PROOFBOOK

Essential Support and Validation for Vollara's Science and Technology

vollara®

DISTRIBUTOR EDUCATIONAL MATERIAL

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This information is being provided to demonstrate the efficacy of our technology and show examples of actual application. The science and testing information in this book is intended for use by Vollara's Distributors.

PROFESSIONALSPORTS

FRESHAIR SURROUND | LIVINGWATER
RE:FUEL | RE:FLEX

Texas Rangers
1000 Ballpark Way
Arlington, TX 76011
March 27, 2011

texasrangers.com

Joseph P. Urso
Chairman
Vollara
Suite 1010
Dallas, TX 75240

Dear Joe:

I want to take this opportunity to express my appreciation for your staff and your Vollara products. Julia and Ron have been great to work with as we introduce your products to our team. Their passion and knowledge of the products have reassured me that I'm providing our players with high quality products.

As a strength coach, I am very selective of the products I supply my players. Right now there are a lot of nsf certified products, but not all provide the results your Vollara products have provided us thus far. We started using the water units this spring and the comments from the staff and players have been great. The water tastes better and the players feel better. I have also introduced the refuel and reflex to our players and the comments have also been very positive.

It is a long season so my strongest focus is on recovery. The refuel and reflex are the most complete products that I've ever tried. I look forward to seeing the long term effects after a long season. I am confident that your products will help my players perform better and recover faster. Last year we came up short so hopefully the Vollara edge will help us bring a world championship to Arlington.

Sincerely,

Jose Vazquez,PT,CSCS
Strength and Conditioning Coach
Texas Rangers

KANSAS CITY ROYALS

April 14, 2011

Joseph P. Urso, Chairman
Vollara
5420 LBJ Freeway
Suite 1010
Dallas, TX 75240

Dear Joe,

I wanted to take a moment to thank you and the Vollara staff for all the help you've given to our organization during both spring training and the regular season. In particular, Julia Chiappetta and Ron Chaves did an outstanding job of working with us to make sure that all of our needs were met. When choosing products that can impact the health, performance and overall well being of our athletes, I always consider the quality and reliability of the products, the cost effectiveness of the products, and the ability of customer service to be as helpful and informative as possible. Once we began to compare Re: Fuel, Re: Flex, and Living Water to the competition, Vollara clearly stood above the rest in all of these categories.

As we move forward through this season I feel confident that the products and support we are receiving from Vollara will help give our players the edge they need to perform at their very best. Thanks again for your role in helping the Kansas City Royals strive for excellence.

Sincerely,

Ty Hill
Strength and Conditioning Coach
Kansas City Royals

KANSAS CITY ROYALS

April 9, 2011

Joseph P. Urso, Chairman
Vollara LLC
5420 LBJ Freeway Suite #1010
Dallas, TX 75240

Dear Mr. Urso,

The Kansas City Royals are constantly searching for ways to improve our athletes' health and performance. This past off-season, our organization conducted extensive research into water ionization technology in hopes to achieve both. After sampling numerous brands, we chose Vollara as our sole provider based upon value and performance.

This Spring Training, we created 100% of the water distributed in our complex in Surprise, AZ and each of our seven practice fields using only two Living Water units. Water consumption was at and all time high, and soft tissue injuries were at an all time low. With Spring Training completed, we have since purchased additional units for each of our Minor League affiliates and our Major League team in Kansas City.

Not all water is created equal and neither are the companies that provide this technology. On behalf of our entire medical staff, I would like to thank you and all the members of Vollara for their assistance in bringing us an edge on the competition.

Thank you,

Ryan Stoneberg
Kansas City Royals
Minor League Strength & Conditioning Coordinator

Toronto Blue Jays Baseball Club
The Bobby Mattick Training Center @ Englebert Comple
1700 Solon Ave.
Dunedin, Florida USA 34698

Joseph P Urso, Chairman
Vollara LLC
5240 LBJ Freeway
Suite 1010
Dallas, TX 75240

Dear Mr Urso,

I am so happy that I had the opportunity to meet Dr. Troy Sanford, Julia Chiappetta, and Ron Chaves, and learn about Vollara products at the 2010 Baseball Winter Meetings.

With over a decade of experience as the Strength and Conditioning Coordinator for the Toronto Blue Jays, I have seen numerous products come and go in the industry—very few have had the potential impact of the water and air purity improvements Vollara offers. I am always looking for an effective long-term solution to the continuing decline in our water source, as well as eliminating the airborne toxins that are present in all of our facilities. Consequently, Vollara's Fresh Air Surround and Living Water units have found a permanent place in our most used and highly populated Bobby Mattick Training Complex in Dunedin, FL.

It's important to note that I would not install any products for athlete use that I would not also use myself. In this case, these products are of particular importance to my family since my two-year-old son Luke was recently diagnosed with Autism Spectrum Disorder. He has many challenges, including a higher susceptibility to pesticides, toxins, and airborne pollutants. I am currently using the Fresh Air Surround and have been transporting Living Water home daily for him to drink. I value these products as another resource in our battle to help Luke overcome the challenges of his disorder and live up to his full potential. There is nothing more important to my wife and me than ensuring we employ all possible means to help him.

I want to personally thank Dr. Troy, Julia, Ron, and all the members of Vollara for their vision and help. I am grateful for the opportunity to implement use of their products for our players, staff, and, especially, my family.

Yours truly

Donovan T. Santas CSCS
Strength and Conditioning Coordinator
Toronto Blue Jays Baseball Club

ROGERS™

nationals.com

Washington Nationals Baseball Club
Nationals Park
1500 South Capitol Street SE
Washington DC 20003-1507

September 25, 2010

Julia Chiappetta
Vollara
5420 LBJ Freeway
Suite 1010
Dallas, TX 75240

Subject: Vollara Products

Dear Julia,

As Head Athletic Trainer for the Washington Nationals I am inundated daily with companies vying for attention and access to our athletes. Mike McGowan, Asst Athletic Trainer and I routinely research these products and discuss them with our team physiciansWe scrutinize these offers and products closely and usually find them to be just another company trying to get attention in our market.

I wanted to thank you for your professional approach in providing us information regarding your products and yourpatience in allowing us to evaluate these products as part of our training regimen.

We currently offer NSF approved Vollara products, Re:Flex and Re:Fuel to our athletes. We are using the Vollara Living Water unit as part of our hydration strategy foour athletes and also utilize the Fresh Air Surround in our athletic training room to provide a clean, fresh atmosphere.

I will recommend your company to my peers and thank you for your accessibility and informational approach regarding your products.

