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Lactobacillus rhamnosus GG conditioned media modulates acute reactive oxygen species and nitric oxide in J774 murine macrophages

Yashaswini Seenappanahalli Nanjundaiah [a], David A. Wright [a], Anwar R. Baydoun [b], William T. O'Hare [a], Zulfiqur Ali [a], Zahangir Khaled [c], Mosharraf H. Sarker [a,*]

[a] School of Science and Engineering, Teesside University, TS1 3BA, UK
[b] School of Life and Medical Sciences, University of Hertfordshire, AL 10 9AB, UK
[c] Methodist Medical Center, Peoria, IL, USA

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ABSTRACT

Phagocytes such as macrophages are capable of detecting and killing pathogenic bacteria by producing reactive oxygen and nitrogen species. Formation of free radicals in macrophages may be regulated by probiotics or by factors released by probiotics but yet to be identified. Thus, studies were carried out to determine whether cell-free conditioned medium obtained from cultures of *Lactobacillus rhamnosus* GG (LGG-CM) regulate production of reactive oxygen species (ROS) and/or nitric oxide (NO) in macrophages. J774 macrophages in culture were loaded with either H_2DCFDA for monitoring ROS or with DAFFM-DA for NO detection. Free radical production was measured on a fluorescence microplate reader and changes were analysed by Cumulative sum (CuSum) calculations. Low concentration of LGG-CM (10% LGG-CM) or LPS did not cause any significant change in basal levels of ROS or NO production. In contrast, high concentration of LGG-CM (75% and 100%) significantly enhanced ROS generation but also significantly reduced NO level. These findings are novel and suggest for the first time that probiotics may release factors in culture which enhance ROS production and may additionally reduce deleterious effects associated with excessive nitrogen species by suppressing NO level. These events may account, in part, for the beneficial bactericidal and anti-inflammatory actions ascribed to probiotics and may be of clinical relevance.

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1. Introduction

Macrophages as professional phagocytes have been considered prominent participants in the response to acute infection and play a significant role in preventing infecting bacteria multiplying and damaging the host environment. Macrophages have complex mechanisms equipped with specialised receptors to recognise their targets. Upon internalisation of receptor-bacterial complex, macrophages initiate killing of the ingested microbes partly through the generation of free radicals species [1]. However, excessive production of ROS and NO during phagocytic activity may also contribute to the inflammatory process which although beneficial in killing bacteria may result in deleterious consequences, especially following an interaction of NO with superoxide radicals to form reactive nitrogen species (RNS) such as peroxynitrite.

There is considerable interest in the role of probiotic bacteria in the modulation of immune functions. It has been shown that probiotic bacteria activate host innate immune function by influencing phagocytic activity of their immune cells [2], and also by regulating release of pro and anti-inflammatory cytokines [3] or free radicals [4,5].

Secretory products from probiotic bacteria or its conditioned medium have also been shown immunomodulatory and providing health beneficial effects on the human gut epithelium both *in vivo* and *in vitro* [6–8]. However the exact mechanism via which the secretory products or conditioned medium cause their actions is currently not known, nor is it clear whether they could regulate ROS and NO production or mediate bacterial killing as is seen with whole cell probiotic cultures. In this study, we have therefore evaluated the influence of cell free *Lactobacillus rhamnosus* GG conditioned medium (LGG-CM) on ROS and NO production by the J774 macrophage cell line, and further evaluated this acutely during their phagocytic process of *Escherichia coli* in real time. Fluorescence analysis was used to monitor ROS or NO generation on a real time basis and the data were analysed using CuSum which offers a simple and rapid method for identifying sustained changes in real time under experimental situations [9,10]. This is however the first study to investigate a real time changes in acute

* Corresponding author.
E-mail address: m.sarker@tees.ac.uk (M.H. Sarker).

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ROS and NO productions using CuSum in *in vitro* experiments. Findings of this study may have clinical implications for balanced ROS and NO production to enhance bacterial killing while protecting against deleterious collateral tissue damage by activated macrophages.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM; 1000 mg/L glucose, 4 mM L-glutamine and 110 mg/L sodium pyruvate) was purchased from Gibco, UK. Foetal bovine serum and penicillin/streptomycin were from Sigma-Aldrich, UK. The LGG was grown from Culturelle® tablets. The fluorescent dyes H_2DCF-DA and DAFFM-DA were purchased from Molecular probes and Santa Cruz biotechnology respectively.

2.2. Cell culture and treatments

The murine macrophage cell line J774 was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Cells were seeded at a density of 50,000 cells/well in special clear bottom 96 well plates (Costar) and allowed to attach overnight.

