UNIMORE
UNIVERSITÀ DEGLI STUDI DI
MODENA E REGGIO EMILIA

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Modena, 03/10/2017

Spett.le Antico Forno s.a.s.

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OGGETTO: Resoconto scientifico progetto Pane Primus 2017 / Consorzio Futuro in Ricerca.

Si allega la relazione scientifica riassuntiva dei risultati ottenuti nel corso del Progetto sul prodotto “Primus” in collaborazione con Consorzio Futuro in Ricerca dal gruppo di lavoro da me coordinato.

Cordiali saluti.

Prof. Vittorio Vellani

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Relazione Scientifica

Abstract. Alcuni consumatori del prodotto Primus, da tempo in commercio, hanno segnalato effetti benefici su diversi stati patologici, che globalmente potrebbero essere riconducibili a un ipotetico effetto anti-infiammatorio del prodotto stesso. La Ditta Antico Forno ha commissionato al Consorzio Futuro in Ricerca uno studio preclinico volto a verificare in un modello animale la reale esistenza e la eventuale portata di tali effetti.
In un modello murino sono stati preparati 4 gruppi di animali identici tra loro, 2 dei quali sono stati alimentati con Primus e 2 con la normale dieta bilanciata per la specie *Mus musculus*. Il trattamento con i due tipi di alimentazione e' durato per 11 giorni. Di seguito, in uno dei due gruppi per ciascun trattamento alimentare e' stato indotto uno stato infiammatorio sistemico tramite iniezione intraperitoneale di lipopolisaccaridi batterici (LPS), mentre agli altri due gruppi hanno ricevuto una iniezione di salina (trattamento di controllo). L'effetto di LPS e' stato mantenuto per 60 ore, con una iniezione giornaliera di LPS. 2 ore dopo l'ultima iniezione gli animali venivano sacrificati e prelevati volumi di sangue da cui veniva estratto il siero. Dal siero sono stati misurati i valori plasmatici di 31 diversi fattori coinvolti nella infiammazione, tra cui:

1) citochine proinfiammatorie (interleuchina IL-1β, TNF-α, IL-6, IL-12, IL-17,IFN-γ)
2) chemochine (MCP1, MIP1, KC, RANTES, IP10)
3) citochine antiinfiammatorie (interleuchina-4, interleuchina-10)
4) citochine ematopoietiche (interleuchina-7, G-CSF, M-CSF, IL-9, GM-CSF)

I risultati sono stati analizzati con test statistici (analisi della varianza e test di Bonferroni) e sono rispettivamente mostrati nelle figure 1-4 della relazione estesa che segue. I fattori misurati non mostrati nelle figure o non hanno avuto differenze riconducibili al trattamento alimentare o sono ancora in fase di valutazione, non essendo direttamente riconducibili a effetti pro-infiammatori o anti-infiammatori.

Dai risultati non e' emerso nessun effetto significativo delle due diverse alimentazioni sui fattori ematici misurati nei due gruppi non trattati con LPS.
Nei due gruppi di animali trattati con LPS si e' verificato, come era atteso, un notevole aumento di tutti i parametri, ma negli animali alimentati con Primus si sono osservate le seguenti differenze significative:

1) significativa riduzione delle citochine proinfiammatorie (tutte tranne la IL-17)
2) significativa riduzione di tutte le chemochine
3) nessuna variazione significativa di IL-10, e una tendenza all'aumento della IL-4 che pero' non raggiunge il livello di significatività statistica
4) delle citochine ematopoietiche vengono ampiamente ridotte la M-CSF e IL-7, nessuna variazione delle altre

Dai risultati ottenuti possiamo affermare che nel modello animale di infiammazione sistemica con LPS la alimentazione con Primus e' in grado di modulare lo stato infiammatorio diminuendo i fattori proinfiammatori e lasciando inalterati i fattori antiinfiammatori. Negli animali non infiammati la alimentazione con Primus non altera i livelli basali di nessuno dei fattori studiati e quindi non sembra alterare l'omeostasi del sistema immune.

Sulla base dei dati ottenuti e' possibile ipotizzare che l'utilizzo di Primus nella alimentazione umana possa contribuire significativamente a ridurre o a prevenire stati infiammatori presenti in numerose patologie. Non e' invece possibile ipotizzare quale o quali componenti presenti nel prodotto Primus o quali sue caratteristiche siano responsabili degli effetti osservati.

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Pane Primus diet controls systemic inflammation in a preclinical model.

