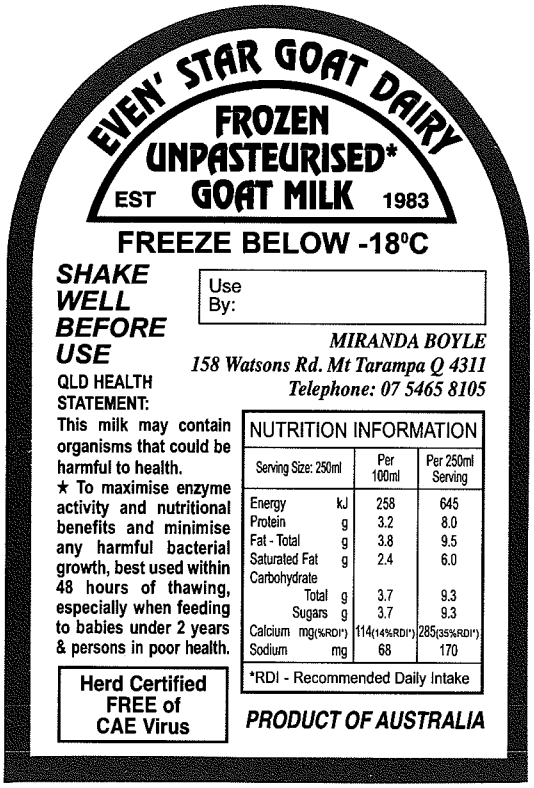

PROPOSAL P1007

PRIMARY PRODUCTION & PROCESSING REQUIREMENTS FOR RAW MILK PRODUCTS (Australia only)

SUBMISSION IN SUPPORT OF LEGISLATION TO ALLOW THE PRODUCTION AND SALE OF RAW GOAT MILK NATIONALLY IN AUSTRALIA

Miranda Boyle

Even 'Star Goat Dairy

Queensland.

27th September 2011

- Attached please find relevant scientific research supporting health benefits of raw milk over pasteurised. As well there are numerous eminent scientific nutritionists who by way of both research and direct experience with patients recommend raw milk over pasteurized, even going so far as to advise against consuming all dairy products if raw is unavailable.

- These experts who work at the coal face of human disease are only too aware of the improvements often experienced by their patients.

- Appendix A is included to demonstrate the exhaustive review carried out in Queensland, which ultimately led to the continued legal production of raw milk under the HACCP scheme and its inclusion in the Milk Act 2003.

- **We the raw goat dairy farmer also witness first-hand the benefits of consuming raw goat milk, yet none of us (doctor, naturopath or farmer) was invited to be representative on the review panel.** I consider this to be extremely undemocratic and biased which can only result in an unbalanced review. * NB at end.

- The vast history of anecdotal evidence supporting the benefits of raw milk over pasteurized has at last received some scientific attention. Yet as was the case for many decades of the various health and medical authorities to deny any differences between cow and goat milk (it was "all in the mind" mothers were told) I expect it will also take a long time for the very important advantages of quality raw milk to be recognised.

- In the meantime the struggle goes on between the peoples' right to have a legal choice of raw milk available to them in the market place, or to seek out illegal sources with no safety checks in place. A retrograde step and not in the interests of public health and safety.

- The misleading advice for mothers that formula feeding was as good if not better for babies health as promoted by large commercial interests and taken up by

government and medical nutritionists has finally been exposed for the big con it was. **It is now universally accepted, mainly thanks to the research conducted, Appendix B, C and D, as well as the volume of anecdotal evidence, that raw breast milk is superior in every way to pasteurized formula milk.**

- Although the scientific research is mainly directed to infant health, it is not unreasonable to apply these same findings to human health in general throughout life. Research and anecdotal evidence overwhelmingly supports this. **Societies who mainly consumed raw milks and products had significantly lower cardio vascular disease and better gut and digestive health, they also lacked many of the auto-immune responses that plague societies today. This is a fact, not a vague belief. (Refer Appendix B, D and C)**

- It is also not an unreasonable conclusion to draw, that if it is safe for a baby to consume raw human milk, which is often manually expressed and transported, and according to major hospital advice, safe to store for up to 6 days in the fridge with no special instruction on hygiene, that it is possible to produce, under hygienic conditions and with a limited shelf life, raw milk from goats that poses minimal safety risks. Many of the pathogens that can be linked to goat milk can also be linked to human milk. It is all to do with the freshness of the milk.