Sincerely,

Lee Kuntz, MA, ATC
Head Athletic Trainer
Washington Nationals

PITTSBURGH PIRATES
PNC Park at North Shore
115 Federal Street
Pittsburgh, PA 15212

www.pirates.com

2/17/11

Joseph P. Urso, Chairman
Vollara
5420 LBJ Freeway
Suite 1010
Dallas, TX 75240

Dear Joe,

It has been a great pleasure getting to know you, Dr. Troy Sanford, Julia Chiappetta, Ron Chaves and the rest of the Vollara family. I very much enjoyed touring the Vollara International Headquarters in Dallas, TX (in November 2010). Witnessing firsthand the company's contagious passion, shared vision and relentless pursuit to be the best in the business was refreshing and impressive.

As the Strength and Conditioning Coach for the Pittsburgh Pirates Baseball Club I am very selective of the supplement and/or wellness products that we bring to the clubhouse for player use. The Vollara line of products that are certified for sport through the NSF are of highest quality. The Reflex and Refuel supplements definitely have a place in the daily routine of a professional athlete seeking optimal performance.

What really gets my heart pumping are the Living Water and Fresh Air units. Not everyone is into the use of nutritional supplements and that's fine. But everyone must drink water and breathe air. The Living Water empowers us to raise alkalinity for healthier water. The Fresh Air Surround cleans surfaces and helps us breath cleaner air that is free of bacteria, mold and other contaminates.

We feel very good about equipping our ball players with Vollara's best in class products. Our job is put them in the best position to use their talents, night after night without missing a beat. Our athletes need to feel great, perform at higher levels, recover faster and compete every night. The smallest edge can lead to a win and ultimately a championship. That's what we are playing for here in Pittsburgh and that is why we feel confident using the Vollara products.

Yours in Optimal Health and Wellness,

Frank Velasquez Jr.
Strength and Conditioning Coach
Pittsburgh Pirates Baseball Club

May 24, 2011

Mr. Joseph P. Urso
Chairman, Vollara LLC
5420 LBJ Freeway
Dallas, Texas 75240

Dear Mr. Urso,

Vollara represents the best-in-class products in air purification, water ionization alkalization and nutritional supplements. The Living Water is having an especially big and positive impact throughout the Royals Organization.

With the amount of stress we place on our bodies each and every day, there is no question that ionized alkaline water will help keep us in prime condition. The lack of sleep, travel and demanding schedule puts a lot of stress on our bodies. The negative ORP and alkalinity are vital to our success on the field by keeping us super hydrated and strong.

The players in our organization have been drinking the 9.5 pH water for a few months now. From my own experience, I have been sleeping better and find my workouts can go a lot harder and longer. We also use the *Super Water* or acid water for cleaning and sanitizing the weight rooms so we don't have to use the harsh detergents and chemicals.

My players are drinking more water then ever before and loving the results. We put the Living Water unit to the test against other machines side-by-side. The new Direct Disk Ionization in the Living Water won each and every time. We know it is the future of water ionization / alkalization. Thank you on behalf of the entire Royals Organization.

Yours in health and fitness,

Joey Greany M.S, CSCS, PES
Strength and Conditioning Coach
Northwest Arkansas Naturals
Double-A Affiliate of the Kansas City Royals

Mr. Joseph P. Urso
Chairman
Vollara
Suite 1010
Dallas, TX 75240

Dear Joe:

I would like to take this time as we turn the page on 2011 and look forward to the prospect of 2012, to thank you for this past year. Not only did you provide us with great products like the ReFlex and the ReFuel, but also an alkaline water unit for the Major League clubhouse that worked great and got a tremendous amount of use. Being at altitude, hydration demands are greater so the alkaline water unit really help our players to stay hydrated this season.

Most impressively has been the knowledge and professionalism exemplified by Julia and Ron. It has been a pleasure working with them this past year and I look forward to working with them in future as well.

Lastly, thank you for providing some of the best products on the market that are safe and effective for all of our players.

I wish you a great 2012 and I am excited for the opportunity to work with you guys in the future!

Sincerely,

Brian Jordan, RSCC,*D
Major League Strength & Conditioning Coach
Colorado Rockies

Colorado Rockies Baseball Club • Coors Field • 2001 Blake Street • Denver, Colorado 80205-2000 • Phone (303) 292-0200 • Fax (303) 312-2116

PROFESSIONAL SPORTS

MEDICALTEAM

VOLLARA MEDICAL & SCIENTIFIC ADVISORY BOARD

Richard G. Urso, M.D.

Dr. Richard Urso graduated from the University of Connecticut. In medical school, Dr. Urso graduated with honors, was an Alpha Omega Alpha member and ranked number one in his class in both medicine and surgery. He completed his ophthalmology residency at the University of Texas Southwestern in Dallas. Dr. Urso then concluded his fellowship in Oculoplastics and Reconstructive Surgery at the University of Texas Medical Branch in Galveston. He has been on the faculty at the University of Texas Medical School at Houston in the Department of Ophthalmology and Visual Sciences for the past 12 years, teaching medical students and residents. For 6 years, Dr. Urso was the leading ocular oncologist at MD Anderson Cancer Center.

Dr. Urso is board certified by the American Board of Ophthalmology. He is affiliated with the Harris County Medical Society, Texas Medical Association, and Texas Ophthalmic Association. Dr. Urso has a patented drug treatment for delayed corneal wound healing and diabetic foot ulcers. He has also written numerous articles and abstracts relating to a variety of topics in the field of ophthalmology. With respect to his knowledge and talent, Dr. Urso is considered to be one of the country's most prominent ocular trauma surgeons.

His honors include Outstanding Teacher in Ophthalmology from UT Houston Medical School, Memorial Hermann Healthcare Hero Award, Dedication and Service Award from Harris County Fraternal Order of Police #39, and America's Top Ophthalmologist in Oculoplastics, Reconstructive Surgery and Refractive Surgery.

Dr. Urso and his wife, Stacy are heavily involved with the West University Softball Association and Little League. They have five wonderful children, Alessandra, Ellie, Catherine, Joey and Anthony.

Martin Schalling, Ph.D.

Dr. Martin Schalling received his Ph.D in Molecular Neurobiology from the Karolinska Institutet. He received his postdoctoral in Molecular Genetics from Massachusetts Institute of Technology. He is currently a Professor of Medical Genetics (Neurogenetics) at the Karolinska Institutet. He is also head and founder of the Neurogenetics group at the Karolinska Institutet and a visiting professor for the Department of Psychiatry at the University of California, San Diego.

Dr. Schalling serves as a scientific advisor for the Scandinavian Clinical Nutrition AB. Scandinavian Clinical Nutrition AB focuses on research and development of nutraceuticals, which are food supplements with clinically proven and scientifically documented efficacy and safety. He has played an important role in establishing SCN and its research and development work.