Overnight cultures of *E. coli* HfrC were prepared by inoculation into nutrient broth (OXOID, UK) and incubated at 37 °C with oscillation at 150 rpm. Quantification of *E. coli* were performed by the agar spread plate method and also by measuring optical density reading at 600 nm.

2.3. The cell-free LGG-CM

The cell-free LGG-CM was prepared as described previously [8]. In brief, LGG were harvested from de Man, Rogosa and Sharpe (MRS) broth by centrifugation and washed twice with phosphate-buffered saline (PBS). Following centrifugation, LGG pellet was incubated in DMEM (10^9 CFU ml^{-1}) and cultured overnight at 37 °C. LGG-CM was produced by filtering the cultured DMEM through a sterile filter of 0.2 μm pore size.

2.4. Determination of ROS and NO production from LGG-CM treated macrophages

Accumulation of intracellular ROS and NO produced by J774 murine macrophages in 96 well tissue culture plates were measured using a fluorescence microplate reader (Biotek). After removing the culture medium, the cells were washed once with pre-warmed PBS. Macrophages were then loaded with fresh medium containing 5 μM H_2DCFDA or 5 μM DAFFMDA for 45 min at 37 °C in 5% CO_2 in a humidified cell culture incubator to measure ROS and NO respectively [11]. The dye solution was then removed and cells again carefully washed twice with pre-warmed PBS. The prepared treatments were added to the cells in the presence and absence of *E. coli* at a multiplicity of 50:1. The fluorescence measurements were taken every two minutes approximately for the first 60 min to monitor ROS or NO production during bacterial ingestion and from 60 min to 280 min to monitor ROS and NO production during the digestion period. The fluorescence was measured at 485 nm excitation and 528 nm emissions.

2.5. Data analysis

Data are expressed as the mean ± standard error of the mean (S.E.M) as indicated in individual experiments. Statistical difference between the means was determined by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison post-hoc test. The analyses were performed using Prism version 3.00 for windows (GraphPad Software, USA).

CuSum was used in the analysis of fluorescence measurements to compare the rate of free radical production. The CuSum methodology is a simple technique that has frequently been applied to elucidate trends in time domain data. It works by comparing each data point to a reference or target value (k) and then cumulatively summing the differences [12,13]. If the underlying mean of the data points is constant the method produces a straight line whose slope is determined by the difference between the mean value and the chosen reference value. Small random variations are smoothed so the technique can reveal differences and changes in the mean value [13].

When analysing the data sets, raw experimental values (test values) were plotted out against time. The average of fluorescence measurements from control macrophages was used as k, the 'target value'. This was then used to calculate the CuSum values. The first CuSum C_o value is calculated using Eq. (1).

$$C_o = F_o - k.......... \quad (1)$$

Where F_o is the first fluorescence reading in the time series and k is the 'target' or reference value. The next and subsequent CuSum values are calculated using Eqs. (2) and (3)

$$C_1 = C_o + (F_1 - k)...... \quad (2)$$

and then

$$C_2 = C_1 + (F_2 - k)......... \quad (3)$$

and so on, where C_n is the nth CuSum value and F_n is the nth fluorescence reading in the time series.

3. Results

3.1. CuSum analysis of free radical and nitric oxide production

Fig. 1A and B show the characteristics of data for ROS and NO generation by J774 cells over an initial 60 min period acquired from controls and from cells treated with either menadione for ROS or L-arginine for NO production. Although there were small differences in the extent of change of fluorescence readings between control and experimental plates, the random variation in the measurements made these differences difficult to follow when the raw data were plotted. Smoothing of the data using the CuSum analysis clarified the difference between control and experimental results. The data in Fig. 1C shows the CuSum values for the same measurements presented as raw data in Fig. 1A. While the control line has no net slope (as F values did not change relative to k) the experimental line has an obvious slope (the F values were greater than k). This is also seen when comparing Figs. 1B and D. The calculated gradient (m values from linear trendline equation $y = mx + c$ from CuSum values) of ROS production from untreated macrophages was 0. However, the gradient of 0.5 mM menadione treated macrophages was 0.3. The gradient of NO production from untreated cells was 0.05 while from 100 μM L-arginine was found to be 0.29.

