Total Bacterial Load within *Echinacea purpurea*, Determined Using a New PCR-based Quantification Method, is Correlated with LPS Levels and *In Vitro* Macrophage Activity

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**Abstract**

Our previous studies indicate that the majority of *in vitro* monocyte/macrophage activation exhibited by extracts of *Echinacea* depends on bacterial components. In the present study, total bacterial load was determined within *E. purpurea* samples and ranged from $6.4 \times 10^6$ to $3.3 \times 10^8$ bacteria/g of dry plant material. To estimate total bacterial load, we developed a PCR-based quantification method that circumvents the problems associated with nonviable/nonculurable cells (which precludes using plate counts) or the coamplification of mitochondrial or chloroplast DNA with the use of universal bacterial primers (which precludes the use of qPCR). Differences in total bacterial load within *Echinacea* samples were strongly correlated with the activity (NF-κB activation in THP-1 cells) and content of bacterial lipopolysaccharides within extracts of this plant material. These results add to the growing body of evidence that bacteria within *Echinacea* are the main source of components responsible for enhancing innate immune function.

**Introduction**

Clinical trials testing *Echinacea*’s (Asteraceae) potential for preventing and/or treating the common cold are difficult to interpret because they have used a wide variety of chemically ill-defined preparations. A review [1] of clinical trials using *Echinacea* found that while many studies have reported some benefit in the treatment of common colds, others have failed to show any efficacy. At present, one of the most problematic factors for conducting such trials is in the selection of *Echinacea* material or extract type. This is due to the incomplete identification of therapeutically relevant components that would be used to standardize these products. In addition, despite decades of research, it is not known if *Echinacea*’s main therapeutic action on colds and the flu is immunostimulatory, anti-inflammatory, or a combination of both. *In vitro* [2–4] and animal research [5–7] indicates that this botanical exerts both therapeutic actions, and *Echinacea* alkylamides represent the major anti-inflammatory component. With respect to the immune-enhancing potential of *Echinacea*, our research [8] suggests that bacterial Braun-type lipoproteins and lipopolysaccharides (LPS) were responsible for over 97% of the *in vitro* macrophage activation exhibited by extracts of *Echinacea* and seven other botanicals traditionally used to enhance immune function. The contribution of bacterial components within extracts to macrophage activation was assessed using agents that targeted bacterial Braun-type lipoproteins (lipoprotein lipase) and LPS (polymyxin B). These biochemical approaches were also used to determine that variations in the content of these two bacterial components were responsible for the up to a 200-fold difference in *in vitro* macrophage/monocyte activation potential exhibited by commercially diverse *E. purpurea* and *E. angustifolia* bulk material obtained from six North American suppliers [4]. It is our hypothesis that differences in bacterial content and/or bacterial type are responsible for the substantial variation in the innate immune-enhancing activity exhibited by this botanical.

The objective of the present study was to directly assess total bacterial load within *E. purpurea* root and herb (aerial) samples, and to determine if differences in bacterial load correlate with *in vitro* macrophage activation of the plant material. To estimate the total bacterial load, we developed a PCR-based quantification method that circumvents the problems associated with nonviable/nonculurable cells (which precludes using plate counts) or the coamplification of plant mitochondrial or chloroplast DNA with the use of universal bacterial primers (which precludes the use of qPCR). Differences in total bacterial load within *Echinacea* samples were strongly correlated with the activity (NF-κB activation in THP-1 cells) and content of bacterial lipopolysaccharides within extracts of this plant material. These results add to the growing body of evidence that bacteria within *Echinacea* are the main source of components responsible for enhancing innate immune function.

**Key words**
- *Echinacea purpurea*
- Asteraceae
- macrophage activation
- lipopolysaccharide
- bacteria
- bacterial load
- PCR method
chloroplast DNA with the use of universal bacterial primers (which precludes the use of qPCR).

