PROJECT REPORT

Chuckling Goat

September 2021

Functional effects of goats' milk Kefir on placental inflammation in vitro

Chuckling Goat currently produces a cultured fermented milk drink, kefir from goats' milk. Kefir is full of 'good' bacteria and is sold as a probiotic. There is ongoing research into specific health benefits of taking probiotics during pregnancy, with some studies claiming that probiotics can be used to treat pregnant women who have bacterial vaginosis, while others suggest that taking probiotics in pregnancy may reduce the likelihood of the baby developing eczema. This collaboration between Chuckling Goat and the Healthcare Technology Centre set out to examine the impact of goats' milk kefir on the biology of the human placenta/trophoblast cells *in vitro*.

Introduction

Kefir is a fermented drink with low alcohol content, it is acidic and bubbly from the fermentation carbonation of kefir grains with milk or water. Kefir varies from other fermented products because of the specific property of its starter culture, the kefir grains [1, 2]. Kefir grains range in size from 1 to 4 cm in length and look like small cauliflower florets in shape (irregular and lobed-shaped) and colour (from white to light yellow) [3]. This gelatinous and slimy structure is comprised of a natural matrix of exopolysaccharides (EPS) kefiran and proteins in which lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB) co-exist in symbiotic connection [2]. The most pre-dominantly found bacterial species in kefir grains are Lactobacillus kefiranofaciens, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Lactobacillus acidophilus, and Lactobacillus delbrueckii subsp. bulgaricus. On the other hand, Saccharomyces cerevisiae, S. unisporus, Candida kefyr, and Kluyveromyces marxianus ssp. marxianus are the predominant yeast species present in kefir [4]. The microbiota of kefir grains may differ depending on the geographical origin of the kefir grains, which are strictly connected to the climate conditions [2].

In recent years, numerous studies on the putative health values of kefir as a natural beverage with probiotic microorganisms and functional organic substances have been reported. According to the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), probiotics refer to live microorganisms which, when applied in sufficient amounts, bestow a health benefit to the host. Additionally, evidence has shown that kefir's exopolysaccharide, kefiran, has very significant physicochemical attributes and biological activities that certainly add value to the products [4, 5-7]. Existing reports have suggested important health benefits from kefir beverage consumption, such as anti-microbial, anti-tumour, anti-carcinogenic, hypocholesterolemic effects, anti-hypertensive, anti-diabetic, immunomodulatory activity, and also improving lactose digestion [8]. All these health-promoting properties are linked to the kefir microorganisms, their interplays, and their metabolic products during the fermentation process [2].

The reported benefits of probiotics are as an adjunctive or alternative treatment to a variety of diseases. In the year 2000, the United Nations Millennium Development Goals committed leaders of the world to eight goals, the fifth of which aimed to improve maternal health [9]. However, due to potential teratogenic effects of common prescription, over the counter, and supplementary medications on

organogenesis and embryonic development, the use of probiotics during pregnancy has become a topic of increasing interest for peripartum healthcare professionals and new mothers alike [10, 11]. In light of the rise of social media and the dramatic spread of information facilitated by the internet, physicians have been faced with more inquiries as families explore the use of probiotics in various pregnancy-related symptoms and complications [12-14].

As a biological process, pregnancy involves several variations in hormones and physiology of the person, which are prerequisites of ensuring appropriate fetal growth and weight gain [15]. The maintenance of maternal-fetal health could be established by proper nutritional supply during pregnancy. In this respect, the composition of the microbiota undergoes natural alterations in several sites of the body, including oral cavity, vagina, gut, breast milk, and placenta [16, 17]. However, there exist limited information on the association between gestational physiometabolic conditions and maternal microbiota.

Chuckling goat based in Llandysul is the UK's number one producer of kefir. To produce the highest grade of therapeutic kefir, Chuckling Goat, use goats' milk instead of cows' milk, which contain the allergenic A1 casein. Prior to the establishment of this collaboration between Chuckling Goat and the Healthcare Technology Centre, the company's kefir had been selected as the only probiotic recommended to over 6, 000, 000 pregnant women by the Royal College of Obstetricians as part of Mum Plus One. Mum Plus One is a magazine produced by B&Y Publishing Ltd in association with the RCOG. Mum Plus One is distributed free to pregnant women around the start of their second trimester and contains a collection of informative editorial features covering pregnancy and newborn issues, making it an essential guide to pregnancy, birth, and baby's first year.