- As stated in the overview of submissions, March 2009, "bacterial problems can occur from time to time in major dairy processors even with the best food safety systems in place....."

- **Statistics show far more incidences of food poisoning involving pasteurized manufactured dairy products and involving much larger number of individuals than incidences reported involving raw goat milk.** (None from legal raw goat dairies since legislation was introduced).

- Although I recognise that pathogens can exist in raw milk even under the most stringent hygiene management system, statistics would support that this is not confined to raw milk alone. Further-more, recent large scale outbreaks of food

poisoning involving raw vegetables in USA and Europe highlights the unreasonable concentration of fear put on raw milk production under modern technologically advanced systems.

- **It is also unjust to use data from societies where refrigeration, testing and knowledge of hygiene practices are virtually non-existent to prop up the pro-pasteurisation argument.** This was typical of the review carried out in Queensland during the 1990's, when the Health Department, in reply to individual submissions, wrote letters in reply, suggesting that typhoid fever etc could be contracted from drinking raw milk.

- If pasteurisation of goat milk becomes mandatory it would be economical and labour wise nigh impossible for the small medium farm to comply and therefore survive. It is the small to medium goat dairy who can offer a localized quality product, meeting consumer demands for standards of animal health, welfare, sustainable farming systems and bio diversity.

- The alternative is what we have now, a single mass produced mono product that is of dubious quality and palatability that relies heavily on pasteurisation as a means of marketing the product.

- Given the statistics of the large number of individuals that can be affected by food poisoning from a single large manufacturer compared to the small number of individuals affected from a small medium dairy operation, in the last 30 years within Australia the false sense of security that pasteurisation is the only way to consume milk, is misleading and biased.

In conclusion:

- **The often repeated argument that the health risks associated with consuming raw milk far outweigh any perceived benefits can <u>no longer be justified</u> for the following reasons.**

- No cases of food poisoning from consuming raw goat milk from legal dairies since legislation was introduced since the early 1980's. (for goat milk production) have been reported. Improved awareness and the introduction of the HAACP scheme has further improved safety levels.

- The benefits of raw milk have never been given due consideration since pasteurisation was introduced , the various scientific and medical bodies simply taking the attitude that if the proteins, fats and minerals are still present, all must be well!. The long term health of studied/researched societies and individual cases of diseases began to show otherwise, and more recently scientific/medical research is giving us the explanations as to why this is so.

- Pasteurisation has become more than just a safety issue and is more used as a means to produce and market large volumes of milk to the large chain retail outlets at an economical and in a convenient manner. A long shelf life of the product is critical to the economy margins involved and higher temperatures, further extending shelf life, are continually being employed.

- The way big business is going and with the support of government regulatory bodies, it is not inconceivable that the only milk available to the consumer of the future would be a UHT type milk.

- This continued effort to suppress and ignore damage to the original product and thus long term damage to human health (e.g. cardio vascular, gut and auto-immune systems) and to over emphasizing the risk factors involving raw milk produced under modern production procedures, is entrenching big business control to the detriment of a robust, competitive, choice of milks to the consumer in the market place, and suppressing the viability of the small to medium localised farmer.

The following suggested practices are put forward for serious and practical consideration:-

- The conditions under which raw goat milk is currently produced using the HACCP scheme has greatly improved the safety of legal raw goat milk. However, if the review panel still considers them inadequate further improvements could be made by ensuring that the milk is frozen within 24hrs of harvest and is consumed within 2-3 days of thawing. This would minimize the opportunity of any pathogens that may be present in the milk to multiply to potentially dangerous levels. This is also important to preserving beneficial enzyme activity and vitamins (e.g. folic acid, Vit. D etc)

- In addition to maintaining the current HACCP scheme legislation, which involves testing a range of pathogens on a fortnightly basis, further on farm testing could be helpful in ensuring a safe product. This could involved twice weekly or even daily testing of milk for SPC, coliforms and staph. aureus using petrefilms and an incubating oven.

