RESEARCH LETTER

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A pilot study: Innate immune modulation reduces F2-Isoprostanes and improves psychological health in a chronically stressed cohort

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1 | INTRODUCTION

Oxidative stress (OS) has become problematic for the biological systems owing to the elevated production of free radicals associated with complex cellular redox processes.[1,2] Furthermore, OS is associated with the aberrant activation of the innate immune system leading to systemic inflammation or stress.[3] Considerable research has been focused on the systemic OS, and its correlation with multiple disease states and acute stress conditions is well-known.[4-6] However, the studies that address lowering the systemic OS levels in humans have been largely inconclusive as they depend on exogenous treatments, such as antioxidants and probiotics.[7-9] Since systemic OS involves the immune system, a potential strategy would involve the development of a targeted treatment that promotes immune balance, thereby helping to lower the global OS profiles endogenously.[10,11] Recently, it has been demonstrated through in vivo *Drosophila* studies that an inactive bacterial biologic (IAB), accompanied with confirmed pathogen molecular associated patterns (PAMPs) to select Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-containing protein (NOD) receptors, lowered the inflammatory targets of the NF-κB signaling cascade.[12] A pilot study was conducted to determine the effect of oral administration of IAB on the systemic OS levels in humans using the OS biomarker, urinary F2-isoprostane (F2-IsoP). F2-IsoP is a stable noninvasive biomarker and an end-stage metabolite of prostaglandin oxidation, present in all fluids and tissues, and has been reported to increase during the oxidative injury (Mayo Clinic Labs; Cleveland Heart Lab, Inc.).[1,2,13] This allows F2-IsoP to be more advantageous than traditional plasma stress biomarkers. This report describes the changes in urinary F2-IsoP levels as well as mental health parameters in a stressed cohort of PTSD combat veterans who received IAB treatment.

2 | METHOD AND MEASURES

2.1 | Description of IAB immune activation and treatment formulation

IAB (ReseT, Labyrinth Holdings, LLC, Houston, Texas) was derived from inactivated *Lactobacillus bulgaricus* and processed to retain the selected TLR and NOD agonists (PAMP activity). The TLR and NOD receptor agonist activity induced by IAB was confirmed using the PRR Ligand Screening Service (https://www.invivogen.com/custom-tlr-screening, InvivoGen, San Diego, California). Screening was performed using a collection of HEK293-blue clones engineered to express individual hTLR and hNOD receptors, and a SEAP-reporter plasmid activated with NF-κB. Cells (50 000 cells/well) was incubated with diluted blinded samples

TABLE 1 In vitro TLR and NOD receptor activation following IAB exposure

TLR/NOD receptor	Positive control		Negative control		IAB powder		Fold induction‡
	Average†	SD	Average	SD	Average	SD	
hTLR2	2.695	0.019	0.052	0.001	2.599	0.025	50.0
hTLR3	2.265	0.009	0.127	0.000	0.292	0.014	2.3
hTLR4	2.522	0.009	0.163	0.027	1.137	0.076	7.0
hTLR5	2.877	0.007	0.066	0.001	0.096	0.003	1.5
hTLR7	2.138	0.059	0.095	0.002	0.104	0.014	1.1
hTLR8	2.120	0.011	0.110	0.003	0.117	0.001	1.1
hTLR9	2.215	0.051	0.156	0.011	0.226	0.003	1.4
NOD1	1.428	0.014	0.070	0.002	0.085	0.000	1.2
NOD2	1.877	0.008	0.073	0.001	1.544	0.046	21.2

Abbreviation: IAB, inactive bacterial biologic.
†Average is calculated based on three replicate assays.
‡Fold induction is the IAB value divided by the negative control value.

(1:70 water) or with the appropriate positive and negative controls. Cell activation was evaluated based on the increase in SEAP activity measured as the absorbance at OD_{650} nm using the Quanti-Blue reagent according to the manufacturer's instructions (InvivoGen, San Diego, California). IAB was incorporated into the oral melt tablets (12 mg per tablet) and supplied for this study (Labyrinth Holdings, LLC, Houston, Texas).

2.2 | Study participants

The study participants were selected from a list of combat veterans experiencing PTSD. This was performed in coordination with the Mental Health America of Greater Houston, Houston, Texas. Subject selection and inclusion criteria included combat veterans who lived in the greater Houston area, were 18 years of age or older, and medically diagnosed with PTSD, but with no other health issues. Subjects were excluded if they were administered probiotics or hospitalized in the past 10 months for any psychiatric-related issue and were classified as vulnerable (ex. prisoners) or could not provide informed consent. Participants were required to read and sign an informed consent form. A physician examined all participants on Day 1, 15, and 70 to assess concerns and confirmed their adherence to the protocol. All procedures involving human participants, consent form, and study ethics were reviewed and approved by the Western Institutional Review Board (WIRB), Puyallup, Washington (#114723). Study was conducted in association with Mental Health America of Greater Houston, Houston, Texas. Seven male participants (combat veterans aged between 28 and 47 years) completed the 70-day study.