He serves as a board member for Scandivir AB. Scandivir is a joint venture between industry and science. Their primary focus is the obesity virus Adenovirus-36 (Ad-36). Believing the virus has contributed to the global obesity epidemic, Scandivir's aim is to create diagnostic kits to make it possible to see if a person is infected by Ad-36.

Dr. Schalling is a member of several societies including the American Society for Human Genetics, the American Association for the Advancement of Science, the International Brain Research Organization and the American Society for Neuroscience. In addition to his numerous scholarships and awards, including number 1 ranking for full professor in Molecular Genetics from Lund University, Dr. Schalling is the author of more than 220 published scientific articles and manuscripts. He serves on the Board of Directors at the Center for Molecular Medicine. He is the founder and board member of SAB Appetite Control, Inc. He serves on the advisory board for Max Planch Institute for Psychiatry and German Network for Depression Research. He also serves on the editorial board for Current Psychiatry Reviews and for Genes, Brain and Behavior.

Dr. Martin Schalling is married to Ellika Schalling. They have three daughters and one son.

Richard L. Atkinson, M.D.

Richard L. Atkinson, M.D. graduated from the Virginia Military Institute and the Medical College of Virginia, Richmond. He took residency and fellowship training in Endocrine-Metabolism at UCLA Harbor General Hospital, Torrance, California, and fellowship training in Endocrine-Metabolism at Walter Reed Army Hospital. He has been on the faculty of the University of Virginia; University of California, Davis; Eastern Virginia Medical School; and the University of Wisconsin, Madison, where he is Emeritus Professor of Medicine and Nutritional Sciences. He currently is Clinical Professor of Pathology at Virginia Commonwealth University; Visiting Professor of Molecular Medicine, Karolinska Institute, Stockholm, Sweden; Director of the Obetech Obesity Research Center, Richmond, VA; and President of Obetech, LLC. He is Editor of the International Journal of Obesity; Regional Vice-President of The Obesity Society; Past President and Co-Founder of the American Obesity Association, Past President of the North American Association for the Study of Obesity, and Past President of the American Society for Clinical Nutrition. NAASO-The Obesity Society established the annual Richard Atkinson-Judith Stern Public Service Award in 2006 to honor his service to the field of obesity. Dr. Atkinson has been a consultant to the National Institutes of Health, National Academy of Sciences, US Department of Agriculture, Department of Defense, Department of Veterans Affairs, Food and Drug Administration, Federal Trade Commission, and numerous companies and foundations. He has been involved in obesity research and treatment for over 30 years. He is interested in obesity policy and has advocated for young investigator programs nationally and internationally. Recently, his research has focused on virus-induced obesity. He and his research group demonstrated that a human adenovirus (Ad-36) produces obesity in animals and is associated with obesity in humans. Dr. Atkinson has published over 175 manuscripts and over 200 abstracts in the medical literature.

James Marsden, Ph.D.

James Marsden joined the ASI faculty in 1994 as the Regent's Distinguished Professor of Meat Science. He has a 100% research appointment. He also serves as the Associate Director of the National Agriculture Biosecurity Center – located at KSU.

His research focus has been on the safety of meat products. This work has included the control of E. coli 0157:H7 in raw ground beef and other processed beef products and Listeria monocytogenes in processed meats. He also acts as the Senior Science Advisor for the North American Meat Science Association and has been involved in food safety training for the meat industry. Dr. Marsden is the author of numerous publications and book chapters on food safety and quality and is the recipient of awards for research and teaching.

He serves on a number of Advisory Boards for companies that provide food safety technologies to the meat industry and is a regular contributor to the television program –"World Business Review with Alexander Haig". He has also appeared on numerous television news programs as a food safety expert.

He enjoys spending time with his wife and five children and two grandchildren. His hobbies include collecting rare books, music and theater.

SCIENTIFICSTUDIES

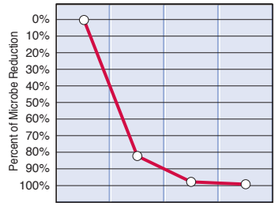
FRESHAIR SURROUND | RE:SIST

Effects of RCI™ Technology

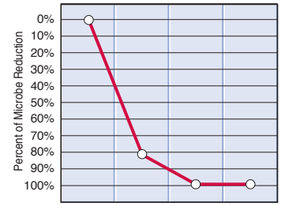
on reducing common bacteria and fungi on **surfaces** in 24-hour testing.

vollara®

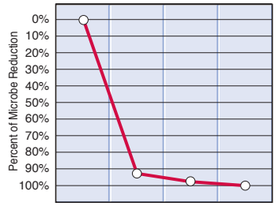
S. aureus Average of two 24-hour tests



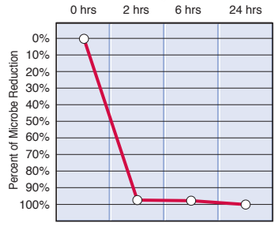
E. Coli Average of two 24-hour tests



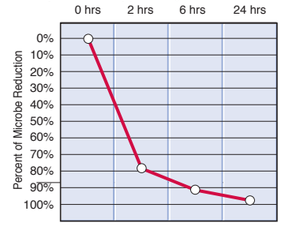
Bacillus spp. Average of two 24-hour tests



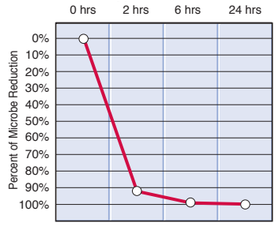
S. aureus r Average of two 24-hour tests



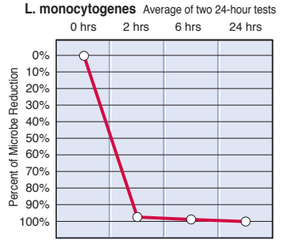
Streptococcus spp. Average of two 24-hour tests



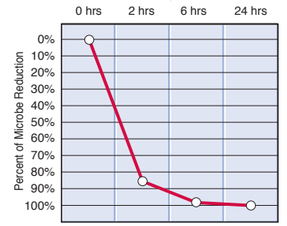
Pseudomonas spp. Average of two 24-hour tests



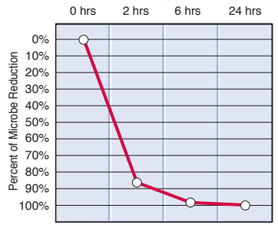
L. monocytogenes Average of two 24-hour tests



C. albicans Average of two 24-hour tests



S. chartarum Average of two 24-hour tests

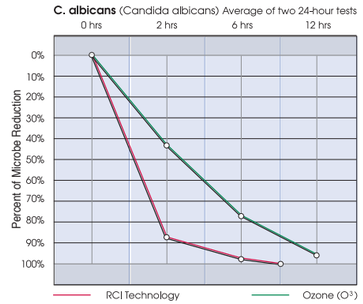


Comparing the Effects of RCI Technology and Ozone Technology

on reducing common bacteria and fungi on **surfaces*** in 24-hour testing.