3.2. Effects of LGG-CM on ROS and NO production in murine J774 macrophages

The raw representative data in Fig. 2 (A, C, E, & G) did not show any clear trends or differences between the treatments and their respective controls. By comparison, the CuSum plots (Fig. 2 B & F)

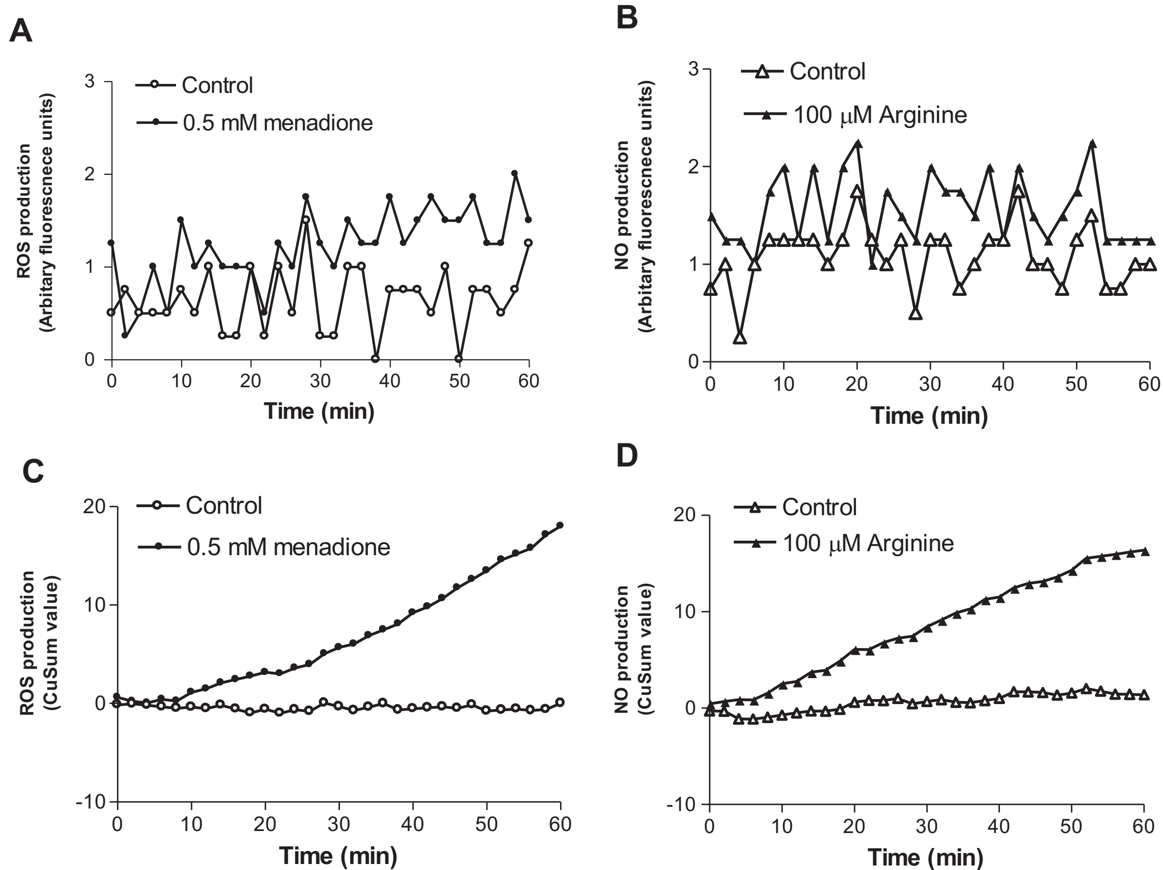

Fig. 1. Measurement of ROS and NO production by J774 macrophages. A illustrates the profile of ROS production from control and menadione treated macrophages and C represents the corresponding CuSum plots. B illustrates the profile of NO production from arginine treated macrophages and D represents the corresponding CuSum plots. The above graphs are representative of at least 3 independent experiments.

show graded responses to LGG-CM. In these studies, calculated CuSum gradient of ROS production in control was 0.0009. There was however an increased rate of ROS production in response to 75% LGG-CM treatment with gradient of 0.275. Cells treated with 20 μg ml^{-1} LPS showed little effect on ROS production (Fig. 2D). In Fig. 2F, NO level was found to be reduced to higher concentration of LGG-CM whereas LPS had little effect on NO level acutely (Fig. 2H).

3.3. Effects of LGG-CM on ROS production in murine J774 macrophages ingesting and digesting E. coli

The effects of LGG-CM on ROS production was investigated in the ingestion and digestion phase of *phagocytosis* by the J774 macrophages. Confirming the previous data above, LGG-CM treated macrophages at concentrations of 75% and 100% significantly increased the rate of ROS production acutely during the first 60 min of incubation (ingestion phase). These effects were not altered in the presence of LPS. Furthermore, treatment with LPS alone or 10% LGG-CM did not induce any changes in the extent of ROS production when compared to control (Fig. 3A). However, the rate of ROS productions during the digestion period (from 60 to 280 min of incubation) was found to be significantly slower than the rate of ROS production during the ingestion period (Fig. 3B). In our experiments the DMEM used to grow the bacteria did not have any significant effect on ROS or NO generation when used independently.