Materials and Methods

**Echinacea purpurea** (L.) Moench plant material
Bulk root and herb (aerial) material for *E. purpurea* were obtained from the following six commercial suppliers: Frontier Natural Products Co-op, lot number 50811.2331, Gaia Herbs, lot numbers 00033874 and 00033507, Glenbrook Farms Herbs & Such, lot numbers not available, Mountain Rose Herbs, lot numbers 11987 and 12066, Richters, lot numbers 21283 and 21580, and Trout Lake Farm LLC, lot numbers EPR-K6041-BCP and EPHS2051-E3P.

Individual plants of *E. purpurea* were cultivated at the University of Mississippi. Root and herb parts were harvested by washing extensively to remove soil and then immediately freeze-dried to prevent postharvest growth or introduction of bacteria. Voucher specimens for all *E. purpurea* plant samples were deposited in the NCNPR repository at the University of Mississippi (voucher numbers NP1019, NP1048, NP2001–NP2006, NP2008–NP2012).

**Alfalfa sprout germination and ampicillin treatment**
Organic alfalfa (*Medicago sativa* L., Fabaceae) seeds (Johnny’s Selected Seeds, lot #25089) were surface sterilized by soaking in 10% chlorox/0.1% Tween-20 for 12 min followed by four rinses with sterile water. One hundred seeds were then transferred to each of two flasks containing either 10 mL sterile water alone or 10 mL sterile water containing ampicillin (30 µg/mL). Seeds were aseptically germinated for 7 days at ambient temperature under natural light and, on days 2, 4, and 6, treatment solutions were replaced with fresh solutions. Sprouts were freeze-dried after removal of hulls and unspouted seeds.

**Extraction of plant material for analysis of activity and content of LPS**
Finely ground plant material (50 mg for *E. purpurea* samples or 20 mg for alfalfa samples) was extracted four times with 95% ethanol (1.0–1.5 mL fresh solvent added and incubated at 75°C for 30–45 minutes for each extraction) to remove anti-inflammatory components. Ethanol extracted plant material was dried at 50–55°C and then extracted with 0.5 mL of water containing 4% SDS at 98°C for 1 h. Following removal of SDS using SDS-out reagent (Pierce) in the presence of 1% octylglucoside, crude extracts were assessed for activity and endotoxin levels.

**Monocyte activation assay and limulus amebocyte lysate (LAL) assay**
The THP-1 human monocyte cell line (American Type Culture Collection) was transected with a luciferase reporter gene construct containing two copies of NF-κB motif from HIV/IGK, and samples were evaluated as described previously [9]. Monocyte activation exhibited by *Echinacea* extracts is reported as an EC50 value that represents the concentration of plant material (µg/mL) required to activate cells to 50% of maximal activation of NF-κB by LPS (10 µg/mL, *E. coli*, serotype 026:B6; Sigma). The amount of LPS (bacterial endotoxin) in the extracts was determined using a limulus amebocyte lysate (LAL) assay [Chromo-LAL test kit with Glucashield® (1–3)-β-D-Glucan Inhibiting Buffer] from Associates of Cape Cod, Inc. Data is reported as endotoxin units (EU) per µg of dried plant and represents the average of duplicate determinations.