Testing by Kansas State University. Field results may vary based on environmental conditions.

C. albicans (Candida albicans) Average of two 24-hour tests



Summary of Test Results – Biological Reductions using RCI
(Ozone at .02 ppm):

- Staphylococcus aureus : ..... **98.5% reduction**
- MRSA - Staphylococcus aureus (Methicillin Resistant): ..... **99.8% reduction**
- Escherichia coli : ..... **98.1% reduction**
- Bacillus spp. : ..... **99.38% reduction**
- Streptococcus spp. : ..... **96.4% reduction**
- Pseudomonas aeruginosa : ..... **99.0% reduction**
- Listeria monocytogenes : ..... **99.75% reduction**
- Candida albicans : ..... **99.92% reduction**
- Stachybotrys chartarum : ..... **99.93% reduction**

*Scientific tests have demonstrated the use of Vollara air purifiers substantially reduce microbial populations on **surfaces** – including but not limited to Escherichia coli, Listeria monocytogenes, Streptococcus spp., Pseudomonas aeruginosa, Bacillus spp., Staphylococcus aureus, Candida albicans, and S. chartarum. Presently Vollara does not make a similar claim with respect to airborne microbials. These statements have not been evaluated by the FDA. These products are not intended to diagnose, treat, cure, or prevent any disease.

Fig. 1 Decontamination of highly polished stainless steel surfaces using the *Radiant Catalytic Ionization (ActivePure) Cell*

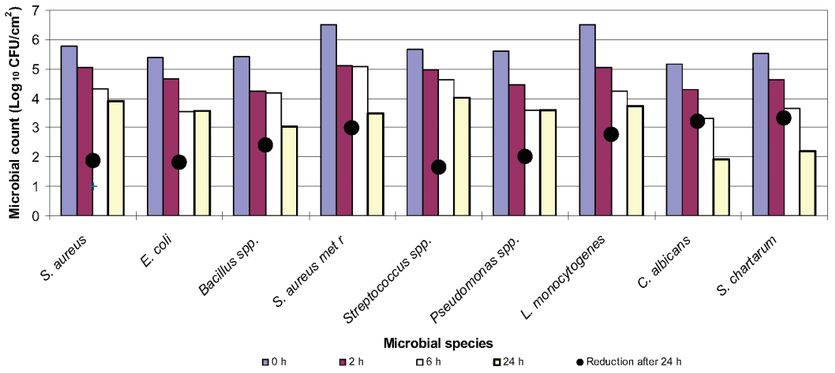
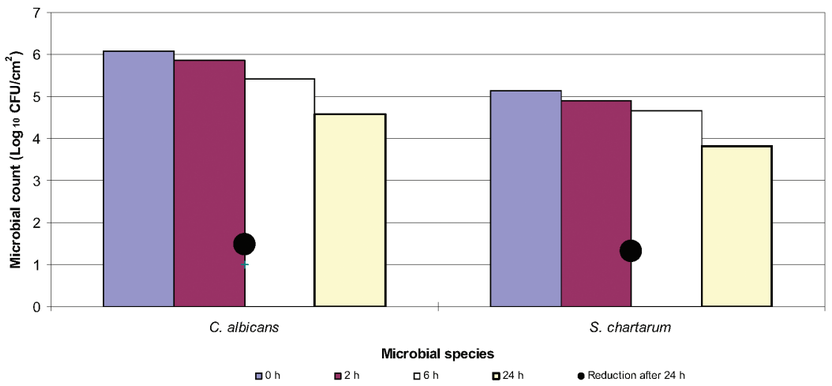


Fig 2. Ozone decontamination on highly polished stainless steel surfaces using the *Breeze AT Ozone* generator



Isolation of Three High Molecular Weight Polysaccharide Preparations with Potent Immunostimulatory Activity from *Spirulina platensis*, *Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa*

Nirmal Pugh[1], Samir A. Ross[1, 2], Hala N. ElSohly[2], Mahmoud A. ElSohly[2, 3], David S. Pasco*[, 1, 2]

[1] Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, Mississippi, USA
[2] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, Mississippi, USA
[3] Department of Pharmaceutics, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi, USA

Received: October 6, 2000; Accepted: March 11, 2001

Abstract: **This research describes the identification of three new high molecular weight polysaccharide preparations isolated from food-grade microalgae that are potent activators of human monocytes/macrophages: "Immulina" from *Spirulina platensis*, "Immunon" from *Aphanizomenon flos-aquae*, and "Immurella" from *Chlorella pyrenoidosa*. These polysaccharides are structurally complex and have estimated molecular weights above ten million daltons. All three polysaccharides are highly water soluble and comprise between 0.5% and 2.0% of microalgal dry weight. Immunostimulatory activity was measured using a transcription factor-based bioassay for nuclear factor kappa B (NF-kappa B) activation in THP-1 human monocytes/macrophages. Using this system the EC_{50} values for these microalgal polysaccharides are between 20 and 110 ng/ml (about 10pM). THP-1 activation was confirmed by measuring immune cytokine mRNA induction using reverse transcriptase-polymerase chain reaction (RT-PCR). Each polysaccharide substantially increased mRNA levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These polysaccharides are between one hundred and one thousand times more active for *in vitro* monocyte activation than polysaccharide preparations that are currently used clinically for cancer immunotherapy.**

Key words: ***Aphanizomenon flos-aquae*** **(Nostocaceae),** ***Spirulina platensis*** **(Oscillatoriaceae),** ***Chlorella pyrenoidosa*** **(Oocystaceae), polysaccharide, THP-1 monocytes, nuclear factor kappa B.**

Introduction

During the last several decades there has been an increasing interest in the commercial production of food-grade microalgae for human consumption. Among the various microalgae that have been explored for their commercial potential, *Spirulina* species, *Chlorella* species and *Aphanizomenon flos-aquae* are three major types that have been successfully produced and that are in widespread use.

Studies on the consumption of food-grade microalgae have reported enhanced immune function in both animals and humans. Oral administration of *Chlorella vulgaris* has been correlated with enhanced granulocyte-macrophage progenitor cells in mice infected with *Listeria monocytogenes* (1). Dietary *Spirulina* species increases macrophage phagocytic activity in chickens (2) and exhibits chemopreventive effects in humans (3). Human consumption of *Aphanizomenon flos-aquae* has been reported to produce changes in immune cell trafficking and enhanced immune surveillance (4). The active components for all these effects have not been conclusively established.