- Also the suggestion that any new producer attend a workshop with an approved laboratory to ensure they have a thorough understanding of pathogens and hygiene etc. before accreditation approval by the relevant authority.

- Because of the high level of quality control and owner operator involvement, Raw Milk production is more suited to a small to medium sized farm operation. The close monitoring of the Dairy Goat's health and general welfare is best achieved in this way.

*N.B. I, personally, as well as Bill carter, the only other existing licensed Raw Goat milk farmer were not even notified that a review of raw milk production was being carried out; either by Safe food QLD, or FSANZ. We only found out by accident in July 2009. During the time - 2007 - early 2009. I had invested almost $1,000,000 in purchasing new land & building a new dairy complex with the sole purpose of producing a raw goat milk under an ideal organic sustainable farming system. I was devastated & very angry to learn that my lifes investment could be jeopardised without notice

Miranda Boyle

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APPENDIX A

Dissenting Report

Queensland Health

Committee of Review

Sale of Unpasteurised Goat milk

Compiled by

Mr. Bill Carter

Mrs Jan Aland Med Lab Tech

Attention: Page 2 App. 2. as highlighted.

DISSENTING REPORT

QUEENSLAND HEALTH

COMMITTEE OF REVIEW

SALE OF UNPASTEURISED GOAT MILK

Compiled by

MR BILL CARTER
MRS JAN ALAND Med Lab Tech

30 JUNE 1997

CONTENTS

PRODUCER REPRESENTATIVES' CONCLUSIONS

1. There is no evidence presented to the Committee that unpasteurised goat milk has even been the cause of an outbreak of disease or serious illness in Queensland.

2. The majority of evidence that supports the Q Health FACT SHEET and the QDA Discussion Paper, appears to be problems involving bovine milk, and almost all was produced before 1992. This does not allow for innovations over the past two years, such as more advanced harvesting procedures and improved quality afforded by the introduction of a strict testing regime, and a stringent Code of Practice for Production of Unpasteurised Goat Milk. The present Code of Practice is not yet a full HACCP system.

3. There is a firm and considered demand for the sale of unpasteurised goat milk in Queensland. The packaging is now clearly marked and both label and container must be approved by Q Health.

4. The shared responsibility for exemption from pasteurisation (Q Health) and production and quality required for sale to consumers (QDA) does not work efficiently and creates a conflict of interest for the QDA.

5. A public health risk does exist whilst unregulated goat dairies exist and are ignored.

6. There is evidence that pasteurisation is not infallible and clinical evidence linking homogenisation with disease (altherosclerosis and diabetes). Also there is need for further research on this issue.

7. There is still need for a balanced educational programme for consumers on the value of milk in the diet.

8. There are serious implications in the mutual recognition policy regarding the sale in Queensland of unpasteurised goat milk produced in other States.

9. There is also evidence that the main reason to ban unpasteurised goat milk in Queensland is based on vested interest and commercial expediency and not health issues.

PRODUCER REPRESENTATIVES' RECOMMENDATIONS

1. The sale of unpasteurised goat milk should be allowed to continue under the provisions of the Food Standards Code H5 and under a Code of Practice based on HACCP already developed by the Queensland Dairy Authority.

2. A system of self-regulation based on the system established in NSW in 1988 should be introduced under the auspices of the Queensland Department of Health.

3. The above system should be managed through a Committee made up of members from the producers' Association, and tworepresentatives from Government nominated by the Minister for Health and the Minister for Primary Industries (if he so desires).

4. Research programmes as identified in the Conclusions and by the Committee, suggested in paragraph 3 above, should be instituted at an early date.