2.3 | Human study design

The study design was a single-arm treatment study, wherein the effects of treatment were assessed and compared to the baseline pretreatment values (Day 1). Once the baseline samples were collected, the study participants were requested to take two 12 mg tablets, sublingually, twice daily (48 mg daily dose) for the duration of the study, and maintain their normal daily routine. Treatment sample collection and the measurements were performed on Days 15 and 70.

2.4 | F2-isoprostane levels

Participants self-collected their first morning urine on Day 1, 15, and 70. Samples were blind-coded, frozen, and stored at $-20°C$. Urinary F2-IsoP levels were assessed using the ELISA-based assay kit, and the values were normalized to the urinary creatinine levels to adjust for dehydration (kit EA85, CR01; Oxford BioMedical Research, Rochester Hills, Michigan). Urine samples were assayed by the service labs (Texas A&M University, College Station, Texas) and spot checked by the Oxford BioMedical Research group.[1,13]

2.5 | Psychological tools

Standardized psychological tools included the Satisfaction with Life Scale,[14,15] Epworth Sleepiness Scale,[16] and Zung Self-Rating Scale for Anxiety[17] and Depression[18] that were self-administered and scored according to the tool's standardized procedure on Days 1, 15, and 70. These measurements were conducted in private and coded to mask the participant's identification.

2.6 | Statistical analysis

The mean and SEM values were calculated using MS Excel and the Student's t test to establish the differences between baseline (Day 1) and treatment time points (Days 15 and 70) using GraphPad Prism software (https://www.graphpad.com, P values, * <0.5; ** <0.01). Excel was used to generate the individual boxplots for each dataset. In the plots, we presented the mean, median, upper, lower, and interquartile ranges, with whiskers highlighting high/low values. Excel was
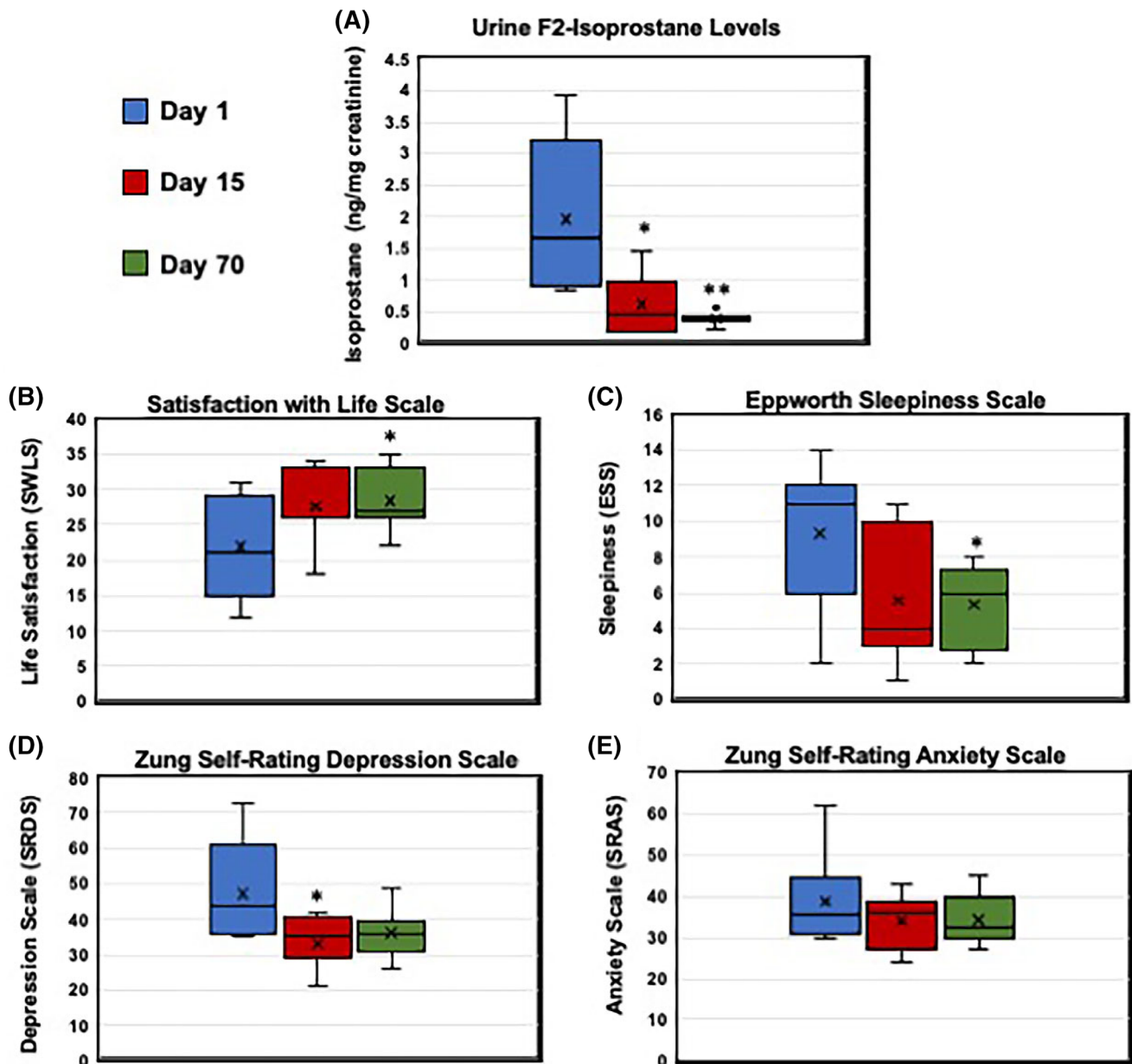
FIGURE 1 Changes to inflammation (F2-IsoP), mood and sleepiness profiles in participants following inactive bacterial biologic (IAB) exposure (1-70 days, n = 7). X marks the mean value for each data set. A, Urinary F2-IsoP levels (ng/mg creatinine). B, Satisfaction with life scale (SWLS). C, Eppworth sleepiness scale (ESS). D, Zung Self-Rating Depression Scale (SRDS). E, Zung Self-Rating Anxiety Scale (SRAS). *P* values * <.05; ** <.01