**Determination of plant total bacterial load**
DNA was extracted from a known mass (100 mg for *E. purpurea* samples or 20 mg for alfalfa samples) of ground plant material using PowerSoil DNA isolation kits (Mo Bio), followed by a clean-up procedure (PowerClean; Mo Bio) to remove potential PCR inhibitors. A portion of the bacterial 16S rRNA gene was amplified from each purified sample using the primer pair 791f (5′-AACMGATTAGATAACCCKG-3′) and 1492r (5′-GTTACCTTGT-TAGCACC-3′) described by Chelius and Triplett [10]. These primers exclude the coamplification of chloroplastic DNA, and potentially yield a bacterial product (approximately 735 bp) and a larger (1090 bp) mitochondrial product when used to amplify DNA extracted from the plant material [10]. Amplifications occurred in 50 µL reactions comprised of a 2 mM MgCl2 PCR buffer, 0.2 mM deoxyribonucleoside triphosphates, 0.4 µM of each primer, 1.0 U Taq polymerase, as well as 2 µL of sample DNA. Reactions consisted of 3 min at 95°C, followed by 30 cycles of 94°C (20 s), 50°C (40 s), 72°C (40 s), and a final extension of 72°C (7 min). Amplification products (5 µL) were visualized on 1.2% agarose gels and the intensity of the bacterial band was determined using a Kodak Gel Logic 200 imaging system running Molecular Imaging Software 4.0 (Eastman Kodak). A strain of *Pseudomonas* (NPC16) that we have previously isolated from freshly harvested *E. purpurea* (data not shown) was used as a reference bacterium to relate band intensity to bacterial load. *Pseudomonas* NPC16 was cultured in trypticase soy broth at 22°C and a sample was taken after 20 h (a time determined by plate counts to correspond to the late exponential phase). The sample was serially diluted, and the bacterial load in the culture was determined as the viable count in cells/mL. A second 20-h sample was taken, serially diluted, and each dilution centrifuged (6000 g, 10 min) to pellet cells, and the pellets were frozen for later DNA extraction. Results from the viable count allowed us to determine the bacterial load in each of these cell pellets, which ranged from 1.0 × 105 to 1.0 × 106 cells. DNA was extracted and amplified from each pellet using the same procedure as for the plant material. Intensity of each of the resulting amplification products in agarose gels was similarly determined to develop a standard curve–relating band intensity to bacterial load in cells.

**Statistics**
Simple linear regressions were used to relate bacterial load to PCR product band intensity for the standard curve samples. These regressions were then used to determine bacterial load in plant extracts based on their PCR product band intensity. Relationships between bacterial load, monocyte activation (as EC50), and the amount of LPS (endotoxin) in *E. purpurea* samples were examined by pairwise linear regressions. Because the values for each variable spanned a range of 2–5 orders of magnitude and were not normally distributed, data for each variable were log10 transformed prior to regressions [11]. All transformations and regressions were conducted in Microsoft Excel 2007.

**Results**
Using molecular methods (PCR, qPCR) to estimate total bacterial cell load is problematic since the use of universal 16S ribosomal bacterial primers is complicated by the coamplification of chloro-
plast and mitochondrial DNA. To overcome this problem, we used bacterial 16S rDNA primers that do not amplify chloroplast DNA 
(1090 bp) on agarose gels and the intensity of the bacterial band is then compared to band intensities of a standard curve generated using PCR products from known bacterial numbers. © Fig. 1a and b provide a representa-
tive standard curve illustrating linearity ($R^2 = 0.9938$) between $1.1 \times 10^6$ and $5.7 \times 10^8$ bacterial cells (concentration range of over two orders of magnitude). © Fig. 1c shows an example that demonstrates the usefulness of this method to determine differences in total bacterial load between bulk commercial *E. purpurea* plant samples that exhibit “low” and “high” monocyte stimulatory activity. An example is also provided that illustrates detection of total bacterial load in *E. purpurea* herb (aerial) and root samples from plant material cultivated at the University of Mississippi. These plant parts were freshly harvested, extensively washed to remove soil and surface bacteria, and then immediately freeze-dried to prevent postharvest growth or introduction of bacteria. To allow accurate detection of monocyte stimulatory components, the plant material was first extracted four times with 95% ethanol to remove alkylamides and other anti-inflammatory substances that are inhibitors of monocyte activation. Ethanol extracted plant material was then further extracted with 4% SDS (98°C, 1 h) to obtain the immune-enhancing components. *E. purpurea* root and herb (aerial) bulk commercial plant material used in our previous study [4] was also included in this research since they exhibit a wide range of *in vitro* monocyte/macrophage activity. The total bacterial load in plant samples was estimated using the PCR-based quantification method described above, and levels ranged from $6.4 \times 10^6$ to $3.3 \times 10^8$ bacteria/g of dry plant material. The results presented in © Fig. 2a show that the activity (NF-κB activation in THP-1 cells) exhibited by *Echinacea* extracts was correlated with the total load of bacterial cells in this plant material ($R^2 = 0.54$, $p = 0.004$). Likewise, © Fig. 2b demonstrates that the content of LPS as determined by the LAL assay was also correlated with the total bacterial load ($R^2 = 0.53$, $p = 0.005$). In support of our previous research [4], © Fig. 2c shows that there is a very strong dependence of monocyte stimulatory activity on LPS concentration in *E. purpurea* plant extracts ($R^2 = 0.88$, $p = 0.000003$).

