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Microbial quality and nutritional aspects of Norwegian brand waters

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ABSTRACT

The microbiological quality of the five leading brands of Norwegian bottled still waters was investigated. All brands were free for the enteric indicator organisms and named pathogens whose absence is demanded in current quality directives. The relatively nutrient-poor agar R_2A revealed large heterogeneous bacterial populations which grew slowly, or not at all, on clinical media specified for use in substrate-utilization approaches to identification. The main approach used for identification was cultivation of microbes on R_2A, followed by amplification and partial sequencing of 16S rDNA genes. The identity of the heterotrophic plate count of the brands differed significantly to that found in many other similar studies with respect to the dominating species. The bacterial flora was dominated by beta- and alphaproteobacteria most of which were psychrotolerant. Several brands contained *Sphingomonas* and large populations of *Methylobacterium* species which have been associated with a variety of opportunistic infections of immunocompromised hosts. Analysis of the isolated strains' nutritional capabilities using the Biolog GN2® system, gave in most instances low positive scores, and strain identifications using the system were generally inconclusive. Measures of assimilable organic carbon in the water revealed that some brands contained levels higher than those which have been associated with biological stability and restricted or no growth of heterotrophs in distribution systems. The relationship between assimilable organic carbon and R_2A plate counts was significant and moderately positive for bottled waters. Assimilable organic carbon correlated strongly with the survival time of *Escherichia coli* when introduced into bottles as a contaminant. Those brands having high values (~100 μg/L) supported protracted survival, but not growth of *E. coli*, whereas *E. coli* quickly became nonculturable in brands with low values. Thus measures of assimilable organic carbon may have a particular value in predicting the survival of this and nutritionally similar species of hygienic relevance. Only small numbers of fungi were found. However, one isolate (*Aureobasidium pullulans*) has been associated with infections of humans.

1. Introduction

Effective marketing and a general scepticism to the quality of tap water have over the past decade resulted in a marked increase in the consumption of bottled/dispenser waters (Liguori et al., 2010). In Norway, an outbreak of *Giardia* disease in Bergen in 2004, detection of this parasite in Oslo's tap water in 2007 leading to a boil water recommendation, and concerns about the poor quality of the distribution net have raised public concerns about tap water quality. There is a general perception that bottled water is purer and safer for human consumption, and targeted groups for marketing have included infants and immuno-compromised individuals (Papapetropoulou, 1998). Current Norwegian legislation governing the quality of bottled waters define natural mineral water (NMW) and spring water (SW) and provide guidelines for the treatment, bottling and microbiological quality of these (HOD, 2004). The Norwegian document adopts the general definitions and guidelines for microbiological quality given in the current European legislature (Directives 80/777/EEC and 96/70/EC of the European Parliament and of the Council). The proven absence of coliforms, *Escherichia coli*, *Enterococcus* sp. and anaerobic spore-forming sulphite-reducing bacteria is demanded. Furthermore, the heterotrophic plate count (HPC) should reflect a source water free from contamination. As neither treatment nor bottling or materials shall otherwise change the natural composition of the water, large bacterial populations usually develop from small initial populations shortly after production (Loy et al., 2005 and references therein). However, few characterizations of the natural heterotrophic plate count microbes (including fungi) exist and to our knowledge there are no such published analyses of Norwegian bottled waters. The HPC as a quality control parameter has several weaknesses: only a small fraction of the total viable microbial population grows on artificial laboratory media (Watkins and Xiangrong, 1997). Furthermore, it is now generally accepted that in the absence of fecal contamination there is no direct relationship between HPC values in ingested water and human health effects in the population at large (EMG, 2003). However, HPC continues to figure in water regulations and guidelines in most countries as an indicator of

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the general microbiological quality and, as such, still requires attention. The test is inexpensive, quick and reliable and the HPC composition can indicate changes in water quality which should be evaluated as a trigger for further investigation (EMG, 2003). Furthermore, specific strains which might be a part of the HPC microbiota can cause infection of immunocompromised people and there is at present insufficient epidemiological data to evaluate the relevance of HPC microbes for these groups (EMG, 2003; Pavlov et al., 2004). The highest HPC values for water are obtained with low nutrient media and long incubation times. Massa et al. (1998) showed that R_2A medium gave plate counts exceeding those on plate count agar used in other studies of bottled waters (eg Pavlov et al., 2004) by more than 300%. Furthermore, *Flavobacterium* spp. and *Arthrobacter* spp. were only recovered on R_2A which the authors subsequently recommend for analysis of NMW. While there have been many studies of the bacterial species found in water, the identification of bacterial isolates from the environment has previously been impaired by a poor database. It is highly probable that many of the identifications reported in the literature over the years are incorrect. Many were made employing clinical systems for which the database was not appropriate for environmental strains. Molecular methods have changed our views of the "species," and we should at least question many of the bacterial identifications in the literature (EMG, 2003).