W K CARTER
J ALAND
Producer Representatives - Review Committee

PRODUCER REPRESENTATIVES' COMMENTS

Overview of Review

We believe that the Review Committee result, as far as the vote to ban unpasteurised goat milk, was predictable. In fact, we wrote to the Minister for Health with this in mind on 21 November 1996. (Appendix 1)

The approach was obviously to build a case that it was financially viable for unpasteurised producers to send milk to the processors, QUF Milk (Suncoast, Nambour), as much as to ensure public safety.

The Committee did not discuss many issues adequately (producers' submissions, consumer letters, present testing procedures, and a considerable volume of formal research projects presented by producers.

The three Government departments, Q Health, QDA, and DPI, made no written nor verbal submissions. Of the "expert" members, one gave a verbal submission, the other (from the minutes) offered only two short contributions of a very general nature.

The time allocated for the third session was hopelessly inadequate and the actual production of the cost benefit analysis was left almost entirely to the Chairman.

Major research projects were ignored and research papers from around the world offered by producers in their submission were ignored, along with consumer letters and consumer participation on the Review Committee.

The submission solicited from the Australian Natural Therapists Association was not recognised despite being tabled at the final meeting. A submission from the pasteurised producers (Appendix 12) was obviously solicited, and letters from the Queensland Dairy Authority and Queensland Dairyfarmers' Organisation, written well after the final meeting of the Committee, were allowed.

We are of the opinion that the case against unpasteurised goat milk, being that unpasteurised goat milk is a potential threat to public health, was a long way from proven.

Furthermore, there is now clinical evidence, which we have included, that pasteurised milk could be injurious to health.

There is also evidence in documents provided to the Committee that some producers, processors, the QDO, and QDA could have been influenced by vested interest in the expansion of production and sale of goat milk, by the removal of unpasteurised milk. Evidence to support these opinions is presented in this report.

PRODUCER REPRESENTATIVES' COMMENTS (Contd.)

We are of the firm opinion that goat milk, produced by approved dairies and experienced goatkeepers, under an approved, stringent hygiene system, offers an acceptable level of protection to the consumer.

Consumer Demand

Sales figures produced to the Committee together with the petition presented in Queensland Parliament, and the letters from consumers of unpasteurised goat milk, prove there is a firm demand in Queensland for unpasteurised goat milk to continue to be sold. In this last year, despite the severe restriction of sales by regulation, about 40% of all sales of goat milk were unpasteurised milk.

Queensland Licensed Raw Goat Milk Producers' Association

This Association represents all producers of unpasteurised goat milk in Queensland.

The aims of the Association are:-

1. To assist producers to provide unpasteurised milk of high quality in order to maintain recognised standards of consumer safety and that will satisfy consumer needs.

2. To continue to develop a Code of Good Practice to meet HACCP requirements.

3. To facilitate product and herd improvement through better management, innovation, and scientific research.

4. To present the view of members through representation.

What is this Submission For?

This document summarises in plain English some key issues to assist a decision by Cabinet on the continued availability of raw goat milk. The document presents succinct facts and introduces new "state-of-the-art" management technology (HACCP) recognised by world health authorities as "appropriate" to augment or even replace pasteurisation as a protective health measure. For any further information contact Bill Carter Licensed Raw Goat Milk Producers Assoc. on (07) 5483 5288.

Pasteurisation Is Not Infallible

- The majority of food-poisoning occurs in food which has been processed (e.g. recent outbreaks in pasteurised cheese, cooked specialty meats/sausage Garibaldi et al.) and not as often in raw foods.
- The primary cause identified is in **handling and processing stages which reflects the need for better management of hazard**, training and monitoring etc (see HACCP notes).
- These outbreaks (and many in pasteurised products and dairy/factories in the past) highlights that pasteurisation (and other processes) are not infallible - are not "set and forget" protections, and conversely ...
- HACCP (management techniques) provides safe protection **without destroying or altering the food or digestive values** of the target product.
- The (Aust.) Office of The Chief Veterinary Officer (OCVO) states that "pasteurisation does not obviate the risk of subsequent recontamination..."[1].
- Pasteurisation of goat milk may increase susceptibility to pathogenic ingress by destroying proteins.†