used to calculate the correlation coefficients between the mean F2-IsoP and mental health values.

3 | RESULTS AND DISCUSSION

3.1 | Innate immune receptor activation

To confirm the biological activity of IAB, the immune activation was confirmed using the HEK293-blue cell culture assay, which monitors the TLR and NOD receptor activation of the NF-κB pathway. IAB exposure resulted in significant activation of cells expressing human TLR2, TLR4, and NOD2 receptors when compared to the negative controls (Table 1). Here,

the induction of >4-fold represents moderate agonist activity, whereas a >10-fold induction represents significant agonist activity. These assays indicate that oral administration of IAB modulates TLR2, TLR4, and NOD2 receptors found in the buccal cavity and intestinal mucosal layers, thereby impacting the immune cellular responses via the NF-κB cascade.[10,11] Previously, in vivo *Drosophila* studies also showed lower basal levels of NF-κB gene targets upon IAB administration, further supporting this conclusion.[12]

3.2 | Oxidative stress profiles

To determine whether the oral administration of IAB impacts systemic OS, urinary F2-IsoP levels were examined on Day 1 (baseline),

15, and 70 of the study. The participants urine F2-IsoP values were normalized to creatinine levels and are presented in Figure 1A. After 14 days of treatment, the average participants F2-IsoP value decreased significantly as did the variability between the samples. Overall, the F2-IsoP mean values were reduced by 68% and 80% by Day 15 and 70, respectively. This reduction in F2-Isop level was rapid and highly significant and has not been reported previously.[7,8]

3.3 | Psychological assessments

The participants psychological measures showed positive changes between Day 1 (baseline), Day 15, and 70 (Figure 1B-E). The graphs demonstrate after 14 days of treatment, most of the participants experienced improved psychological health that were consistent with reduced urinary levels of F2-IsoP (Figure 1A). In case of depression profiles, there was significant improvement observed by Day 15 (Figure 1D). However, with further treatment, the participants continued to exhibit improvement, with significant improvement in the metrics of life satisfaction (Figure 1B) and daytime sleepiness (Figure 1C) by Day 70. The correlation coefficients between the F2-IsoP and mental health parameter values were −0.99, 0.99, 0.95, and 0.96 for life satisfaction, sleepiness, depression, and anxiety, respectively.

4 | CONCLUSION

In this study, we conducted sublingual administration of IAB, which is a PAMP-based technology that modulates the innate immune system receptors TLR2, TLR4, and NOD2, as a potential treatment to lower the systemic OS endogenously and improve the mental health parameters in a stressed cohort (PTSD veterans). IAB administration to the participants significantly reduced the systemic OS ($P < .01$) as assessed by the changes in the urinary F2-IsoP levels (Figure 1A). Simultaneous clinical and psychological improvements were also detected across this chronically stressed cohort of individuals (Figure 1B-E), suggesting a correlation between improved OS levels and mental health. However, for better understanding, further controlled studies investigating the potential effects of IAB on stress-related physiological processes, and psychological profiles are warranted. Moreover, the future study design would include large number of participants, the addition of placebo control groups, and assessment of other inflammatory and stress biomarkers, to broaden our mechanistic understanding of the therapeutic efficacy of IAB treatment on chronic stress.

AUTHOR CONTRIBUTIONS
Conceptualization: John Marcellus
Formal analysis: Kim Finley
Investigation: Kim Finley, John Marcellus
Methodology: Kim Finley, Beth Jones
Writing—Original draft: Kim Finley
Writing—Review and editing: Kim Finley, Beth Jones, John Marcellus
    All authors have read and approved the final version of the manuscript.
    Kim Finley had full access to all of the data generated in this study, and takes complete responsibility for the integrity of the data and accuracy of the analysis.

TRANSPARENCY STATEMENT
Kim Finley affirms that this manuscript is an honest, accurate, and transparent account of the study being reported, and no important aspects of the study have been omitted. Moreover, any discrepancies from the study as planned have been detailed in the text and methods section.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study can be availed from the corresponding author upon reasonable request.

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