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Visualization of microbe-dietary remnant interactions in digesta from pigs, by fluorescence *in situ* hybridization and staining methods; effects of a dietary arabinoxylan-rich wheat fraction

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ABSTRACT

This study investigated how the addition of a specific hydrocolloid, arabinoxylan (AX), to the pig diets containing red meat, affected the gut microbiota in terms of changes to the bacterial community dynamics. Fluorescence *in situ* hybridization (FISH) was used to enumerate both broad groups and particular bacterial species, and showed systematic differences in pigs fed AX. In addition, the role of bacteria in the fermentation of dietary substrates was studied by visualizing direct associations between the bacteria and substrate particles. To achieve this, novel methods of combining histological staining or enzymatic labelling with FISH protocols were established. In this way, undigested and/or unfermented remnants of meat, endogenous mucin, and several plant cell wall hydrocolloids (cellulose, lignin and arabinoxylan) were distinguished, together with large intestine microbiota under brightfield and/or confocal laser scanning microscopy (CLSM). It was evident that no apparent direct associations occurred between specific classes or groups of bacteria and meat, mucin, lignin or AX remnants. In contrast, bacteria belonging to *Clostridium* clusters XIVa and XIVb formed a strong ($P < 0.01$) direct association with cellulose remnants in the pig digesta in the caecum.

1. Introduction

The human digestive tract harbours a complex microbial community, which has an important role in mediating the health of the gastro-intestinal tract (GIT) and, ultimately, the individual (Durbán et al., 2011). To support this community, energy and nutrients must be available to the microbiota, in the form of undigested food remnants, endogenous secretions such as pancreatic enzymes and mucins, and sloughed off epithelial cells (Walker et al., 2008).

Major energy sources available to GIT bacteria from undigested food include two types of hydrocolloids. One is proteins, which have transited the stomach and small intestine without being completely digested by mammalian digestive enzymes. The second is carbohydrate components that are not susceptible to human intestinal enzymes, primarily in the form of polysaccharide derived from plant cell walls (PCW) i.e. dietary fibre. These carbohydrates vary in their structure depending on their origin and function, and include cellulose, pectins, xylans, glucans and other polymers (Bird, Conlon, Christopherson, & Topping, 2010). These carbohydrates may be susceptible to degradation by enzymes produced by resident bacteria in the GIT.

Given that wheat is the most commonly consumed cereal grain in the developed world, AX is of specific interest. AX is a PCW polymer commonly found in the aleurone layer of wheat and rye, which comprises a xylan backbone of 1–4 linked xylose units substituted with 2, 3 or 2,3-linked arabinose residues (Saulnier, Guillon, & Chateigner-Boutin, 2012). Highly substituted AX tends to be more soluble in aqueous solutions, whereas AX with high numbers of ferulic acid linkages tends to be less water extractable from the PCW, both important features for the gut lumen (Saulnier et al., 2012). Broekaert et al., (2011) summarised a range of health benefits associated with the consumption of AX and AX-oligosaccharides, in human studies, together with trials involving mice, rat and chicken models, rather than pigs as used in this study. These benefits include increased caecal and faecal *Bifidobacteria* numbers for all animal models stated and human studies, reduced

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Escherichia coli, *Streptococcus*, *Staphylococcus* and *Lactobacillus* numbers, and increases in production of short chain fatty acids (SCFA) such as acetate, butyrate and propionate. *In vivo* human studies of gut microbiota are often limited to faecal samples, whilst animal models provide the opportunity for samples to be taken from along the length of the gut.

Pigs in particular are an appropriate animal model for research in the area of nutrition and health. They are monogastric and omnivorous, and from a metabolic viewpoint, pigs and humans have in common a range of bacterial species, metabolic pathways, and similar skin, kidney, cardiovascular and digestive physiologies (Guilloteau, Zabielski, Hammon, & Metges, 2010).

To date, little research has been reported on the identification of undigested food remnants in digesta at specific monogastric GIT sites. Nor have links been established between specific bacterial groups and their ability to attach to these remnants. This is, at least in part, due to difficulties in applying staining protocols traditionally used on histological thin sections, to samples that have undergone the processes of digestion and fermentation, which result in heterogeneous material structures.

Fluorescent studies of bacterial assemblages have a relatively long history in microbiology, with FISH being a useful tool since the mid-1990's to visualize microbes in different environments. In a complex environment such as GIT digesta, one limitation of FISH is the presence of background fluorescence that could mask the cells and particulate matter under investigation. To minimise this problem, fluorescent markers were used to highlight the different classes of dietary substrate particles, thereby decreasing the masking effect so that any bacterial–substrate association could be visualized.

This study investigated the interactions between bacteria and dietary remnants in large intestine digesta from pigs fed a 'Western-style' diet containing either 23% or 30% red meat, with and without the addition of AX. The objectives of this study were to (i) quantify, in terms of proportions, the changes to the bacterial community in response to the different diets being fed, (ii) establish specific histological stain and enzyme-linked fluorophore methods for the visualization of the respective dietary remnants in the GIT of the pig, and (iii) optimise these methods in conjunction with a FISH protocol to reveal specific direct associations between bacterial groups and hydrocolloid dietary remnants.

