

# Evaluation of bovine-derived lacteal complex supplementation on gene expression in BALB/c mice

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**Abstract:** We conducted an evaluation of gene expression associated with innate and adaptive immunity in a double-blind ex vivo mouse study using a bovine-derived dietary ingredient (Ai/E<sup>10</sup><sup>®</sup>, Health Technology Resources, Inc., Scottsdale, AZ, USA). BALB/c female mice (5–6 weeks of age) were fed chewy granola bars supplemented with (Test) or without (Control) Ai/E<sup>10</sup> for 10 days. After the feeding period, the animals were sacrificed and spleen cells were isolated and incubated with lipopolysaccharide and phorbol myristate acetate-ionomycin. RNA was extracted and mRNA expression of 84 genes involved in innate and acquired immunity was determined with real-time PCR arrays. Numerous genes associated with innate and adaptive immunity were upregulated in the Test group when stimulated with mitogens. Significant upregulation was observed in 30% (25 of 84) of genes upon lipopolysaccharide stimulation and in 14% (12 of 84) of genes upon phorbol myristate acetate + ionomycin stimulation in the Test group relative to Controls. This study illustrates that Ai/E<sup>10</sup> supplementation results in significant and specific upregulation of genes associated with innate and adaptive immunity in mice. Notably, this effect was observed only in stimulated cultures.

**Keywords:** dietary supplementation, immunomodulation, mice

## Introduction

A healthy immune system offers protection from the deleterious effects of infectious agents such as bacteria, viruses, fungi, and parasites and is involved in the identification and elimination of tumor cells and the response to injury and trauma. The immune response is influenced by a number of factors including age,<sup>1,2</sup> stress,<sup>3</sup> and gene variability<sup>4,5</sup> and imbalances in this response can lead to disease or predisposition to disease.<sup>6</sup> The immune system can be categorized into two broad overlapping categories of defenses. Innate immunity offers a second nonspecific defense soon after the appearance of and activation by an antigen in the body. The adaptive immune response is more complex and is antigen-specific. That is, the antigen is first recognized and then immune cells specifically designed to attack that antigen are produced. Adaptive immunity also makes future responses to an antigen more efficient via immunologic memory.

Immune reconstitution therapy via immune modulation is an emerging field in the treatment of disease. Immune modulation works via the introduction of agents into the body that can strengthen and/or support the immune system. Mounting evidence suggests that such agents may lead to fewer infections and other forms of disease.<sup>7</sup> Numerous clinical trials have studied the effects of consumption of dietary supplements to support immunity.<sup>8–11</sup> The field of nutrigenomics, which studies the effect of

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diet on health by altering the expression on an individual's genetic make-up, has grown in popularity in recent years.<sup>12,13</sup> Nutrients can affect gene expression directly or indirectly by acting directly as ligands for transcription factor receptors, by causing changes in the concentration of substrates or intermediates involved in gene regulation or cell signaling, and by altering signal transduction pathways and signaling.<sup>14,15</sup> This has been substantiated in numerous clinical trials that have demonstrated gene expression alterations with supplementation of selenium on immune function,<sup>16</sup> antioxidants on cardiac endothelial function,<sup>17</sup> and zinc supplementation on bone metabolism,<sup>18</sup> to name a few.

The beneficial physiological effects of dairy products and products derived from bovine milk are well known.<sup>19,20</sup> Bovine milk-derived products exhibit beneficial properties for human health, including the immune response,<sup>21–23</sup> although this area has not been extensively studied. This study was designed to examine the effects of supplementation with a bovine-derived dietary ingredient (Ai/E<sup>10</sup>®, Health Technology Resources, Inc., Scottsdale, AZ, USA) on expression of immune-related genes in BALB/c mice.

## Methods

Ai/E<sup>10</sup> is a <100 kD whey extract derived from bovine milk collected from cows via a patented process. The animals receive an infusion of an array of specially prepared antigens including *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Escherichia coli*, and *Salmonella* via their teat canals into the udder. The natural reaction of the udder, when infused in this manner, produces an array of molecules reactive to the infused antigens, which are then harvested and concentrated. Examination by amino acid sequencing and gel electrophoresis has determined that Ai/E<sup>10</sup> contains over 60 immune communication peptides and other molecules, primarily defensin, granulysin, immunoglobulin production stimulating factor, cathelicidin, transfer factors, granulins, perforins, chemokines, minicytokines, and granulocyte-macrophage colony stimulating factor.<sup>22,23</sup> Ai/E<sup>10</sup> has been self-affirmed GRAS (generally recognized as safe).