In the present study we have identified robust macrophage stimulating activity in the crude extracts of *Spirulina platensis*, *Aphanizomenon flos-aquae*, and *Chlorella pyrenoidosa*. Our objective was to isolate and characterize the compound(s) responsible for this activity. Macrophage activation was evaluated using a luciferase reporter gene based bioassay where luciferase expression is driven by the binding of NF-kappa B. The activation of transcription factor NF-kappa B coordinates gene expression and regulates many immune and inflammatory responses in activated monocytes/macrophages (5).

Materials and Methods

Materials

Freeze-dried microalgae were purchased from the following sources: *Spirulina platensis* (Lot No. B16933, MISS accession No. 63118) from Triarco Industries, Inc. (Wayne, NJ), distributed through General Nutrition Corporation; *Aphanizomenon flos-aquae* (Lot No. 0110FA, MISS accession No. 63116) from Cell Tech (Klamath Falls, OR); and, *Chlorella pyrenoidosa* (Lot No. VP0978, MISS accession No. 63117) from Sun Chlorella (Torrance, CA). MISS accession numbers refer to voucher specimens deposited at the Pullen Herbarium (MISS), Department of Biology, The University of Mississippi, University MS 38677. Bacterial lipopolysaccharide (*E. coli*, serotype 026:B6) and polymyxin B were obtained from Sigma Chemical Co. Carrington Laboratories Inc. (Irving, TX) provided two different preparations of acemannan: *Aloe vera* mucilaginous polysaccharide (AVMP, Lot. No. 11586) and Manapol (Lot. No. 116018). Schizophyllan polysaccharide was a gift from Dr. David Williams. The polysaccharide lentinan was also a gift from Dr. Yukiko Maeda (Lot. No. 2L832). JHS Natural Products (Eugene, OR) generously provided the polysaccharide krestin (PSK).

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THP-1 human monocytes were obtained from American Type Culture Collection (Rockville, MD). LucLite™ luciferase reporter gene assay kit was purchased from Packard (Downers Grove, IL). NF-kappa B plasmid construct (pBIIXLUC) was a gift from Dr. Riccardo Dalla-Favera that contains two copies of NF-kappa B motif from HIV/IgK (6). Reverse Transcriptase (RT)-PCR kits were obtained from Promega (Madison, WI) and for RNA isolation the TRI Reagent® system was used (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR primers for IL-1β, TNF-α and GAPDH were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Isolation procedure

Freeze-dried microalgae (35 g *Spirulina platensis*, 125 g *Aphanizomenon flos-aquae* and 35 g *Chlorella pyrenoidosa*) were extracted three times with 70% ethanol at 40 °C, 4 hours each time. Ethanol extracts were evaporated to dryness and then solvent partitioned between water and chloroform (1 : 1), followed by further partitioning of the water layers with *n*-butanol (water : *n*-butanol, 63 : 37). The water layers from the second solvent partition was subjected to alcohol precipitation (water : methanol : ethanol, 1 : 2 : 3) at –80 °C for 24 hours. Precipitatable materials were passed through an ultrafiltration device with a 100,000 molecular weight cut-off polyethersulfone membrane (Centricon Plus-20 from Millipore, Bedford, MA). The retentates were subsequently washed several times with 3% KCl (w/v) to remove impurities that adhered (probably through ionic interaction) to the large molecular weight materials.

The high molecular weight retentates were analyzed using size exclusion chromatography (SEC). The set-up consisted of a Model 600E system controller, UK6 injector, Model 600 solvent delivery system, Model 401 differential refractometer and a Model 3396A Hewlett-Packard integrator. Analyses were performed at a flow rate of 1 ml/minute using HPLC grade water and a Shodex Ohpak KB-805 SEC column (300 mm length × 8 mm ID) held at 30 °C. The high molecular weight retentates from each microalgae contained predominantly one peak that eluted in the void volume: "Immulina" for *Spirulina platensis*, "Immunon" for *Aphanizomenon flos-aquae* , and "Immurella" for *Chlorella pyrenoidosa* . Estimation of the molecular weight for each peak was achieved by comparison with retention times for dextran standards (12,000, 0.1 million, 1.66 million and 5 – 40 million daltons).

Structural characterization

Carbohydrate content of the purified polysaccharides (Immulina, Immunon and Immurella) were estimated using a colorimetric assay based on reaction with phenol (5% w/v in water) and concentrated sulphuric acid. Absorbance was determined at 450 nm and 490 nm (7). Elemental analyses for carbon, hydrogen, nitrogen and sulfur was performed by Galbraith Laboratories, Inc. (Knoxville, TN). Glycosyl composition and glycosyl linkage analyses were performed by The University of Georgia, Complex Carbohydrate Research Center. The glycosyl composition was determined using GC-mass spectrometry analysis of the TMS-methyl glycosides. In order to identify the O-methylated sugars detected during the TMS-methyl glycoside procedure, glycosyl composition was also determined using the alditol acetate procedure (8). Glycosyl linkage analysis was performed using the Hakomori procedure (9), in combination with carboxyl-reduction in order to detect uronic acid linkages (10).

Macrophage assay

Macrophage activation was measured using a luciferase reporter gene assay in THP-1 human monocytic cells as previously described (11). This assay measures immunostimulatory activity as indicated by increased expression of an NF-kappa B-driven luciferase reporter. THP-1 cells are transiently transfected using DEAE-dextran and the pBIIXLUC reporter plasmid containing two binding sites for NF-kappa B. Activation is reported as a percentage relative to maximal activation of NF-kappa B by 10 μg/ml LPS.

RT-PCR for IL-1β , TNF-α and GAPDH

Detection of mRNAs for IL-1β and TNF-α was performed as previously described (11). In brief, total RNA was isolated from THP-1 cells using the TRI Reagent® method and RT-PCR reactions were run using kit reagents from Promega. Sequence for the primers were described in Su et al. (12). Total RNA amounts used in the reactions were not saturating.

Results and Discussion

The luciferase reporter gene bioassay for activation of NF-kappa B in human THP-1 cells was used to guide purification of the immunostimulatory polysaccharides. For all three microalgae, the same isolation procedure was used for purification. A crude extract for each microalgae was prepared by extracting the freeze-dried material with 70% ethanol. Extraction with 70% ethanol allowed for efficient separation of the active substance from the bulk of other inactive polysaccharides that would be isolated if a typical hot water extraction was employed (refer to additional details below). Crude extracts at 50 μg/ml (*Spirulina platensis*), 10 μg/ml (*Aphanizomenon flos-aquae*) and 25 μg/ml (*Chlorella pyrenoidosa*) increased NF-kappa B directed luciferase expression to levels 50% of those achieved by maximal concentrations (10 μg/ml) of LPS.