Beyond Pasteurisation - World Authorities use "space age" standards

- The HACCP (Hazard Analysis Critical Control Point, *[pron. "hassip"]* technique is a risk assessment and risk management control procedure recognised by world regulatory bodies including US Food & Drug Administration[2], International Dairy Federation, World Health Organisation, et al.
- The Codex Committee on Milk and Milk products[3] considered a proposal that "Pasteurisation, or an equivalent measure approved by the official agency having jurisdiction, shall be used to achieve the appropriate level of public health protection."
- "Several delegations strongly resisted the specific reference to pasteurisation" ... because revised standards, and "Application of the HACCP System negated the need to specify any one processing step as being necessary for health protection".
- The **Australia New Zealand Food Authority (ANZFA)** recognises that "for goat milk it was recognised that an appropriate HACCP based code of practice such as is in place in NSW and WA could be an effective alternative way of assuring product safety and the **potential for exemption by local authorities**"... (from requirement to pasteurise) ... "should remain"[4].
- HACCP procedures are summarised in Attachment A - these include many practices already implemented under a strict Code Of Practice adopted by Licensed Raw Goat Milk Producers Assoc. including **large-letter warning labels that product is unpasteurised, on-farm testing** and **monitoring** of product stages. The presence or absence of pathogens is not the real issue, but the quantity and/or type (at given stages)! The presence of some pathogens does not present a risk to public health. (Ref Footnotes 3 & 4) Food standards[4] must be the guide, and testing, monitoring, and ongoing recording be done. HACCP "permits more efficient and effective government oversight".[2]
- Due to its **unique characteristics, consumers of raw goat milk** use the product with full knowledge and intent - **choice of unpasteurised milk is deliberate and well-considered**, so with appropriate **warnings on packaging (as part of total Code/HACCP plan)** an "appropriate level" of protection for raw goat milk consumer will exist - this may be different to the level of protection appropriate for "raw cow milk" as this product clearly may be confused by consumers†††.

Goat Milk is a Natural Product - naturally protected, and protective!

- †Goats have a natural immune system in the udder to resist Salmonella and E-coli . Raw milk contains (heat-sensitive) natural antibodies and "protective proteins" or enzymes which provide excellent nutrition, but also a natural immune system, especially helping maintain health in the lower human gut. This natural system is destroyed by pasteurisation, which alters milk and negates its rôle in the immune/digestive systems. These are scientific world-recognised facts![5]

Strong Community Support And Demand For Raw Goat Milk Option

- Three thousand (plus) signatures[6] on petitions of support from community, Cairns to Gold Coast (despite limited publicity of intended withdrawal of raw goat milk.) Hundreds of people wrote letters of support for raw goat milk to Minister/s and local Members (and continue to write to Government).
- Unpasteurised (raw) Goat milk is used to treat ulcers, stroke, cardio-vascular/heart problems, psoriasis, eczema, bowel disorders, the aged, lactose intolerant infants etc, by doctors, homoeopaths, naturopaths, and independent practitioners (eg chiropractors) world-wide[7]!
- Consumers want and have the right to choose to consume raw and pasteurised goat milk. One person, or a small group, surely has no right to do more than to provide "an appropriate level" of protection for them - after the warnings and due care have been taken, an individual surely may have

the freedom of choice - especially to consume a healthy product respected and consumed for centuries by the educated and ignorant alike - indeed, a vital food resource to many Australians!

Industry and Market Factors

- The majority market demand is for raw goat milk - not for pasteurised goat milk. This is demonstrated by raw milk sales currently being roughly equal to pasteurised milk sales, **despite extreme Gov't restriction on marketing raw milk** only from farm gate, not in "mainstreet"!
- Internationally, withdrawal of raw milk (e.g. Florida USA) has led to severe industry decline! In Queensland there were 70 goat milk producers in 1982, however severe regulation of goat milk marketing (driving up overheads and direct costs) has left us with only 12 farms, and diminished herds. This in turn can lead to diminished gene pools, reduced product volumes leading to cost-ineffectiveness etc, which becomes a lose-lose situation for consumers, farmer, and our economy!
- Withdrawal of the licensed, safe product will open up a dangerous "black" market for backyard milk in which real dangers arise, as no code of practice and HACCP controls and monitoring will be possible.
- Goat farming and goats, are very different to cow farming and cows[†††] - it seems inappropriate that the peak body governing Licensed Raw Goat Milk Producers Assoc. is a cow-dairy body, Queensland Dairy Authority (QDA) which we perceive to lack expertise on goats and to represent vested interest!
- [†††]Further to the above, **goat milk and cow milk are extremely different** and need separate consideration. HACCP procedures and product characteristics of one do not match the other. The hands-on intensive nature of goat-farming, and the close monitoring of individual goats and batches of milk, together with strict codes of practice and the planned new HACCP processes are "appropriate" protection **given that food poisoning by raw goat milk is a real rarity!!**

