thresholds suggested to indicate SARA were not reached in the present study (e.g., 330 min/d of pH < 5.6, [16]; or <5.8, [49]). However, the definition of rumen acidosis in terms of rumen pH thresholds is still under discussion and rumen pH cannot be seen as the sole marker for this digestive and metabolic disorder [11]. In addition, the absence of a marked decrease in pH during the challenge could also be related to the measurement system used: pH was recorded at the reticulum, and previous research has shown limited comparability between pH recorded at the reticulum and the rumen [58]. Mensching et al. [59] found a difference of about 0.4 points pH higher in the reticulum than in the rumen. Moreover, the pH in the reticulum is more stable compared to the pH in the rumen [60]. Other signs of SARA, all of which were seen in the present study, include milk yield decrease, milk fat depression, and inversion of fat-protein ratio [12,61]. All these reasons could be a limitation of our study.

Some immune and metabolic markers increased over the dietary challenge: EOS, CER, SAA, ILs, GGT, PON, and ROM. Acute phase proteins are produced mainly in the liver and are considered sensitive markers of inflammation. The positive APP, including CER and SAA, have a protective role against pathogens, e.g., in neutralizing enzymes, scavenging free hemoglobin and radicals, and in modulating the host’s immune response [62]. The increase in CER (Figure 4b), even if not specific, is an expression of a systemic and innate reaction of the organism to inflammation triggered by external (pathogens, toxins, etc.) or internal (tissue damage, etc.) stimuli [63]. Moreover, SAA (Figure 4c) is reported as a marker of the ruminal LPS translocation in cattle [17,20]. This increase could be related in our study to the dietary challenge, probably because of a translocation of LPS out of the digestive tract into the portal circulation [17,63]. The increase in positive APP was also seen in previous nutritional challenge trials [64,65], in which an increase in plasma concentrations of positive APP were observed when rumen pH was below 5.8 for at least 6 h a day. On the contrary, ALB, a negative APP, slightly decreased over the dietary challenge (Figure 5a) which is likely the consequence of a shift towards production of positive APP in the liver [66,67]. CHOL, another marker of cow wellbeing, diminished in the challenge phase of our study (Figure 5b) and this supports the impact of the dietary challenge on immune function and metabolism. Previous studies [68] reported a lower level of plasma CHOL in farms with high prevalence of SARA; CHOL was used in that study as an index of the CHOL binding protein, a negative APP related to an inflammatory response.

In addition, the challenge diet was associated with a decrease in red blood cell (RBC) parameters, mostly HG, HTC, ERT, MCV, MCHC, and RWI (Figure 2), highlighting the
stress experienced by the animals. During chronic stress mature red blood cell forms decrease and more immature forms (RET) are released [69]. RET usually have higher volume and lower content in HG, which are likely the reasons for the decrease observed in MCHC and the increase in RWI in this trial. Altogether, the changes observed in rumination parameters and production, and in metabolic and immune markers suggest that the diet fed during the SARA challenge was effective in creating the intended nutritional, metabolic and immunological stress.

Regarding the effect of the immunomodulant product in treated animals limited interesting results has been collected. Cows in the OMN treatment tended to have a higher percentage of RET throughout the nutritional challenge (Figure 2d), suggesting a prompter response in in replacing damaged ERT with immature red blood cells (RET). Mezzetti et al. [29] observed similar effects on RBC in transition cows fed OMN. As reported by other authors, OMN exerts effects on white blood cells [15,24,33,70]. In the present study, we observed a change in the proportion of white blood cells species in OMN treated cows, with a decrease in LYM (Figure 3c) compensated by an increase in NEU (Figure 3b). These results are consistent with previously reported findings related to an increase in NEU and phagocytic activity of polymorphonuclear cells upon feeding OMN [15,71,72]. Other studies have reported positive effects of this product on leukocyte function and gene expression of L-selectin [24,73,74]. CORT, another stress marker, was greater in the OMN treatment, another finding that under certain circumstances has been previously reported for this product by other authors [24,71,72] and suggests some modulation of the innate immune system [75,76]. On the other hand, on some previous studies the supplementation of OMN was associated by equal or lower levels of CORT [29,33,74]. These differences on CORT recorded levels could be related with the high variability of this parameter and low sampling frequency applied in this research. Moreover, it is proposed that the functional metabolites, organic acids, vitamins, and antioxidants present in the yeast cells’ wall, one of the active ingredients in OMNG, may either be used as nutrients by the rumen and gut microbiota or act as signaling molecules affecting interactions between microbes and the immunological response [77].

During the SARA challenge BHB gradually decreased in all animals, probably because of the highly energetic diet. However, this reduction was more evident in OMN fed cows during the second half of the challenge, becoming significantly greater than in CON cows at d 21 and 28 (Figure 5c). This suggests a better energy balance of OMN fed cows, as previously suggested by Wu et al. [15,78] and could be explained by the energetic cost of inflammation. Inflammation changes the prioritization of nutrients, affecting energy balance and performance [22]. At the same time, depletion of energy storages, excess of NEFA and BHB result in fatty liver and ketosis which have negative effects on the immune function [21,79] and productive performance [80] of early lactating cows. GGT, a marker related with the liver function and index of cholestasis [81] was lower in OMN fed cows during the challenge, suggesting healthier liver function in those cows (Figure 5d).

As antioxidant capacity directly depends on liver activity [22], dysregulation on the liver functions reflects on blood concentrations of such biomarkers. Among oxidative status markers, PON, which is also a negative APP, was lower in the OMN treatment (Figure 6a) although levels observed were higher than the minimum indicated by Trevisi and Minuti [62], suggesting no depletion of this compound. ROM had no overall variation due to the treatment (Figure 6c); different results were reported by Mezzetti et al. [29] where this marker was found at lower levels in OMN fed cows. Finally, FRAP was not affected by the treatment (Figure 6b); this compound is known to exert antioxidant activity and provides a measurement of antioxidant power via blood concentration of bilirubin, uric acid, proteins, and vitamins C and E [82].

Finally, in literature the effects of the tested immunomodulatory feed additive are greater during the transition period [15,28,29], a really risky phase for dairy cows [4]. In the present research, the enrolled cows were, at the beginning of the challenge period,
around 100 DIM. In this phase their physiological status is more stable and less susceptible to external stressors [1].

5. Conclusions

In conclusion, these results show evidence of the nutritional stress induced by feeding a high-starch, low-NDF challenge diet, with measurements of digestive, metabolic, and immunological markers. The digestive impact was markedly seen as a decrease in rumination time and a shift in acetate and propionate proportions, even if reticular pH was barely impacted. Not all metabolic and immunological markers were impacted to the same degree but CER, GGT and ROM increased while ALB and BHB decreased along the challenge, reflecting the metabolic and immunological impact of this type of diet. Cows fed OMN showed a modulated metabolic and immune response to the challenge diet, as reflected by hematological changes compatible with a more reactive regeneration of red blood cells, a greater proportion of neutrophils in WBC, higher CORT, and lower PON, GGT, and BHB.

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