Originally this collaboration was planned as an ambitious investigation, examining the impact of Chuckling Goats, goats' milk kefir in the immune response of pregnant women with a comparison made to non-pregnant women to determine i) if there was a broad immunomodulatory effect of the kefir and ii) if this differed in pregnancy. Furthermore, the investigation would extend to investigate any immunomodulatory effect on the human placenta, using primary human placental organ cultures. Following the March 2019 closure of the university due to the COVID-19 pandemic and the resulting limitation placed on research and access to clinical samples, the proposed collaboration was required to be reworked to ensure a feasible work plan was undertaken. This required the removal of all clinical samples and a shift to the use of immortalized trophoblast cell lines.

Trophoblasts (from Greek to feed: *threphein*) are cells forming the outer layer of a blastocyst, which provides nutrients to the embryo, and develops into a large part of the placenta. They are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg. Villous trophoblasts have two cell populations: undifferentiated cytotrophoblasts and fully differentiated syncytiotrophoblasts. The syncytiotrophoblasts are a continuous, specialized layer of epithelial cells. They cover the entire surface of villous trees and are in direct contact with maternal blood [18]. In terms of fetal-maternal communication, it is mainly the syncytialized trophoblasts that orchestrate the complex biomolecular interactions between the fetus and mother. Not only do placental trophoblasts provide structural and biochemical barriers between the maternal and fetal compartments during pregnancy, they also serve as an important endocrine organ that produces numerous growth factors and hormones that support and regulate placental and fetal development and growth [19-22]. Therefore, trophoblast cell biology is integral to understanding placental development and pregnancy-related diseases.

The aim of the collaboration described within this report was to investigate the impact of Chuckling Goats, goats' milk kefir on the biology of the human trophoblast, with particular focus on the trophoblast inflammation.

Methodology

To determine any potential immunomodulatory effect of the kefir on trophoblast cells, one work package was designed and undertaking.

Ideally, primary trophoblast isolated from placental tissue would be the ideal choice for such studies, however, due to COVID-19 availability was restricted. To overcome this limitation a variety of trophoblast cell lines have been developed and are commonly used by researchers when there is restrictions on access to primary material [23]. Among these cell lines, there are naturally immortalized cell lines obtained from choriocarcinoma tissue such as BeWo [24], JAR [25] and JEG-3 [26]. Other cell lines such as AC1M-2 [27] and ACH-3P [28] have been generated by the fusion of the AC1-1 cell line (a JEG-3 mutant [29]) with primary term or first-trimester trophoblast, respectively. In addition, trophoblast cell lines such as HTR-8/SVneo [30] and SGHPL-5 [31] have been generated by SV40 large T antigen transfection. While trophoblast cell lines represent a valuable tool for studying placental function in vitro, it is essential that these models are thoroughly characterized.

Choosing the ideal trophoblast cell line for this investigation was required. Despite several reports in the literature of their use in various settings, several discrepancies are found in what is reported, in particular the inflammatory response of these cell lines. To overcome this, the cell lines choriocarcinoma trophoblast cell lines BeWO, JAR, JEG-3 and the SV40 transfected trophoblast cell line HTR-8/SVneo were used for preliminary investigations. These cell lines were chosen above the others as they were the only ones commercially available to us at the time of this project. Preliminary investigations focused on determining which if the four cell lines generated the most roboust inflammatory response when challenged. Of the four cell lines available, HTR-8/SVneo was chosen for all experiments involving the kefir.