In Fig. 3C and D similar findings as above were observed when *E. coli* was added to all the above treatments. In the presence of *E. coli*, higher concentration of LGG-CM (75% LGG and 100% LGG) caused a significant increases in free radical production during the first 60 min of incubation (ingestion phase) as well as in the digestion phase. ROS production during the digestion period (from 60 to 280 min of incubation), however, shows a diminished rate as found above with higher LGG-CM treatment alone (in absence of *E. coli*).

3.4. LGG-CM decreases extent of nitric oxide in murine J774 macrophages

Macrophages were exposed to 20 μg ml^{-1} LPS, 100 μM arginine and various dilutions of LGG-CM or combinations of respective LGG-CM and LPS. Continuous measurements of NO carried out for the first 60 min (ingestion period) showed that there were significant reductions in the extent of NO in response to higher concentrations of LGG-CM treatments of macrophages ($P \leq 0.05$; Fig. 4A). There was no further change in NO level when *E. coli* was added to the treatments (Fig. 4C). LPS together with low concentration of LGG-CM (10%) alone and in combination of *E. coli* had no effect on NO production by J774 cells. However, there was an elevated level of NO production from macrophages treated with 100 μM arginine (Fig. 4A and B).

Measurement of NO by J774 macrophages was also measured between 60 and 280 min (digestion period) to see if there was any change in levels of NO during this period. No further significant change of NO were observed to various treatments with LGG-CM during the digestion period. However, there appeared to be a small increase of NO level to LPS or *E. coli* and low LGG-CM and *E. coli* treatments during the digestion period (Fig. 4D).