The reasons for the development of a HPC in waters after bottling are still a matter of debate. The influence of materials, the relative contributions of attached (eg, biofilms) to unattached microbes, and of regrowth (from small initial populations) as opposed to resuscitation of existing microbes show the complexity of the issue (see discussion in EMG, 2003). An aspect which so far has received little attention, is a possible correlation between the development of a HPC in bottles and measures of the fraction of dissolved organic carbon which can be used in the production of bacterial biomass. The assimilable organic carbon (AOC) analysis (Van der Kooij et al., 1999) involves the addition of 1–2 heterotrophic stock strains to pasteurized water samples. The combined metabolic capabilities and carbon assimilatory capacity of the strains should, ideally, result in a growth response which gives a good indication of the amount of carbon that can be converted to biomass by indigenous species. The units are carbon equivalents derived from growth of the assay strains on model carbon sources, usually acetate and oxalate. It has been observed for groundwater distribution systems that when AOC values approached 10–15 μg C/L little or no regrowth of heterotrophic bacteria occurred and the HPC remained low at <100 CFU/mL (Van der Kooij et al., 1999 and references therein). It has also been reported that waters with AOC levels of >100 μg C/L gave 82% more positive coliform samples (LeChevallier et al., 1999). AOC measurements have not been previously used in the analysis of bottled waters, but bacterial growth during storage might by comparison to tap water be related to high AOC values. If so this might provide the analyst with a tool for predicting the development of a HPC population and more importantly the survival of enteric pathogens.

The numbers, types and resistance profiles of fungi causing infections have increased dramatically in recent years. There are increasing numbers of severe fungal infections, particularly among immunocompromised people by commensal or fully saprophytic species (Enoch et al., 2006). The role of drinking water in the dissemination of fungal pathogens is still a matter of debate (EMG, 2003). There is thus a need to evaluate the fungal fraction of drinking waters and to monitor any changes in its status. The explicit requirement for the absence of pathogenic organisms in all bottled water types (HOD, 2004) would naturally encompass fungal pathogens. However, fungi as a group are not mentioned in the Norwegian drinking water directives. Methods for detection are not provided and to what extent fungi are tested for is not known.

The main focus of the current study is to investigate the microbiological quality of the leading brands of Norwegian bottled waters. It seeks to characterize the bacterial and fungal HPC using genotype-based methodologies. Factors, particularly nutritional capabilities of the HPC and water AOC-values which might relate to the growth and survival of microbes, including enteric pathogens, are discussed.

2. Materials and methods

2.1. Water types included in the study

The study includes the 5 leading brands of Norwegian bottled still waters: Brand 1, Brand 3 and Brand 4 (NMW in plastic bottles), Brand 2 (SW in plastic bottles), Brand 5a (SW/"artesian water" glass bottles) and Brand 5b (SW/"artesian water" plastic bottles).

2.2. Determination of the bacterial heterotrophic plate count (HPC) at 22 ± 2 °C

In order to account for putative inter-batch variations, analyses were performed on a minimum of 3 bottles from different production batches of each brand in the period October 2008–November 2009. Each brand is produced at a single factory production site and from a single source water. Samples of 0.1 mL were removed in triplicate, diluted where required and spread onto R_2A agar (Oxoid, UK). Plates were packed in plastic to prevent drying and incubated in the dark at 22 ± 2 °C. The colony count was determined at 14 days.

Brand 1 water alone was available directly after bottling such that the development of the bacterial flora could be followed. Three bottles (1.5 L) were received at the laboratory within 24 h of bottling; samples were maintained at <4 °C during transport. Upon arrival, each bottle was tested for the initial HPC. Thereafter two bottles were maintained at room temperature. A third bottle was refrigerated (4 ± 1 °C). Bottles were sampled at intervals using carbon-free pipette tips over a period of 6 months in order to follow the development of a HPC.

2.3. Detection and enumeration of coliforms, E. coli, Enterococcus, Pseudomonas aeruginosa and spore-forming anaerobic sulphite-reducing bacteria

Samples (250 mL), were filtered through a membrane filter (0.45 μm nitrocellulose) for cell capture. Filters were placed onto MacConkey agar (coliforms, *E. coli* and *Enterococcus*) or *Pseudomonas* cetrimide agar (*P. aeruginosa*). Plates were incubated at 37 ± 1 °C and examined for colonies at 24 h and again at 48 h. Brilliance *E. coli*/coliform chromogenic medium (Oxoid) on which *E. coli* produces purple colonies and other coliforms pink colonies, was used to further characterize colonies appearing on MacConkey agar. Samples of 50 mL were filtered as described earlier and incubated anaerobically at 37 ± 1 °C (48 h) on sheep's blood agar for the initial isolation of spore-forming anaerobic sulphite-reducing bacteria. Culture collection strains (*E. coli* ATCC 25922, *P. aeruginosa* DSM 9027, *Enterococcus faecium* ATCC 19434 and *C. perfringens* ATCC 13124) were used to confirm the suitability of the chosen methods. Three bottles representing different production batches of each bottle water brand were tested.

2.4. Determination of the optimal growth temperature and nutrient concentration of bacterial isolates

Colonies from primary plates were streaked out on R_2A (relatively nutrient poor) and tryptone-soya agar (relatively nutrient rich). Plates were incubated at 4 ± 1 °C, 8 ± 1 °C, 22 ± 2 °C and 37 ± 1 °C and read for growth (presence/absence) over a period of 14 days.