BALB/c female mice 5–6 weeks of age were fed exclusively with chewy granola bars (J. Schuette, Inc., Chicago, IL, USA) containing (Test group, n = 5) or not containing (Control group, n = 5) Ai/E<sup>10</sup> (Table 1). The animals were grouped and housed according to the different treatments. Cages were placed in a temperature- and light-controlled room so that it was dark only during the night. One bar a day was provided to each animal. Water consumption was ad libitum and supplied through a

**Table 1** Composition of cereal bars

Ingredient	Test bar	Control bar
Total fat (g)	3	3
Saturated fat (g)	1.5	1.5
Trans fat (g)	0	0
Sodium (mg)	75	75
Total carbohydrate (g)	18	18
Dietary fiber (g)	1	1
Sugars (g)	7	7
Protein (g)	1	1
Ai/E <sup>10</sup> (mg)	10	–

feeding bottle. After 10 days of daily supplementation, the animals were sacrificed by cervical dislocation and their spleens were extracted. Spleen cells were selected for analysis because the spleen plays a critical role in both innate and adaptive immune processes and is a key site for antibody production, phagocytosis, and hematopoiesis.

Spleen cells were obtained by passing the spleen through a cell-strainer filter (BD Falcon 2350, BD Biosciences, Bedford, MA, USA), and cells were separated by density-gradient centrifugation (Ficoll, Organon Teknika Corp., Durham, NC, USA). The cells were washed twice with phosphate-buffered saline (Organon Teknika Corp., Durham, NC, USA) and counted with a trypan blue exclusion test.

Five × 10<sup>5</sup> freshly isolated spleen cells were incubated for 3 hours with: (1) medium alone (buffer saline plus fetal bovine serum, L-glutamide, and streptomycin); (2) medium plus 2 µg/mL lipopolysaccharide (LPS); or (3) medium plus 50 ng/mL phorbol myristate acetate (PMA) and 1 µg/mL ionomycin (PMA + Iono). RNA was extracted from basal and mitogen-stimulated spleen cells using the acid guanidinium thiocyanate–phenol–chloroform method.<sup>24</sup> First-strand cDNA was reverse transcribed from the mRNA (RT<sub>2</sub> First Strand Kit, SABiosciences, Frederick, MD, USA).

Innate and adaptive immune signalling pathways were analyzed using a set of optimized real-time PCR primer arrays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA).<sup>6</sup> This approach permitted the analysis of mRNA expression levels for 84 genes related to the innate and adaptive immune activation pathway, along with 5 housekeeping genes. Controls were also included on each array for assessing genomic DNA contamination, RNA quality, and general PCR performance. Targets that showed marked differences between Test and Control group animals were retested using standard real-time PCR on each individual sample to confirm the results of the real-time PCR array experiments (data not shown).

Results were analyzed by a web-based PCR array data analysis program that performs  $\Delta\Delta C_t$  based fold-change calculations from the uploaded raw threshold cycle data and displays the fold difference in the expression of each gene between the Test and Control samples. Statistically significant upregulation was defined as a fold-change  $> 2$  and down-regulation was defined as a fold-change  $< -2$ . Statistically significant differences between the Test and Control groups were defined as a relative mean-fold difference  $> 5$ .

## Results

No changes in gene expression were observed following supplementation in either treatment group under basal conditions (results not shown). A broad immune stimulation was observed in the mitogen-stimulated cell cultures, most notably in response to LPS stimulation. Of the 84 genes tested, 25 (30%) were significantly upregulated in Test cultures stimulated with LPS. In cultures stimulated with PMA + iono, 12 (14%) genes were significantly upregulated. Genes that were upregulated in the LPS-stimulated cell culture group are functionally related to genes involved in the host defense to bacteria and in the interleukin-1 type 1 receptor (IL-1R) pathway, innate immunity, and septic shock (Table 2). The same expression pattern was observed for the PMA + iono stimulated cells, with the exception of those involved in innate immunity. In addition, the *IL-10* gene was significantly upregulated only after PMA + iono stimulation. The highest fold change comparing Test with Control groups in response to LPS was recorded for *Serpine1*, a septic shock-associated gene (relative mean-fold change = 87).

Following PMA/iono stimulation, we observed a significant increase in *Proc* expression, another gene associated with septic shock. Furthermore, *Il1rap12*, *sftpd*, *defb4*, *Ilfnb1*, *C8a*, *CRP*, *Il1f8*, *Il1R1*, *Il1R2*, *pglyrp*, *Hc*, and *serpine1* expression levels were robustly augmented. In contrast, LPS stimulation resulted in much stronger upregulation in twice as many genes compared with PMA + iono stimulation (Table 2).