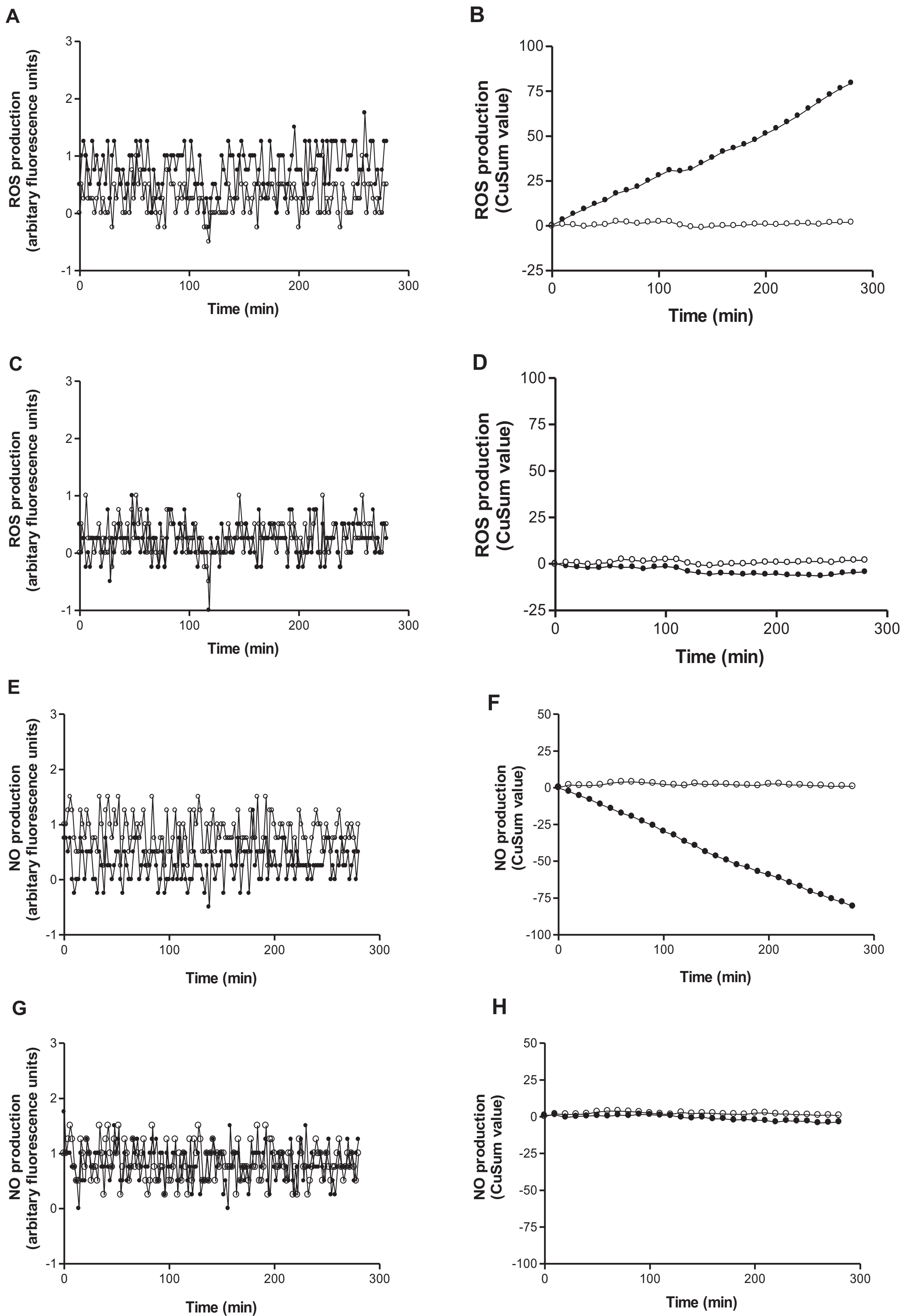

Fig. 2. ROS and NO production from J774 macrophages in the presence of LPS and LGG-CM. A, C, E, and G represents raw data of ROS and NO production recorded over 280 min for control cells and cells exposed to 75% LGG-CM & LPS 20μg ml^{-1}) and B, D, F and H represents their corresponding CuSum plots. The random variation evident in these figures make it very difficult to detect differences between control (o) and the treatments (●) to estimate the extent of ROS or NO production. However, their corresponding CuSum plots clearly demonstrate the gradient of ROS and NO production to different treatments.