Introduction

The inflammatory response is beneficial as an acute, transient reaction to harmful conditions, facilitating the defence, repair, turnover and adaptation of many tissues.

However, chronic and low grade inflammation is likely to be detrimental for many tissues and for normal functions. In the last years it is progressively emerging that inflammatory processes plays a role in most pathologies and diseases, including cardiovascular and neurodegenerative diseases. Indeed, there are many studies reporting strong links between inflammation, morbidity and mortality.

Moreover low grade, chronic inflammatory state is a phenomenon that accompanies ageing, that is characterised by an increase in the concentration of a number of pro-inflammatory molecules in the circulation, a phenomenon that has been termed “inflammageing”. There are numerous studies showing that the circulating concentrations of many markers and mediators of inflammation are higher in old than in young adults.

Of interest, chronic low-grade systemic inflammation can be also caused and modified by diet. There is a growing body of evidence indicating that the combination of food quantity and quality, mainly dietary patterns with high calorie intake or low in micronutrients, and genetic susceptibility are able to influence the chronic inflammatory state . Consequently, the recognition of the emerging role of diet-induced inflammatory process in disease development has been accompanied by efforts to identify dietary factors and dietary patterns that may promote or inhibit the inflammatory process, thereby affecting disease risk and severity.

The role of dietary patterns, specific foods and individual nutrients and non-nutrients in influencing low grade inflammation has been extensively reviewed in the context of overweight and obesity.

Healthy eating patterns as described by the healthy eating index, the alternative healthy eating index, vegetarian diets, and the Mediterranean diet are all associated with lower circulating concentrations of inflammatory markers including CRP and several cytokines .Among the components of a healthy diet, higher intake of whole grains, vegetables and fruits, nuts, and fish are all associated with lower

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inflammation.

For these reasons we have decided to perform a preclinical study in order to investigate the effect of a diet consisting of Pane Primus on circulating cytokines in an experimental model of inflammation in the mouse.

Experimental protocol

In order to induce an inflammatory responses in the mouse, animals were treated 3 times with 3 mg/Kg of lipopolisaccaride (LPS) by intraperitoneal injection, and killed for cytokine evaluation 2 hours after the last LPS administration.

This is the most trusted and used model for inducing systemic inflammation in the mouse, and the gravity of the inflammatory responses can be controlled by the amount of LPS injected. LPS is the major component of gram-negative bacterial membrane, and it activates TLR4 signaling cascade ending up in the activation and nuclear translocation of NF-κB , which further leads to the upregulation of a battery of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α along with other molecules such as, COX-2, E-Selectin, MCP-1 and iNOS.

In our model we chose a medium-low dose of LPS, able to mimic a mild inflammatory status.

Experimental scheme is reported in figure 1.

Animals were fed with a Pane Primus diet, starting 11 days before the first LPS injection. Control animals were fed with the usual pellet diet. The experiment consisted of 4 groups (6 animals each):

Pellet- fed mice injected with saline

Pellet -fed mice injected with LPS

Pane Primus -fed mice injected with saline

Pane primus -fed mice injected with LPS

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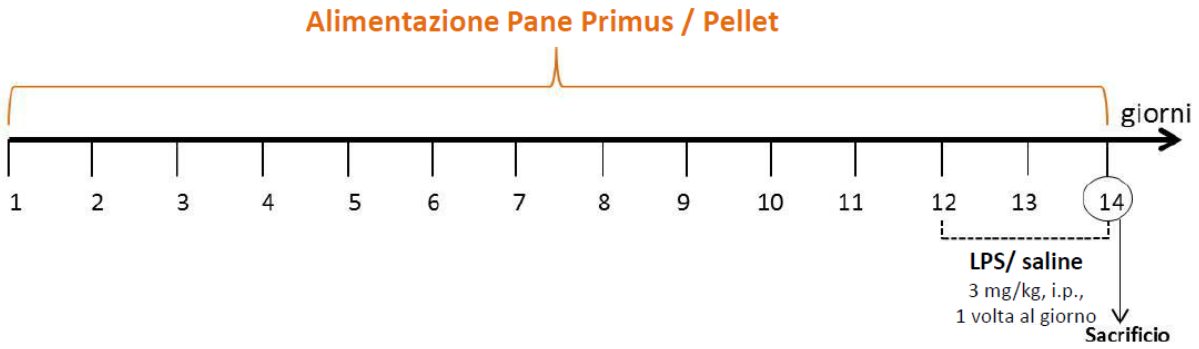
Figure 1



A panel of pro and anti-inflammatory cytokines and other molecules were measured in plasma with a multiplex ELISA analysis (Millipore). This method allows the detection of multiple molecules also with a very small sample volumes.