Recommendations for Cabinet Decision

The **Licensed Raw Goat Milk Producers Association** recommends:.

1. **Support freedom of choice! Take a decision to assure both raw and pasteurised goat milk availability - if necessary by legislation, or at least by commitment to continuing exemptions (enabling ongoing investment in the industry which is currently in real doubt and dismay). Raw milk would be labelled with prescribed warnings, worded and in lettering of a standard required size/type.**

2. **Ensure that both products are fully protected by appropriate HACCP practices, and Code/s Of Practice (currently in use and open to ongoing improvement). To achieve this for raw goat milk consider the establishment of an industry body** (see 3.)

3. **Being guided by the NSW "model" for management particularly, and considering WA and SA methods, permit self regulation by the raw goat milk industry of all production and marketing standards and activities. These would be obliged to inaugurate and maintain (monitor, report etc) HACCP standards and ensure adherence to a Code of Practice, under a peak body reporting to government and having authority over (and assuming responsibility for) all raw goat milk marketing in Queensland. An officer of DPI and Health department would be in close liaison and costs and risk to government lessened.**

4. **Through 1 - 3 (above) support the continuing improvement of herds, gene pools and milk product and by-products, ensuring to some degree a return to a more stable industry able to revive and possibly expand to take advantage of a world market for quality goat milk, and goats.**

References

[1]OCVO - Report on Microbial Food Safety Issues, Chapter 4, Principles of Pathogen Control refer : <http://www.dpie.gov.au/ocvo/pub/mfsi>

[2]FDA Consumer Reprint--**HACCP: A State-of-the-Art Approach to Food Safety (lists BENEFITS of HACCP** see Attachment A - an FDA document (www) <http://vm.cfsan.fda.gov:80/~lrd/fdahaccp.txt>)

[3]The proceedings of the 29th Session, **Codex Committee on Food Hygiene** (Milk and Milk products) (Washington DC , 21-25 Oct, 1996) (document available upon request - not attached due to bulk)

[4] ANZFA STANDARD H5 - refer also to ANZFA Manager Product Standards Program Mr Simon Brooke-Taylor on Ph: 61 6 271 2222. (Fax 61 6 271 2278)

[5]**Protective Proteins in Milk - Biological Significance and Exploitation.** (International Dairy Federation Bulletin #191, 1985)

Also, **Antimicrobial Systems in Milk - NATURAL ANTIMICROBIAL SYSTEMS** (Part 2 of Symposium, Bath Uni. UK 1985).

[6] Petition lodged with Minister for Health on November 15th, 1996.

[7] refer Nov 96 submission to Health Minister, by A.Milliane, also Australian Natural Therapists Association et al. (cites numerous cases, comments by health practitioners, etc., includes learned papers on applications for raw goat milk in healing and health.)

Attachment A - Submission to Cabinet & Members - Licensed Raw Goat Milk Producers Assoc.

FDA Consumer Reprint--HACCP: A State-of-the-Art Approach to Food Safety

The Food and Drug Administration is adapting a food safety program developed nearly 30 years ago for astronauts for much of the U.S. food supply. The program focuses on preventing hazards that could cause food-borne illnesses by applying science-based controls, from raw material to finished products. Traditionally, industry and regulators have depended on spot- checks of manufacturing conditions and random sampling of final products to ensure safe food. This system, however, tends to be reactive, rather than preventive, and can be less efficient than the new system.