When investigating the immunomodulatory potential of an adjuvant, in this case, the kefir, there must be an adequate inflammatory response generated by the cell of interest. Under *in vitro* experimentation, this typically involved stimulating the cells of interest with a prototypic inflammatory stimulus such as a fragment of a bacterial cells wall. For the preliminary investigations three inflammatory stimuli were chosen, Lipopolysaccharide (LPS) a preparation of the *E. coli* cell wall, Poly IC, a synthetic double-stranded RNA and Curdlan, a beta-glucan from *A. faecalis*, representing bacterial, viral, and fungal stimuli respectively. Initially, each of the four cell lines were exposed to a dose range of each of the

three stimuli and cytokine production was measured following 24 hours. Cytokines are small proteins crucial for signalling between cells and are release following activation to drive inflammation.

For cytokine analysis, the concentrations of interlukin-1β (IL-1β), interlukin-1 receptor antagonist (IL-1RA), interlukin-10 (IL-10), interlukin-6 (IL-6), interlukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) kits (Duoset, R&D Systems) according to the manufacturer's instructions. Following completion of the assay, absorbance is read at 450nm using a BMG PolarStar Omega plate reader. The ELISA is an immunological assay commonly used to measure antibodies, antigen, protein, and glycoproteins in biological samples. An overview of a general ELISA protocol can be seen in Figure 1.

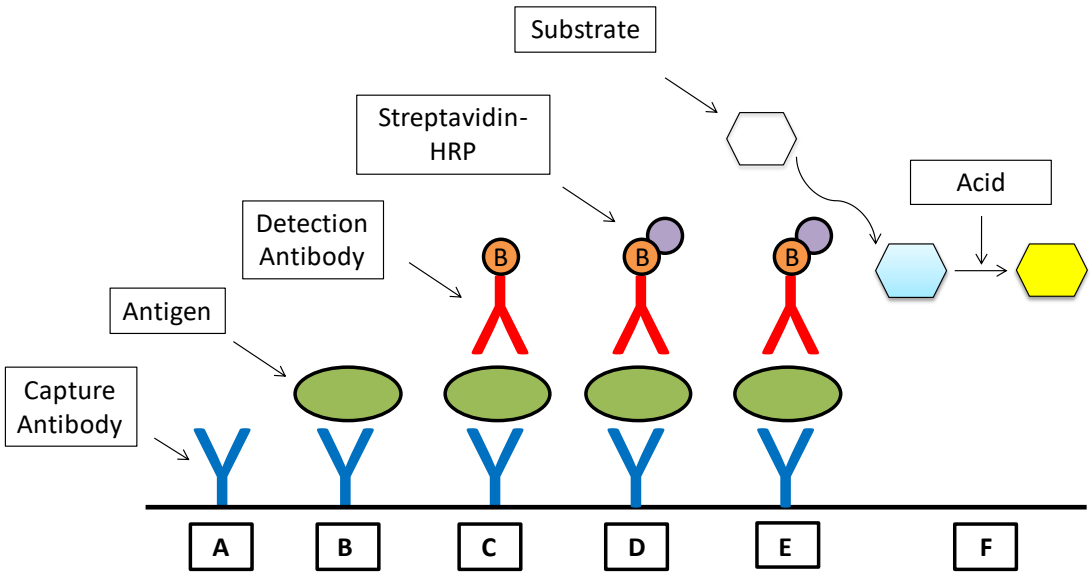

Figure 1. Overview of ELISA protocol.

(A) Capture antibody is immobilised on plastic. (B) Antigen is added and binds to the capture antibody. (C) Biotin-conjugated detection antibody is added and binds the antigen. (D) HRP linked streptavidin binds to biotin on the detection antibody. (E) TMB is added and catalysed by the HRP producing a blue colour. (F) Acid is added, stopping the reaction producing a yellow colour.

Results / Conclusions

Following the completion of preliminary experiments to determine which trophoblast cell line was the most appropriate, the cell line HTR-8/SVneo was chosen for all subsequent experiments involving the keifr.

Initial investigation examined whether kefir had a beneficial or negative effect on the ability of trophoblast cells to proliferate in vitro. To achieve this, one million trophoblast cells were seeded and cultured for 24 hours and 48 hours in the presence of an increasing concentration of keifr (0.5%, 1% and 2.5%). Total cell counts were then performed with the results shown in Figure 2. Regardless of the kefir percentage in the culture media, no significant increase or decrease in total cell counts were observed when compared to a control (no kefir). This data suggests that when exposed to kefir in vitro, there are no detrimental effects to trophoblast survival.