Molecules evaluated.

We studied inflammatory and antinflammatory biomarkers and selected based on their availability and relevance. Included markers are as follows:

Proinflammatory cytokines:, interleukin IL-1β, TNF-alpha, IL-6, IL-12, IL-17,IFN-gamma

Antinflammatory /inhibitory cytokines: IL-10, IL-4

Hematopoietic cytokines: IL-7,G-CSF, M-CSF, IL-9, ,GM-CSF

Chemokines: MCP1, MIP1, KC, RANTES, IP10

Statistical analysis of results was performed with One way ANOVA, followed by Bonferroni test for multiple comparisons. Significant was set at $p<0.05$.

Results and Discussion

Figures 1 reports the levels of proinflammatory cytokines, figure 2 the levels of chemokines, figure 3 the levels of antinflammatory cytokines, and figure 4 those of hematopoietic cytokines.

As expected from this experimental model, the administration of LPS induced a highly significant systemic

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inflammation. Figures 1-5 report that in animals treated with LPS there is a relevant increase of all factors considered.

However it is also evident that a Pane Primus diet is able to modulate the levels of cytokines.

As reported in figure 1, in animals fed with Pane, it is present a significant reduction of IL-1, TNF, IFNg, IL-6 and IL-12, in comparison with pellet-fed animals.

IL-1, TNF,IL-6 and IL-12 are the more representative proinflammatory cytokines and elevated levels are often associated with chronic inflammation and autoimmune diseases. IL-6 is also the cytokines more implicated in the subtle low grade inflammation that is present during aging . Also IFN-g is related to immune system activation.

Chemokines are inflammatory and are released from a wide variety of cells in response to bacterial infection, viruses and agents that cause physical damage. Inflammatory chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage. Although they are fundamental for a correct immune activation, elevated levels of chemokines are linked to chronic inflammatory conditions and autoimmunity. As reported in Figure 2, the levels of the chemokines MIP-1, KC and IP-10 are lower in animals fed with Pane primus .

The antinflammatory cytokines are endogenous regulators of immune homeostasis and have the role of preventing immune overshooting. As reported in Figure 3, no significant effect of Pane is evident on the stimulated levels of the antinflammatory cytokines IL-10 and IL-4.

Finally, also some hematopoietic cytokines that are activated by LPS, are reduced in animals fed with a Pane Primus diet. In particular a significant decrease is present for IL-7, that is a growth factor for cells of the lymphoid series, and for M-CSF, that is a s a hematopoietic growth factor that is involved in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. Locally produced M-CSF in the vessel wall contributes to the development and progression of atherosclerosis, while the chronic activation of monocytes can lead to multiple metabolic, hematologic and immunologic abnormalities.

While, as described, in animals treated with Pane Primus there is a significant modulation of LPS-induced

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cytokines, in the same figures it also emerges that no difference is present when cytokines are measured in normal animals. In normal animals plasmatic levels of cytokines are generally low, but they could be measured by our ELISA system. No effect for any cytokine was evident in animals fed with Pane Primum in comparison with the pellet diet. This interesting result suggests that the modulation of Pane is exerted only when there is an inflammatory/pathological condition, without affecting the basal homeostasis of immune response.

Conclusion

From the data reported we conclude that a Pane Primus diet is able to modulate the inflammatory status in an experimental model. A significant reduction of the amount of the main proinflammatory cytokines and chemokines is in fact present in animals that were fed with Pane rather than with the conventional pellet diet. Interestingly the reduction appears specific only for the proinflammatory cytokines and not for the antinflammatory cytokines, whose role is to endogenously counteract inflammation. The balance of pro/anti-inflammatory cytokines in Pane mice is therefore clearly shifted towards an antinflammatory direction.

Moreover, the modulation is observed only when cytokine production is stimulated by LPS, without affecting the basal homeostasis of the immune system responses.

At the moment we do not know which of the factors in the Pane Primus recipe are the main involved in the observed effects.

From the data reported we can suggest that a diet including Pane Primum may help/participate in controlling the inflammatory status that negatively affects many aspects of human health.