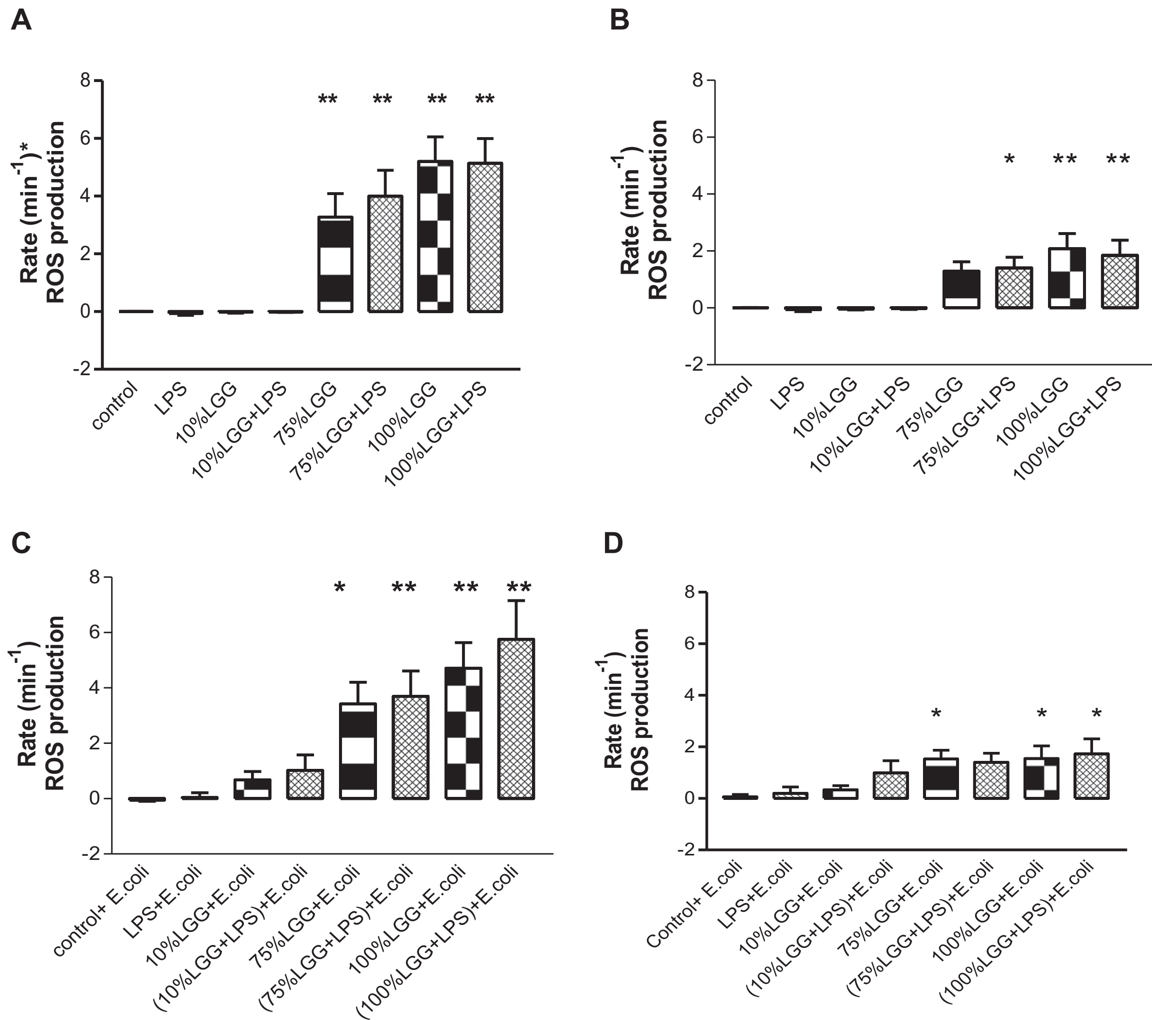

Fig. 3. Effect of LGG-CM on ROS production in J774 macrophage in the absence and presence of *E. coli*. Experiments were performed to investigate ROS production during the course of ingestion phase (A and C) and digestion phase (B and D) of phagocytosis. Macrophages were treated with cell free LGG-CM both in the presence (C and D) and absence of *E. coli* (A and B). The rate is estimated as average slope of the CuSum curve. Each value is a mean ± S.E.M (n=7). A * denotes $p < 0.05$ and ** denotes $p < 0.01$.

4. Discussion

Much interest has been generated in recent years regarding the role of probiotics in immunomodulation. In this study LGG (a probiotic strain of human origin with widely evidenced health effects) was used. Cell free LGG-CM has also been shown to have immunomodulation effectiveness similar to live bacteria [14] and this effect is thought to be due to the presence of low molecular weight proteins [15,16] or other soluble factors such as cell membrane polysaccharides [17] and glycoproteins [18] released by the probiotic bacteria into the culture medium. The LGG-CM has also been shown to modulate macrophage activity by enhancing phagocytic digestion of *E. coli* acutely [19]. The factors that may be present have not yet been identified nor has there been any clear demonstration that conditioned medium from cultures of probiotics regulates ROS and/or NO production or mediate bacterial killing. Studies were therefore initiated to address these issues using the fluorescent probes H_2-DCFDA and DAFFM-DA to detect ROS and NO respectively in cultured J774 macrophages. More importantly, measurements were carried out in real time and in the presence and absence of live bacteria (*E. coli*) to establish whether or how free radical generation may be influenced during the phases of ingestion and digestion of bacteria. In this study LPS (20 μg ml^{-1}), LGG-CM and *E. coli* HfrC had no effect on macrophage growth within the experimental period (unpublished data). LGG-CM medium did not show any influence on the growth of *E. coli* up to 24 h.

In this study, CuSum analysis was adopted due to the noisy nature of the data generated in real time using fluorescence probes. This technique has proved useful in monitoring the acute changes in the rate of free radical generation from pre-existing levels. The interpretation is most robust when detecting a subtle change or irregular fluctuations or shifts in free radical production as seen in the measurement of circadian change of Blood pressure for 24 h in hypertensive subjects [10] or in sustained change in air pollution level over few months [20]. The CuSum method is quite simple as it perceives the variation without assuming any functional form of the data. If the measured fluorescence value of free radical production from the experiment is equal to the target value (average fluorescence from untreated cells) the slope would be zero. But if the measured fluorescence value of free radical production from the experiment is greater than the target value, the slope will be a positive and vice versa. The greater the difference between the measured fluorescence value and the target value then the greater will be the slope. The slope of the plot over any given time period (the change in the CuSum over that period divided by the period duration) indicates the average deviation of free radicals generation from mean control ROS generation during that period. The CuSum plot facilitates the calculation of relative rate of ROS or NO production and the derivation between