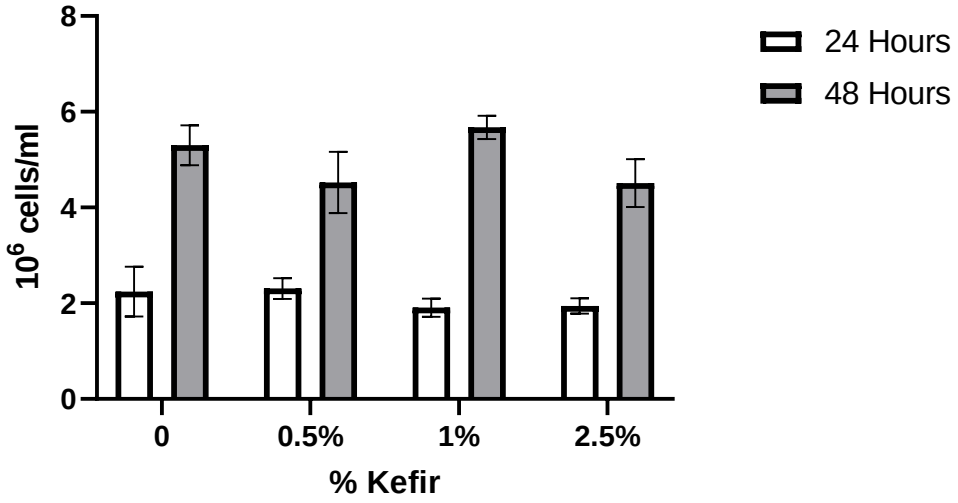

Figure 2. Total cell counts of HTR-8/SVneo cells when cultured with increasing concentrations of Kefir.

Graph represents total HTR-8/SVneo cell counts (n=3) at 24 and 48 hours following in vitro culture with wither 0%, 0.5%, 1% or 2.5% Kefir. Results represented at 10^6 cells/ml. Statistical significance was determined by 2-way ANOVA.

To determine if the kefir had any immunomodulatory effect, HTR-8/SVneo cells were cultured in the presence of increasing concentration of keifr (0.5%, 1% and 2.5%) +/- either LPS (100 ng/ml), Poly IC

(10 ug/ml) or curdlan (100 ug/ml) as a prototypic inflammatory stimulus. The impact of the kefir on stimulus-induced cytokine production was investigated using a panel of six cytokines; four pro-inflammatory cytokines, IL-1β, IL-6, IL-8 and TNFa and two anti-inflammatory cytokines, IL-1RA and IL-10. Cytokine outputs were chosen as a standard panel of cytokines when investigating immunomodulatory potential of an adjuvant. In the presence of increasing concentrations of kefir, a dose-dependent decrease in stimulus-induced IL-6 production is observed, however, this was not statistically significant (Figure 3). This decrease is more apparent when trophoblast cells are stimulated with LPS, compared to trophoblast stimulated with either Poly IC or curdlan, likely reflecting the different cellular pathways used to respond to bacterial infection compared to either viral or fungal infections. Similarly, IL-8 also demonstrated a dose-dependent decrease with increasing concentrations of kefir when added to the culture (Figure 4).

No detectable levels of IL-1β, IL-1RA, IL-10 or TNFa were present in the experimental model.