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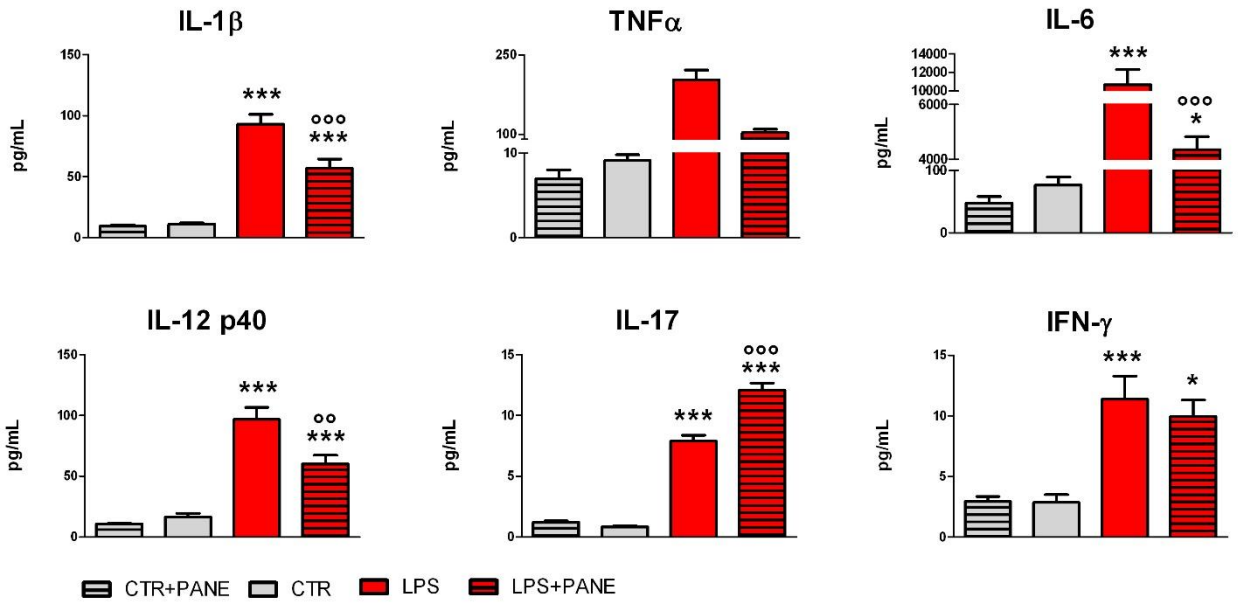