## Discussion

The goal of this study was to assess whether supplementation with Ai/E<sup>10</sup> using a food matrix results in the modulation of innate and adaptive immunity-associated gene expression without provoking an aspecific stimulation of the immune system. The analyses of immune-response genes in a non-stimulated condition showed that baseline functionality of the immune system is not altered by supplementation, which implies that product use does not result in an unspecific overactivation of

**Table 2** Mean fold-changes in gene expression over the 10-day supplementation period with LPS or PMA + iono stimulation

Functional group	Gene	LPS		PMA + iono	
		Control	Ai/E <sup>10</sup>	Control	Ai/E <sup>10</sup>
Host defense to bacteria	Colec12	1.2	8.2		
	C8a	2.1	83	-1.9	8.1
	Hc	3.6	157	-1.2	12
	Defb4	1.3	103	-3.1	6.0
	Lalba	4.4	46		
	Lbp	3.6	15		
	DMb71	3.1	21		
	Pglyrp	3.4	33	-1.3	5.4
	CCL	1.7	24		
	CRP	2.5	78	-2.0	8.9
IL-1R/TLR	Ilfnb1	1.7	23		
	Il1f5	3.3	17		
	Il1f6	2.4	71		
	Il1f10	4.5	32		
	Il1f8	2.5	68	-1.7	4.9
	Il1R1	2.9	33	-1.6	4.7
	TLR3	3.3	31		
	Il1rap2	1.6	95	-1.8	5.3
	Il1R2	-1.8	30	-1.8	3.5
	Sftpd	4.5	102		
Septic shock	Mapk8	1.2	6.7		
	Proc	4.2	201	-1.0	24
	Il6	2.4	13		
	Il10			8.3	23
	Serpina1a	5.3	67		
Serpine1	6.5	564	1.1	21	

**Note:** Values are listed only for genes with significant mean-fold change of Ai/E<sup>10</sup> vs Control (relative mean-fold change  $> 5$ ).

**Abbreviations:** LPS, lipopolysaccharide; PMA, phorbol myristate acetate; ILR-1, interleukin-1 type 1 receptor; TLR, Toll-like receptor.

the immune response that could alter its physiological function. Upon stimulation with both LPS and PMA + iono, spleen cells isolated from mice that consumed bars containing Ai/E<sup>10</sup> showed a significant increase in the expression of genes directly involved in innate and adaptive immune response.

The genes selected for analysis in this study are involved in the direction and regulation of nearly all aspects of the immune response. These include genes associated with the host response to bacterial infection and sepsis, those related to the detection of pathogens and supportive of the signaling pathways, and genes involved in the acute-phase response, complement activation, the inflammatory response, and the antibacterial humoral response. Genes involved in the innate immune response and septic shock were also selected for study to provide a broad assessment of the immune supportive properties of the supplement ingredient.

Ai/E<sup>10</sup> is a collection of immune communication molecules differentiated by their very small size ( $< 100$  kD) and their specific reactivity to proprietary antigens infused into the udder of

the producing dairy cows. Molecules of this size are generally not present in the food chain or in dietary supplements and their unique reactivity differentiates this product from compounds such as milk and colostrum. The precise mechanism of action has not yet been identified, but the measured effects in this study suggest the molecules in Ai/E<sup>10</sup> directly supplement components of the cytokine pathways, enabling more complete and efficient responses to challenges. It is known that cytokine communication pathways are damaged by stress, toxin, and other factors and this ingredient appears to support minimizing the effects of such damage.<sup>22</sup> Aside from the current study, a true modulation benefit has not been reported by other supplement ingredients, dairy or otherwise, to the authors' knowledge.

The current study provides additional evidence for immune modulation with Ai/E<sup>10</sup> supplementation. Advanced study of the technology used to produce Ai/E<sup>10</sup> yielded a single antigen complex that was evaluated in vivo using a mouse model of MRSA-induced peritonitis and resulted in 83% survival versus 0% survival in untreated animals.<sup>23</sup> In addition, a clinical study of 12 adults who consumed 100 mg Ai/E<sup>10</sup> twice a day for 90 days reported a significant increase in natural killer activity (30 to 101 lytic units,  $P = 0.001$ ).<sup>22</sup>

Overall, the results of this study suggest that Ai/E<sup>10</sup> supplementation may contribute to strengthening the immune response through the activation of different immune system components. Additional clinical trials in animals and humans are warranted in order to substantiate these promising results.

## Disclosure

The authors report no competing financial interests.

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