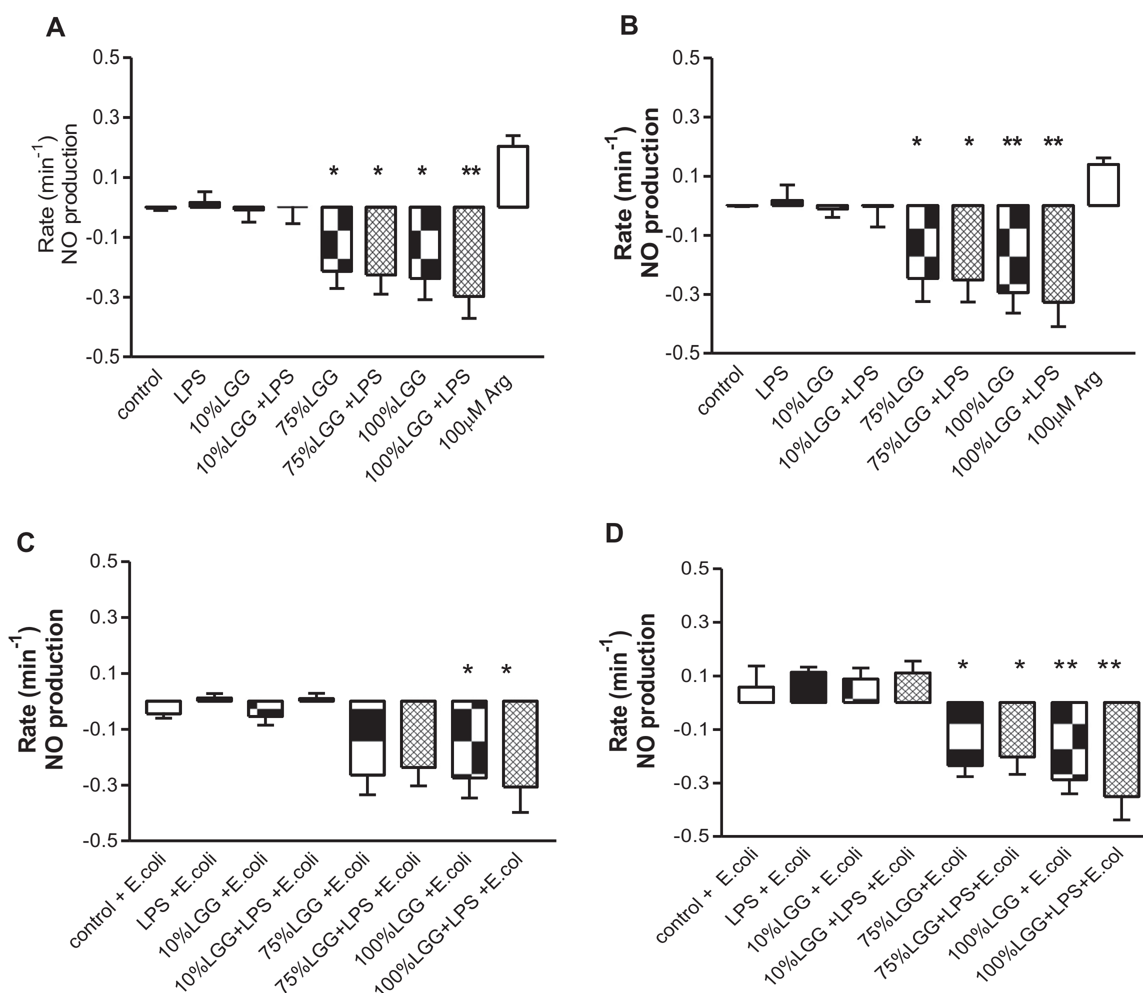

Fig. 4. Decrease in NO from J774 macrophages to LGG-CM. Experiments were performed to investigate the NO production during the course of bacterial ingestion Phase (A & C) and digestion phase (B & D). Macrophages were treated with cell free LGG-CM, both in the presence and absence of *E. coli*. Each value is a mean ± S.E.M of 3–7 experiments.* denotes $p < 0.05$ and ** denotes $p < 0.01$.

treatments. This was therefore considered an appropriate tool to use to analyse data generated from our studies.

In the present study, LGG-CM significantly increased the rate of ROS production and reduced the rate of NO production acutely in macrophage. The rapid increase of ROS generation was significantly higher within the first 60 min of treatment followed by lower rate of ROS production for the further 280 min. This pattern of ROS production was also maintained when macrophages were exposed to both non-pathogenic *E. coli* HfrC and LGG-CM together. LPS or diluted LGG-CM (10%) did not induce any significant ROS or NO production acutely. Both menadione and L-arginine were used as positive control for ROS and NO respectively. The 20 $\mu g\ ml^{-1}$ LPS, *E. coli* HfrC and LGG-CM were found to be well tolerated by J774 macrophage during the study period as the MTT assay did not show any evident of cytotoxicity (unpublished data).