Semi-pure microalgal polysaccharides were obtained by a combination of solvent partitioning and alcohol precipitation. Final purification was accomplished by removal of all material less than 100,000 daltons using an ultrafiltration device (refer to experimental section). The high molecular weight polysaccharides were analyzed using size exclusion chromatography and were found to contain one peak: "Immulina" for *Spirulina platensis*, "Immunon" for *Aphanizomenon flos-aquae*, and "Immurella" for *Chlorella pyrenoidosa*. These polysaccharides have retention times between 5.2 and 4.8 minutes (estimated molecular weight above 10 million daltons) and are very water soluble at 10 mg/ml. By comparison, immunostimulant polysaccharides such as acemannan and β-glucans are difficult to dissolve even at low concentrations. Polysaccharides Immulina and Immurella comprise between 0.5 and 1.0% of the dry weight of *Spirulina platensis* and *Chlorella pyrenoidosa*, respectively. The percent composition of Immunon is higher and represents about 2.0% of the dry weight of *Aphanizomenon flos-aquae*.

In addition to our active polysaccharides, microalgae hot water extracts contain substantial amounts of other high molecular weight material (size exclusion chromatography, data not shown). Removal of these contaminating substances from our active polysaccharides could be difficult and time consuming. Hence, the initial extraction procedure using 70% ethanol provides an elegant method whereby the active polysaccharides can be separated from potentially interfering substances that would be present with the hot water extraction.

Fig. **1** presents a dose response for both LPS and the isolated microalgal polysaccharides. The EC_{50} (50% of maximal LPS induction) values for NF-kappa B directed luciferase expression were as follows: Immulina at 110 ng/ml, Immunon at 20 ng/ml, Immurella at 80 ng/ml, and LPS at 250 ng/ml. To confirm THP-1 macrophage activation by purified microalgal polysaccharides, mRNA levels of proinflammatory cytokines IL-1β and TNF-α were measured using RT-PCR (Fig. **2**). Treatment of THP-1 cells with either LPS or microalgal polysaccharides resulted in a dramatic increase in both IL-1β mRNA (810 bp) and TNF-α mRNA (444 bp), as compared with the control. This was not the case for the mRNA of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1000 bp) (Fig. **2**).

It is possible that the observed NF-kappa B activation by Immulina, Immunon and Immurella was due to endotoxin contamination of the preparation. To address this possibility two experiments were conducted. First, polymyxin B (10 μg/ml) was added in combination with each polysaccharide (0.1 to 1 μg/ml) to observe whether there was any abrogation in NF-kappa B activation. Polymyxin B is a polycationic antibiotic known to block many of the biological effects of LPS by binding to the lipid A portion of the molecule. All three microalgal polysaccharides were insensitive to polymyxin B addition (data not shown). Addition of polymyxin B to LPS (10 μg/ml) suppressed NF-kappa B activation by 75%. The second experiment used to examine possible endotoxin-mediated effects was to look for the presence of 3β-hydroxymyristate in the glycosyl composition analysis. In sample preparations of Immulina and Immurella there were no detectable levels of 3β-hydroxymyristate. Thus, it is unlikely that the observed macrophage activation by Immulina and Immurella is due to endotoxins.

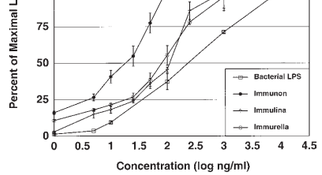

Fig. 1 Dose response for Immulina polysaccharide, Immunon polysaccharide, Immurella polysaccharide, and bacterial LPS activation of NF-kappa B in THP-1 monocytes/macrophages at 4 hours. Samples run in quadruplicate. Means ± standard deviation.

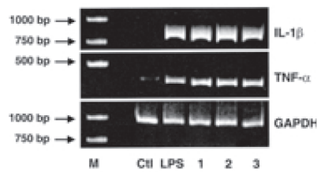

Fig. 2 Microalgal polysaccharides Immulina, Immunon and Immurella enhance proinflammatory cytokine mRNA production. RT-PCR results for IL-1β mRNA, TNF-α mRNA and GAPDH mRNA in THP-1 cells at 2 hours: (M) PCR marker, control, bacterial LPS at 10 μg/ml, (1) Immunon polysaccharide at 0.5 μg/ml, (2) Immulina polysaccharide at 0.5 μg/ml, and (3) Immurella polysaccharide at 0.5 μg/ml.

Using a colorimetric assay (7) with phenol-sulphuric acid at 450 nm and 490 nm, the carbohydrate content of each isolated microalgal polysaccharide was estimated to be between 90% and 100%. This further supports the view that these compounds are predominantly polysaccharides. Treatment of Immunon and Immulina preparations with either heat (100 °C for 30 minutes) or one of the following enzymes (0.1 mg/ml at 37 °C for 1 hour): DNase 1, RNase A, trypsin, proteinase K, papain and α-chymotrypsin did not alter their EC_{50} values for macrophage activation. The activity of Immurella was not influenced by most of these enzymes, but it was reduced by 50% with heat treatment and by 25% with proteinase K. This suggests that although the biological activity of Immunon and Immulina is not due to nucleic acid or proteins, Immurella may contain either a protein contaminant or a peptide component to its structure that contributes to its activity. Coomassie blue based protein determinations indicate 2% protein for Immurella. Enough material was available for Immunon that elemental analysis was also performed and was found to contain the following elements: 49.1% carbon, 40.8% oxygen, 7.62% hydrogen, 2.46% nitrogen and trace amounts of sulfur.

However, in two different sample preparations of Immunon, small amounts of 3β-hydroxymyristate (0.6% of total peak area) were detected. In order to determine how much "endotoxin-like" material was present, six samples of *Aphanizomenon flos-aquae* were analyzed using the Limulus amebocyte lysate (LAL) assay (analysis performed by BioWhittaker, Walkersville, MD). The amount of LAL positive material detected using this assay represented 0.002% of microalgal dry weight. By comparison, the percent composition of Immunon is about 1000 times greater (2.0% of microalgal dry weight). This means that at the concentration required to produce maximum NF-kappa B activation by Immunon (100 ng/ml), the total amount of potential LAL positive material present would be 100 pg/ml. This concentration of endotoxin would not be detectable using our THP-1 assay system. Therefore, the stimulatory effect of Immunon on macrophage activation is not due to endotoxin contamination.