We have previously used [7,8] alfalfa sprouts as a model system since extracts of this plant material exhibit high monocyte stimulatory activity and germination/harvest conditions are easily controllable. High levels of activity are observed in extracts of sprouts germinated under aseptic conditions using surface sterilized seeds and the appearance of this activity is suppressed by treatment with ampicillin and other antibiotics [8]. The results presented in © Fig. 3 provide further evidence that the activity detected in this system is of bacterial origin. In agreement with our previous research [8], extracts from sprouts grown in the presence of ampicillin for seven days exhibited undetectable activity at all concentrations tested as compared to control sprouts. © Fig. 3 insert shows that ampicillin treatment also substantially suppresses total bacterial load during the germination of alfalfa seeds. Total bacterial load estimates in cells/g dry weight of control sprouts versus ampicillin-treated sprouts were $7.7 \times 10^8$ and $6.3 \times 10^6$, respectively (a difference of 122-fold). The content of LPS was undetectable in both control sprouts and ampicillin-treated sprouts. Since only gram-negative bacteria produce LPS, this result suggests that most of the bacteria present were gram-positive. Ampicillin treatment did not influence the final biomass or outward appearance of the sprouts (data not shown).
We have previously reported [4] that the LAL-determined LPS shield®. Glucashield® reagent blocks the contribution of (1 → 3)-β-D-glucans in the LAL reaction. The use of this reagent is crucial during the analysis of plant extracts since trace levels of glucan are present from cellulosic material and from fungal/bacterial origin. When the Echinacea extracts were rerun in the presence of Glucashield®, endotoxin units decreased by several orders of magnitude (data not shown). Maximal in-ducing concentrations of LPS gives 100 RLA. (Insert) DNA was extracted from freeze-dried sprouts, amplified with bacterial 16S rDNA primers, and PCR products were visualized on an agarose gel ("B" and "M" signify bacterial and mitochondrial bands, respectively).

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**Discussion**

In the present study, we have developed a method to assess total bacterial load (culturable and nonculturable/nonviable bacteria) within both freshly harvested plants and commercially diverse dried bulk material. Using this method, we show that the activity (NF-κB activation in THP-1 cells) and content of LPS exhibited by Echinacea extracts is strongly correlated with the estimated total bacterial load of these plant samples.

In our initial experiments to estimate total bacterial load in Echinacea plant material, we used culture-dependent techniques. However, we found that less than 1% of the activity exhibited by extracts of the plant material was accounted for by the activity of extracts from the culturable bacteria (data not shown). This suggested that a culture-dependent method was problematic due to nonviable/nonculturable bacteria and/or potential cell loss during processing (filtering of homogenate before plating). Plant-derived immunostimulatory agents could not account for the observed discrepancy in activity since we have shown that the majority of the activity from this plant material is due to bacterial components [8]. Further experiments attempting to determine bacterial load using flow cytometry were also ineffective because of plant mitochondria interference (data not shown). Since molecular methods (PCR, qPCR) using standard universal bacterial primers are complicated by the coamplification of chloroplast and mitochondrial DNA, we developed a PCR quantification method using 16S rDNA primers that do not amplify chloroplast DNA. Bacterial PCR products are separated from mitochondrial products on agarose gels and the intensity of the bacterial band is then compared to band intensities of a standard curve using known bacterial numbers (PCR amplified using the same primers). This simple method has not been previously described and can be used to estimate total bacterial load in both dried and freshly harvested plant material (Fig. 1).