Production of ROS and NO by macrophages are essential factors for macrophage mediated host defence mechanisms. Following phagocytosis, macrophages kill pathogens in the phagosome through ROS, generated by the activation of NADPH oxidase. Upon activation, the NADPH oxidase transfer electrons from cytosolic NADPH to molecular oxygen releasing superoxide into the phagosomal lumen. Inside the phagosome, oxygen free radical then converted rapidly to hydrogen peroxide (H_2O_2) by superoxide dismutase which in turn reacts with iron to generate highly reactive hydroxyl radicals (HO^-)$\cdot H_2O_2$ can also be converted by myeloperoxidase into hypochlorous acid and chloramines. Collectively, these highly reactive, toxic ROS and other reactive nitrogen species are shown to be highly effective as antimicrobial agents [21].

In macrophage NO is produced due to activation of inducible nitric oxide synthase (iNOS). However, unlike superoxide, NO is synthesized on the cytoplasmic side of phagosomes and diffuses across membranes to reach intraphagosomal targets where it can interact with ROS to generate a range of RNS, including nitrogen dioxide, peroxynitrite, dinitrogen trioxide, dinitrosyl iron complexes, nitrosothiols and nitroxyl radicals [22]. In the phagosome ROS and RNS synergize to exert highly toxic effects on microorganisms leading to inhibit their replication and ultimately death.

Excessive ROS and RNS generation causes oxidative stress which has been implicated in many disease processes. Disproportionate or prolonged ROS and RNS generation may result in non-specific oxidation of biomolecules and contribute to tissue injury. High ROS production has also been implicated to cause deleterious suppression of NF-κB mediated cell survival factors [23]. In gut epithelial cells, LGG has been shown to induce an increased ROS production through NADPH activation. However, this increase in ROS has also been suggested to play a role as an anti-inflammatory agent by inhibiting NF-κB through oxidative inactivation of the key regulatory enzyme Ubc12 [24,25]. Similar suppression of NF-κB activation was also demonstrated in liver cells to probiotic treatment [26]. However, this suppression was due to reduced activity of Jun N-terminal kinase (JNK); a

regulatory enzyme found to be modulated by ROS [27]. It seems that ROS mediated suppression of NF-κB may account for the physiological regulation of inflammatory susceptibility of the cells to native commensal bacteria.

Farnell et al. demonstrated that formalin killed LGG produces increased oxidative burst in chicken heterophils [28]. They found that this oxidative burst to probiotics bacteria was rapid and occurs in less than an hour. LGG has also been shown to produce ROS within 30 min of exposure in murine immature intestinal epithelia cell line [25]. It is plausible that dynamic changes of ROS production would be involved in the activation of signalling process. Detection of oscillating ROS signals in plant root hairs clearly demonstrates such possibility [29]. Mittler postulated that maintaining a nontoxic steady-state level of ROS, while allowing for the transient accumulation of ROS in particular subcellular locations could have physiological significance and could act as signals [30].

Previous studies have reported differential kinetics of ROS and NO production by macrophages in response to parasite infections. Gantt et al. detected ROS production 40 min after addition of opsonised promastigote to murine or human macrophages, whereas they were unable to detect any NO production even after 4 h of infection [31]. They were only able to detect NO after 48 h of infection. These observations are consistent with our studies where we also observed a rapid ROS production from macrophages, but failed to detect any enhanced acute NO production. Reduced rate of NO production from the control in our study is probably due to inactivation of NO by ROS. Interestingly there is evidence in the central nervous system that SOD could increase the toxic effects of NO by inhibiting the superoxide mediated inactivation of NO [32]. Nitric oxide can rapidly react with superoxide to generate the stable peroxynitrite anions. This pattern of elevated level of ROS leading to decrease in NO bioavailability has been described in endothelial cells [33]. Scavenging of superoxide by SOD has also been shown to increase the half-life of endothelium-derived relaxing factor (EDRF/NO) and to preserve the biological activity of NO [34]. Thus, it is likely that the acute reduced rate of NO production to LGG-CM in our study is probably due to enhanced ROS generation in macrophages.

In summary we have shown for the first time that by using CuSuM it has become possible to detect subtle changes in the trend in free radical production from macrophages treated with various treatments and thus can be used as a successful tool in analysing noisy *in vitro* data.

It is clear that LGG-CM may have some soluble components which activate macrophage to significantly increase the rate of ROS production both in the presence and absence of *E. coli*. In contrast, macrophages treated with LGG-CM demonstrated a significant decrease in the rate of NO accumulation. This skewing modulation of ROS and NO production is probably beneficial to the host as excessive production of NO might not only be useful to accelerate bacterial killing mechanism but could also potentially damage the host tissue. The ability of probiotic to differentially regulate NO and ROS generation may have clinical implications in several pathological conditions associated with bacterial infection and may prove useful in improving intestinal homeostasis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.003.

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