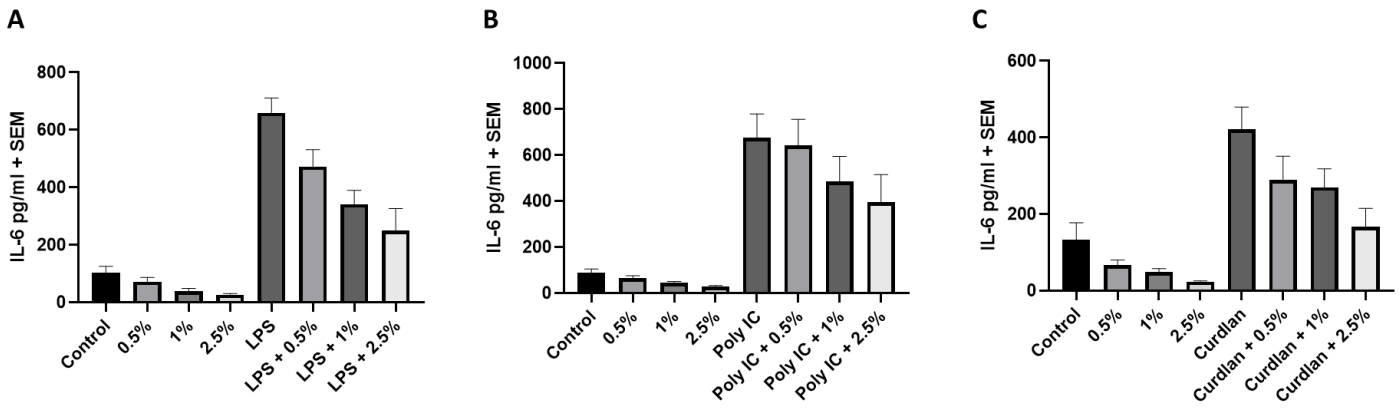

Figure 3. Impact of increasing concentrations of Kefir on the IL-6 response of HTR-8/SVneo cells
Graphs represent IL-6 response of HTR-8/SVneo (n=4) stimulated with (A) LPS, (B) Poly IC and (C) curdlan in the presence of absence of increasing concentrations of kefir (0%, 0.5%, 1% or 2.5%) for 24 hours Kefir. Results represented as pg/ml + standard error of the mean (SEM). Statistical significance is determined by Friedman's test.

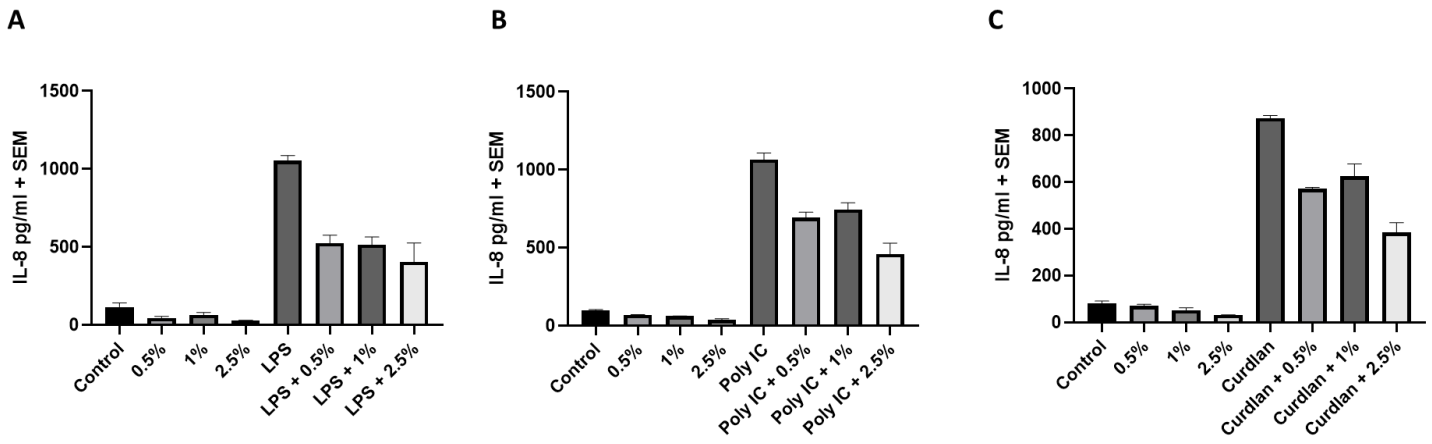

Figure 4. Impact of increasing concentrations of Kefir on the IL-8 response of HTR-8/SVneo cells Graph represents IL-8 response of HTR-8/SVneo (n=4) stimulated with (A) LPS, (B) Poly IC and (C) curdlan in the presence of absence of increasing concentrations of kefir (0%, 0.5%, 1% or 2.5%) for 24 hours Kefir. Results represented as pg/ml + standard error of the mean (SEM). Statistical significance is determined by Friedman's test.