We have previously reported [4] that the LAL-determined LPS content of Echinacea extracts was correlated with in vitro macrophage stimulatory activity. In the present study, these extracts were reanalyzed using the Chromo-LAL test kit with Glucashield®. Glucashield® reagent blocks the contribution of (1 → 3)-β-D-glucans in the LAL reaction. The use of this reagent is crucial during the analysis of plant extracts since trace levels of glucan are present from cellulosic material and from fungal/bacterial origin. When the Echinacea extracts were rerun in the presence of Glucashield®, endotoxin units decreased by several orders of magnitude (data not shown). Maximal in-ducing concentrations of LPS gives 100 RLA. (Insert) DNA was extracted from freeze-dried sprouts, amplified with bacterial 16S rDNA primers, and PCR products were visualized on an agarose gel ("B" and "M" signify bacterial and mitochondrial bands, respectively).
Although it is our hypothesis that the bacterial load detected in around the roots or from the leaf surface [13, 14]. The isolation associated with animals, most plants are colonized by bacterial In ways that are analogous to the commensal bacteria naturally 

vation exhibited by extracts of E. purpurea (1.2 × 10^7 and 3.1 × 10^7 bacterial cells/g of dried root and herb, re-

study indicates that it is possible to obtain a high bacterial load (more than 10 000-fold) from extracts of different bacterial iso-

mune the extent that the type of bacteria in addition to its preva-

lates (unpublished data). Future research is required to deter-

regression by Akao et al. reported that oral consumption of a hot water Spirulina extract reduced tumor growth in mice through enhancement of NK cell activity via a TLR2- and TLR4-dependent 

process [19]. The high levels of TLR2 (Braun-type lipoproteins) and TLR4 (LPS) agonists in Echinacea may contribute to the therapeutic action of this plant material. For example, these bacterial components may explain the enhanced NK cell activity observed in mice by dietary administration of Echinacea [6, 20].

Oral ingestion of Echinacea plant material containing a high bacterial load may have effects similar to those reported in studies using probiotic bacteria. A recent study using human subjects evaluated the in vivo intestinal mucosal gene expression profile six hours after oral administration of heat-killed Lactobacillus plantarum [21]. Ingestion of dead bacteria was found to induce gene expression mainly involved in innate and adaptive immune responses. These effects may be most pertinent to the potential effect of bacteria within Echinacea since the majority of bacteria within dried plant material are nonviable.

The average bacterial load within the Echinacea plant material evaluated in this study is comparable to the daily therapeutic dose of live bacteria used in studies evaluating the effect of probiotics on disease resistance. We found that the average bacterial load in E. purpurea was 7 × 10^7 bacterial cells/g of dried plant material.

Touring a typical recommended dose [22] of 2.7–3.0 g Echinacea plant material/day, containing this average bacterial load would result in a daily dose of about 2 × 10^9 bacterial cells. This bacterial load within Echinacea is comparable to the daily dose of probiotics (between 5 × 10^7 and 2 × 10^10 CFU) that have been used in studies reporting statistically significant effects on various parameters related to the common cold and flu infections (reviewed in [23]). For example, a recent double blind, placebo-controlled study on the consumption of probiotics in 326 children (3–5 yrs old) reported statistically significant reductions over placebo during a six-month period with respect to incidence of fever (73%), coughing (62%), rhinorrhea (59%), and antibiotic use (84%) [24]. Additional research is required to evaluate whether the bacteria associated with Echinacea plant material can impart similar therapeutic effects on colds and flu infections.
to that observed in studies using oral consumption of live probiotics.

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Conflict of Interest

All authors declare that there are no conflicts of interest.

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