Glycosyl composition and glycosyl linkage analysis for each polysaccharide is summarized in Tables **1** and **2**. Due to the high volatility of terminal residues, especially deoxyhexoses and pentoses, reported values for these components in Table **2** may be lower than the actual levels. Based on their glycosyl

compositions, glycosyl linkages and molecular weights, all three microalgal polysaccharides are new compounds that have not been previously reported. Interestingly, all three polysaccharides contain high levels of both methylated carbohydrate residues and deoxyhexoses (e.g., rhamnose and fucose) which may explain their extractability with 70% ethanol. Due to the complex nature of these polysaccharides having a variety of glycosyl linkages (refer to Table **2**), the anomeric configurations for each linkage have not yet been determined.

Neither the chemical structures nor the macrophage stimulating activity of our microalgal polysaccharides have been reported in the scientific or patent literature. Various other compounds have however been isolated from the microalgae studied in this paper. From *Spirulina* and *Chlorella* species a number of polysaccharides have been characterized for their antitumor, antiviral and immunostimulating activity (13), (14), (15), (16). In contrast, no such compounds have been isolated from *Aphanizomenon flos-aquae* showing any biological activity.

From *Chlorella* species a number of polysaccharides have been identified that possess biological activity. In U.S. Patent 4,533,548 an acidic polysaccharide was isolated from *Chlorella pyrenoidosa* that exhibits antitumor and antiviral activity (13). The glycosyl composition for this polysaccharide was mostly rhamnose, with minor amounts of galactose, arabinose, glucose and glucuronic acid. This glycosyl composition is distinctly different from Immurella which contains arabinose, galactose and rhamnose as the major components. Another polysaccharide, isolated from marine *Chlorella minutissima*, reported in U.S. Patent 4,831,020 appears to have tumor growth-inhibiting effects. However, no molecular weight or glycosyl composition was reported (14).

From *Spirulina* species several different types of polysaccharides have been isolated that exhibit biological activity. For example, the sulfated polysaccharide calcium spirulan exhibits antiviral properties and is composed of rhamnose (52.3%), 3-*O*-methylrhamnose (32.5%), 2,3-di-*O*-methylrhamnose (4.4%), 3-*O*-methylxylose (4.8%), trace amounts of other sugars and sulfate (15). The molecular weight of calcium spirulan (74,600 daltons) is about 100 times less than Immulina (above 10 million daltons).

In U.S. Patent 5,585,365 an antiviral polysaccharide was isolated using hot water extraction from *Spirulina* species with a molecular weight between 250,000 and 300,000 datlons (16). This polysaccharide is composed of rhamnose, glucose, fructose, ribose, galactose, xylose, mannose, glucuronic acid and galacturonic acid. Both the glycosyl composition and molecular weight of Immulina is different than this polysaccharide.

Pharmaceutical development of Immulina, Immunon and Immurella as immunostimulants may reveal a significant potential for immunotherapy. These polysaccharides have superior macrophage stimulatory activity compared with clinically used polysaccharide preparations. There are three major fungal polysaccharide immunostimulants in clinical use for a variety of human cancers: schizophyllan, lentinan and krestin (17). These pharmaceuticals are used primarily in Japan either alone or in combination with chemotherapy and/or radiotherapy. Another polysaccharide, acemannan (Carra Vet®, isolated from *Aloe vera*), is licensed by the United States Department of Agriculture for the treatment of fibrosarcoma in dogs and cats (18). In our macrophage bioassay these four commercial polysaccharide immunostimulants (schizophyllan, lentinan, krestin and acemannan) were at least one thousand times less active than our microalgal polysaccharides (data not shown). These results agree with *in vitro* studies demonstrating that these clinically used polysaccharides have weak/modest effects on macrophage function (18), (19), (20). Successful development of these microalgal polysaccharides would add to the arsenal of available agents for immunotherapy in the treatment of cancer and infectious diseases.

Table 1 Glycosyl composition for isolated polysaccharides from *Spirulina platensis* (Immulina), *Aphanizomenon flos-aquae* (Immunon) and *Chlorella pyrenoidosa* (Immurella). Data obtained from one experiment

Immulina Polysaccharide		Immunon Polysaccharide		Immurella Polysaccharide	
Glycosyl Residue	Mole %	Glycosyl Residue	Mole %	Glycosyl Residue	Mole %
Rhamnose	35.4	Mannose	16.0	Arabinose	31.6
Glucuronic acid	9.7	Glucose	13.1	Galactose	26.8
Fucose	7.7	4-Me-Mannose	11.2	Rhamnose	12.4
Galactose	7.1	Rhamnose	10.3	Glucose	5.4
2-Me-Rhamnose	5.9	2-Me-Rhamnose	8.1	3-Me-Arabinose	3.0
Xylose	5.5	Galactose	8.0	3-Me-Mannose	2.5
3-Me-Rhamnose	4.2	Fucose	7.0	Xylose	2.4
3-Me-Xylose	4.2	*N*-Acetyl-galactosamine	7.0	4-Me-Arabinose	2.4
4-Me-Rhamnose	3.9			Mannose	2.3
Glucose	3.6	*N*-Acetyl-glucosamine	5.8	Ribose	1.9
Mannose	2.4	Xylose	4.8	2,4-di-Me-Arabinose	1.3
Galacturonic acid	2.0	2-Me-Fucose	3.1	3-Me-Galactose	1.2
3-Me-Galactose	2.0	3-Me-Galactose	2.6	3-Me-Xylose	0.9
Arabinose	1.8	3-Me-Arabinose	1.8	3-Me-Rhamnose	0.9
amino sugar	1.5	Arabinose	1.6	3,5-diMe-hexose	0.9
2,3-diMe-Fucose	1.2	2,3-diMe-Arabinose	1.2	6-Me-Galactose	0.7
N-Acetyl-glucosamine	0.9			Glycerol	0.5
2-Me-Glucose	0.5			2-keto-3-deoxy-Octulosonic acid	0.5
Glycerol	0.4			2,3,6-triMe-Mannose	0.4
				3,6-diMe-Mannose	0.4
				2,3-diMe-Mannose	0.4
				2-Me-Galactose	0.4
				N-Acetyl-galactosamine	0.3
				N-Acetyl-glucosamine	0.3
				amino sugar	0.3

Note: Methyl groups are represented by "Me".