Cytokine production at the maternal-fetal interface is a part of normal pregnancy. Changes in cytokine production occur with term and preterm labor; whether such changes precede labor and might serve as targets for therapeutic intervention in adverse obstetric outcomes remains to be determined [32]. Evidence supports inflammatory processes: heterogeneous proinflammatory profiles reflecting different underlying causes of pre-term birth, including infection. Targeting common inflammatory pathways might curtail the inflammatory cascade before myometrial contractions and cervical changes occur to prevent preterm delivery [32]. The data generated during this collaboration is in keeping with other in vitro investigations using other probiotics. These studies both demonstrate an anti-inflammatory effect of the probiotic on the LPS induced inflammatory response by primary trophoblast cells [33, 34]. Here we observe a decrease in both IL-6 and IL-8 production in response to the increasing concentrations of kefir, however, the exact mechanism associated with this was beyond the scope of this pilot study. Previous studies suggest that probiotics increase the production of anti-inflammatory cytokines by trophoblasts [33, 34]. It has been previously noted that anti-inflammatory cytokines such as interlukin-4 (IL-4) and interlukin (IL-13) might offer therapeutic potential in LPS stimulated placental tissue, by decreasing the level of pro-inflammatory cytokines produced [32]. Whether the kefir increases the levels of these anti-inflammatory cytokines by trophoblast is still to be determined. Investigating this could be the basis of future collaborations.

Impact

It's estimated that over 800, 000 women of all ages in England and Wales are pregnant each year. Growing evidence suggests that maternal diet influences pregnancy outcome, for example, dietary patterns characterized by a high intake of vegetables, fruits, and vegetable oils, which is associated with reduced risk of preeclampsia and preterm delivery [35-38]. Probiotics are defined as 'live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host' [39]. It has been shown that orally ingested probiotics have the potential to colonize the vagina [40] and normalise the bacterial flora in the lower genital tract [41] the anti-inflammatory effect of orally ingested probiotics has also been shown in vivo [42, 43].

The ambition of this collaboration was to demonstrate whether goats milk kefir had an immunomodulatory effect on the human trophoblast. The data generated here shows in vitro that kefir has an anti-inflammatory effect as noted by the decrease in inflammatory cytokine production (IL-6 and IL-8) without having any negative impact on trophoblast proliferation and growth. This is in keeping with other in vitro studies with other probiotics [33, 34], further adding to the body of literature suggesting a beneficial effect of probiotic consumption during pregnancy. However, much work is still required in this field to determine the how and the why a beneficial effect is observed.

Innovation Audit Tool

Through this collaboration, Chuckling Goat have increased their links with Academia and now have a relationship with Swansea University Medical School. There is potential to continue working together following this successful project and if suitable funding is sourced to further expand on the research, development, and innovation.

Figure 5. Innovation Audit Tool.

Dark orange represents pre-delivery and post-delivery by the light orange.

Recommendations

- To continue further investigate the effect of the impact of kefir on trophoblast cells including molecular mechanisms associated with mode of action. This could be extended to include primary human trophoblasts and placental organ cultures.

- To revisit the original concept of the collaboration, by investigating the impact of kefir on the inflammatory response of pregnant women *in vitro*. This can be achieved by incubating blood with different percentages of kefir +/- a prototypic inflammatory stimulus with cytokine production, cell death and cell activation status considered as output. A comparison of the response of non-pregnant women would also be needed as a control group.

- If the above studies are favourable the line of inquire could be expanded to *in vivo* models of pregnancy to investigate the impact on specific pathophysiological outcomes or a double-blind randomized controlled trial.

- To investigate the benefit of kefir in other settings either *in vitro*, *in vivo* or by trial.

- To continue to collaborate with Swansea University Medical School to undertake more research in this area e.g., joint grand application, to further understand the mechanisms behind the therapeutic benefit. This could directly feed into the teaching of BSc and MSci degree scheme including research projects.

IN VITRO

The presence of kefir reduced prototypic stimulus induced inflammation in a dose dependent manner

RECOMMENDATION

To revisit the originally conceived plan post-COVID-19

IAT

↑ links with academia

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