2. Materials and methods

2.1. Digesta sampling and preservation

All animal procedures were approved by the Animal Ethics Committees of the University of Queensland (AEC Approval Number SAS/181/09/CSIRO-NF). Zhang et al. (2015) describes in detail the experimental design, diets, and digesta sampling procedures. In brief, the four diets composed of red meat (either 23.6% or 30%), with or without an enriched wheat fraction (AXRF) that contained soluble AX, starch (adjusted according to the starch content of the AXRF), wheat bran (containing insoluble AX), oils, fats, salt, vitamins & minerals. All formulations were calculated to meet necessary dietary requirements, and as isocaloric as possible. Digesta contents of eight pigs were investigated in samples collected from the terminal ileum, caecum and distal colon. In brief, the pigs were fed a diet with two cooked red meat contents, with or without the addition of AX, for four weeks. When investigating the bacterial community composition, caecal digesta was used for the proportional counts. Unless otherwise stated, caecal samples were used for bacteria-substrate association protocol development.

A volume of 400 μL of digesta was fixed in 1.2 mL of 4% paraformaldehyde (PFA) solution (Sigma–Aldrich, Sydney, Australia) for 2–4 h at 4 °C. After fixation, samples were centrifuged at 5000 × *g* for 3 min, the supernatant discarded and 1 × phosphate-buffered saline (PBS) was used to wash the pellet twice. Thereafter, the pellet was resuspended in a final mix of absolute ethanol and 1 × PBS (50:50), and fixed samples were stored at −20 °C.

Undigested diet components were used as positive controls in establishing the substrate staining or fluorophore-linked enzymatic protocols, with the exception of protein and mucin. The source of protein for the positive control was supermarket purchased topside steak, which was browned on a hotplate and finely diced. To achieve a powdered form, the meat was then ground using a mortar and pestle with liquid nitrogen and stored at −20 °C. Porcine stomach mucin Type III (Sigma–Aldrich, Sydney, Australia, product number M1778-100G) was stored at 4 °C as purchased, and made into a 1% w/v mixture with 1 × PBS. This mixture was then mixed 2:1 v/v with the dry feed components and stored at −20 °C. All positive controls were duplicated once concentrations for working solutions were established. The use of the non-diet components (steak protein and commercial porcine stomach mucin) was ceased once the protocols were refined and finalized.

2.2. Fluorescence in situ hybridization protocol

The FISH protocol as previously described (Amann, 1995; Amann, Ludwig, & Schleifer, 1995), was modified as follows. A formamide concentration of 25% was chosen for the hybridization buffer based on preliminary trials, with sufficient fluorescence signal having been obtained from each probe against positive control digesta samples.

Teflon-coated 8-well slides were chosen and silanized (see below) for better sample adhesion due to the washing steps required for the various protocols.

A volume of 6 μL of digesta was added to each well and air dried. The slides were then placed in an ethanol dehydration series of 50%, 80% and 99.9% ethanol/water mixtures for 3 min at each step, removed and air dried.

Hybridizations were performed using 8 μL of hybridization buffer per well at 46 °C for 1 h, with the hybridization buffer containing 360 μL of 5 M NaCl, 40 μL 1 M Tris–HCl, 500 μL formamide, 1098 μL distilled water and 2 μL 10% SDS. The probe concentration was 50 ng/μL with 0.5 μL added per well. After hybridization, slides were washed for 15 min at 48 °C in a washing buffer, containing 1490 μL of 5 M NaCl, 1000 μL 1 M Tris–HCl, 500 μL 0.5 M EDTA and 50 μL 10% SDS, made up to 50 mL with distilled water.

After washing, the slides were rinsed in 4 °C distilled water for 2–3 s to remove any salts, and were then dried using compressed air. To each well, 5 μL of Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, CA) was added to prevent photobleaching, a cover-slip applied and sealed with nail varnish before visualization with a Zeiss LSM-700 Confocal laser scanning microscope.

The oligonucleotides used in this study are listed in Table 1. The universal probes EUB338, Eub338 II and EUB338 III were combined as described (Daims, Brühl, Amann, Schleifer, & Wagner, 1999) to form an equimolar mix, herein referred to as EUB338Mix.

The probes Bac303, Bif164, CFB286, Chis150, Erec482 and Lab158 are probes commonly used in human health research and have broad coverages of Class, Order or Family. The final probe Fprau0645 is a species specific probe for *Faecalibacterium prausnitzii*, a bacterium whose absence from the gut microbiota may be associated with the presence of Crohn disease (Sokol et al., 2008).

No pre-treatment of the sample by lysozyme or similar methods to enhance hybridization of Gram-positive bacteria was used due to possible loss of cell membrane integrity of Gram-negative bacteria.

A minimum of 15 random images per well per slide were