Table 2 Glycosyl linkage analysis for isolated polysaccharides from *Spirulina platensis* (Immulina), *Aphanizomenon flos-aquae* (Immunon) and *Chlorella pyrenoidosa* (Immurella). Data obtained from one experiment

Immulina Polysaccharide		Immunon Polysaccharide		Immurella Polysaccharide	
Glycosyl Linkage	% total area	Glycosyl Linkage	% total area	Glycosyl Linkage	% total area
3-Rha + T-GlcA	25.8	2-Man + 3-Man	13.4	T-Galactose (*f*)	12.2
4-Galactose	7.8	4-Rha + T-Man	10.6	2-Glucose	9.2
4-Glucuronic acid	7.3	2-Rhamnose	7.6	6-Galactose (*p*)	8.6
3,4-Glucuronic acid	6.9	T-Rhamnose	7.5	2,3-Rhamnose	8.4
2-Rhamnose	5.7	3-Rhamnose	6.9	T-Glucose	5.9
3-Fucose	5.1	2-Glucose	5.3	T-Arabinose (*f*)	5.5
2,3-Rhamnose	4.9	2-Galactose	4.8	2-Arabinose (*f*)	5.4
T-Xylose (*p*)	4.8	2-Fucose	4.7	3,6-Galactose	5.1
4,6-Galactose	4.3	3,4-Fucose	4.5	2,3,6-Galactose	4.9
T-Rhamnose	4.2	4-Glucose	4.4	T-Man + 3-Rha + 4-Rha	3.7
3,4-Fucose	3.1	3-Xylose	4.4	2,3-Arabinose (*f*)	3.3
3,4-Galacturonic Acid	2.4	4-Fuc + T-Gal	4.3	T-Arabinose (*p*)	2.8
2-Man + 3-Man	2.2	T-Xylose	3.2	6-Gal (*f*)	2.6
4-Fucose	2.2	unidentified	2.7	3-Hexose (*f*)	2.4
T-Fucose	2.2	T-Fucose	2.5	3-Galactose	2.3
3,4-Rhamnose	2.1	4-Mannose	2.2	2-pento (*f*)	2.1
2-Glucose	1.5	2-Arabinose (*p*)	2.1	4-Glc/2,4-Ara(*p*)/2,5-Ara(*f*)	2.1
2,3-Mannose	1.4	4-Galactose	2.1	T-Xylose (*p*)	1.8
3-Glucose	1.2	2,3,6-Galactose	2.1	4,6-Galactose	1.9
3-Galactose	1.1	3-Galactose	1.4	4-Galactose	1.9
4-Mannose	1.0	3,5-Ara(*f*)/3,4-Ara(*p*)	1.3	3,4-Galactose	1.7
6-Mannose	0.8	2,6-Glucose	1.2	T-Galactose (*p*)	1.4
2,6-Glc + 4,6-Glc	0.8	6-Mannose	0.6	3-pentose (*f*)	1.3
3-Xylose	0.7			3,4-Rhamnose	1.1
4-Xylose	0.6			2-Mannose + 3-Mannose	1.1
				3-Arabinose (*f*)	1.0
				2,6-Glucose	0.5

Note: All glycosyl linkages are also 1-linked unless otherwise specified. Glycosyl abbreviations represent the following: "Man" for mannose, "Rha" for rhamnose, "Glc" for glucose, "Fuc" for fucose, "Ara" for arabinose, "Gal" for galactose, "GlcA" for glucuronic acid, "T" for terminal linkage, "*f*" for furanose, and "*p*" for pyranose. Presence of two or three glycosyl units indicates co-elution of components during analysis.

Acknowledgements

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COMMUNITYCONTRIBUTION

Defense Department Tokens of Appreciation for ActivePure (RCI) technology.

Two Department of the Army DSS-W coins were originally presented in appreciation for the donation of ActivePure air purification equipment following the attack on the Pentagon on September 11, 2001.

“Like the rest of the country, we saw the terrible destruction and wanted to help,” said field leader Mike Jackson. “With some help from contacts within the government, we learned that the smoke and fire damage left lingering odors in the Pentagon.”

“That is tough, because people still had to go work in their offices the next day. That’s where we knew we could help.” The ActivePure technology in FreshAir is scientifically proven to eliminate smoke and odors in the air. By installing the equipment, employees at the Pentagon immediately noticed a difference.

“We received a letter thanking us for the donation, along with the Defense Supply Service coins. That meant a lot to us,” Jackson said. “But a month later, we received another letter from an Army Colonel letting us know the products were really helping, and we were most touched. As he put it, he ‘witnessed the tremendous improvement in the air quality in the offices.’ That meant a lot to us, because we really wanted to help make that environment more livable.”

DIRECT SELLING ASSOCIATION
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April 26, 2011

Mr. Joe P. Urso
Chairman & CEO
Vollara, LLC
5420 LBJ Freeway, Suite 1010
Dallas, TX 75240

Dear Joe:

Since its founding in 1910, membership in the Direct Selling Association has symbolized a commitment to the highest standards in business ethics. When a company can display the DSA logo, it means the company and its leaders have made an investment in the success of each individual who does business with the company – be it a customer of the products or a customer of the opportunity.

Through its DSA membership and requisite pledge to abide by a rigorous Code of Ethics, Vollara, under the leadership of Joseph Urso, has committed itself not only to maintain a high standard of excellence in its own operations, but also to promote those practices industry-wide.

The Code sets the bar at a high level, but for those who believe in the benefits of ethical leadership, there is no alternative. Throughout his involvement in direct selling, and now at Vollara, Joseph Urso has been a constant champion of this position. Through mentorship of other direct selling executives, service on DSA's Board of Directors and his belief in empowering people to achieve their full potential, Joe has exhibited his commitment to ensuring direct selling remains a viable path for those who seek independence, flexibility and personal growth.

The appeal of direct selling is often rooted in what one can do for oneself as well as what one can do for others. There is great satisfaction in personal achievement as well as in helping others achieve their goals. The opportunity and products offered by Vollara have Mr. Joe P. Urso certainly made this a reality for many, and under Joe's leadership there are sure to be many more people who achieve this same success.

Sincerely,

Neil H. Offen
President and CEO
Direct Selling Association

NHO:mlr

88 Hamilton Avenue
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AmeriCares®

October 31, 2011

Mr. Joseph P. Urso
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Dear Mr. Urso:

Thank you for Vollara's generous donation which will help AmeriCares in fulfilling our mission to save lives and restore health and hope to those in need around the world.

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Your extraordinary donation of two tractor-trailer loads – **1,223 cases** of high quality products for women – has already been placed on shipments to our partners in Ghana, Honduras, Nicaragua and the Philippines. I know that we have more shipments building. Your donation will have a huge positive impact on thousands and thousands of people around the world.

Thank you for caring and sharing your wealth and hope with people in need.

Sincerely,

Emanuela Chiaranda
Associate Director, Corporate Relations

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Kevin Hickey, Executive Vice President & Chief Operating Officer
Vollara
5420 LBJ Freeway, Suite 1010
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Best regards,

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