Figure 2

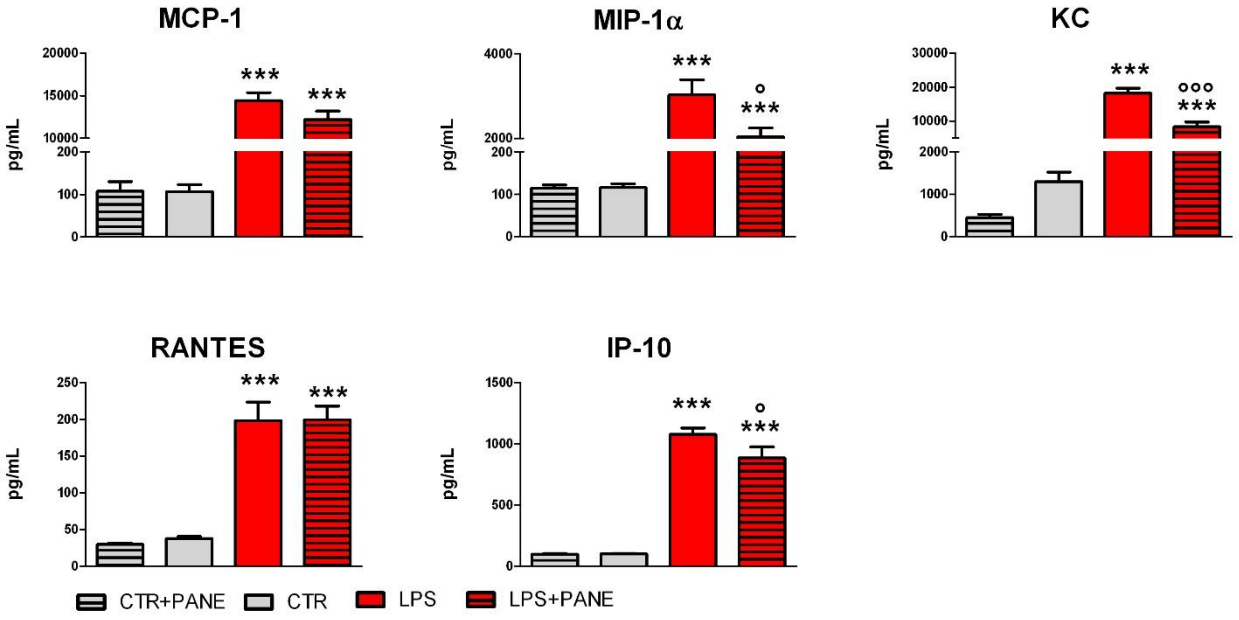

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Figure 3

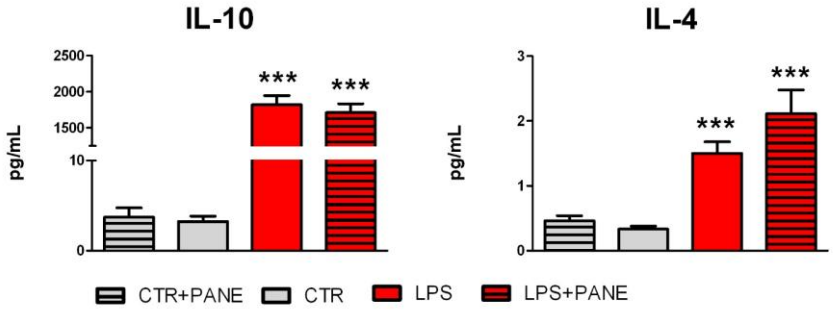
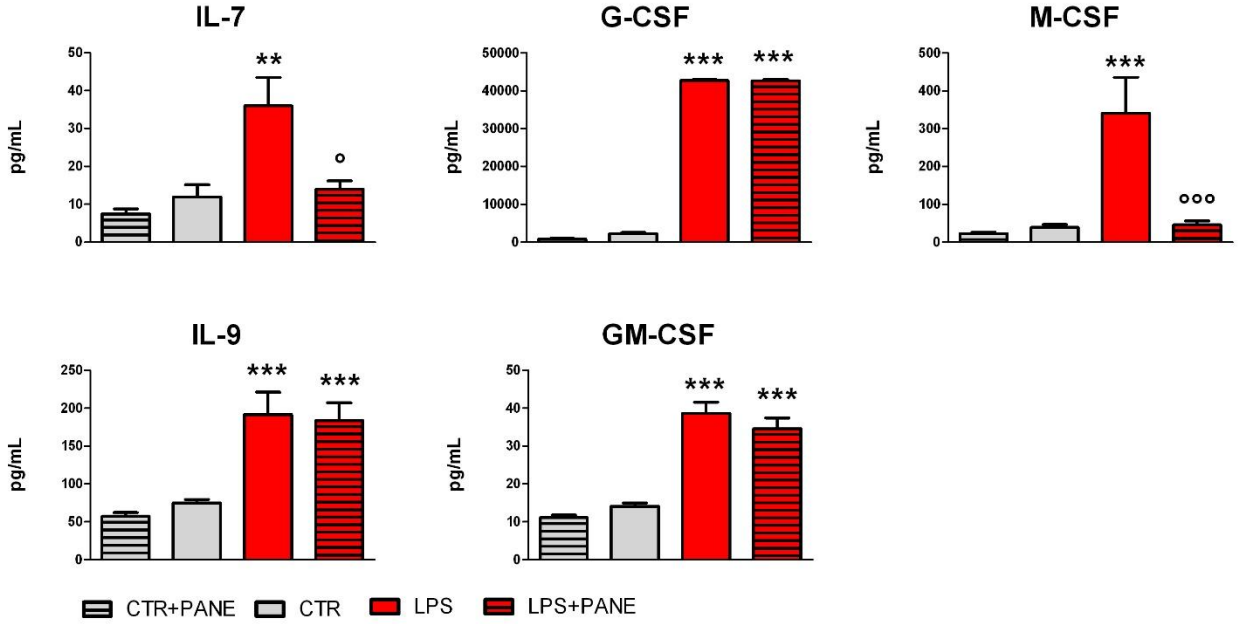


Figure 4



Values are mean ±SD of 6 animals in each group
When not elsewhere indicated, animals received the standard pellet diet

- P<0.05 ** <0.001 *** p<0.001 vs CTR;
- ° p< 0.05, °° p<0.01, °°° p<0.001 vs LPS