The new system is known as **Hazard Analysis Critical Control Points, or HACCP** (pronounced hassip). Many of its principles already are in place in the FDA-regulated low-acid canned food industry and have been incorporated into the most recent revision of FDA,s Food Code . The Food Code serves as **model legislation** for state and territorial agencies that license and inspect food establishments in the United States. In January 1994, FDA proposed regulations that would establish HACCP for the seafood industry. FDA issued its final rule on HACCP for seafood in December 1995. A number of U.S. food companies use the system in their manufacturing processes, and the U.S. Department of Agriculture has proposed HACCP for the meat and poultry industry. (USDA regulates meat and poultry; FDA all other foods.) It is already in use in other countries, including Canada. Recently, **FDA began steps that could result in this state-of- the-art food safety system becoming the standard for food safety** in the United States.
...edited extraneous text at this point , not affecting context nor meaning of article...

HACCP has been endorsed by the **National Academy of Sciences**, the **Codex Alimentarius Commission** (an international food standard- setting organization), and the **National Advisory Committee on Microbiological Criteria for Foods.**

What is HACCP? HACCP involves seven steps:
<*> **Analyze hazards.** Potential hazards associated with a food are identified. The hazard could be biological, such as a microbe; chemical, such as mercury; or physical, such as ground glass or metal.
<*> **Identify critical control points.** These are points in a food's production -- from its raw state through processing and shipping to consumption by the consumer-- at which the potential hazard can be controlled or eliminated. Examples are cooking, chilling, handling, cleaning, and storage.
<*> **Establish preventive measures** with critical limits for each control point. For a cooked food, for example, this might include setting the minimum cooking temperature and time required to ensure a safe product. The temperature and time are the critical limits.
<*> **Establish procedures to monitor the control points.** Such procedures might include determining how and by whom cooking time and temperature should be monitored.
<*> **Establish corrective actions** to be taken when monitoring shows that a critical limit has not been met --for example, reprocessing or disposing of food if the minimum cooking temperature is not met.
<*> **Establish effective recordkeeping** to document the HACCP system.
<*> Establish procedures **to verify** that the system is working consistently --for example, testing time-and-temperature recording devices to verify that a cooking unit is working properly. Each of these steps would have to be backed by sound scientific knowledge: for example, published microbiological studies.

Need for HACCP New challenges to the U.S. food supply have prompted FDA to consider adopting a HACCP-based food safety system. One of the most important challenges is the **increasing number of new food pathogens.** For example, between 1973 and 1988, **bacteria not previously recognized** as important causes of food-borne illness-- such as Escherichia coli 0157:H7 and Salmonella enteritidis--became more widespread. There also is increasing public health concern about **chemical contamination** of food: for example, the effects of lead on the nervous system. Another important factor is that the **size of the food industry** has grown tremendously--in the amount of domestic food manufactured and the number and kinds of foods imported. At the same time, FDA and state and local agencies have come under severe resource constraints in ensuring food safety.

Advantages HACCP offers a number of advantages over the current system.

Most importantly, HACCP:
<*> focuses on preventing hazards from contaminating food
<*> is based on sound science
<*> permits more efficient and effective government oversight, primarily because the recordkeeping allows investigators to see how well a firm is complying with food safety laws over a period of time rather than how well it is doing on any given day
<*> places responsibility for ensuring food safety appropriately on the food manufacturer or distributor
<*> helps food companies compete more effectively in the world market.

BACKGROUND

The producers of unpasteurised goat milk (Class D licence issued by Queensland Dairy Authority) were not notified of the QDA Board decision not to issue licences for the production of unpasteurised goat milk for the year 1996/7 until sometime after the expiry of licences for the year 1995/6, and then only in answer to telephone enquiries regarding the late issue of licences. The licences were eventually issued after intervention by the Minister for Health in late August 1996.

In a letter which accompanied the licence, producers were informed that the QDA had decided not to issue licences to producers after 30 June 1997. **No reason was given for the decision.**

A report of the QDA Review held in 1995/6 has not been made available to producers, however, we believe a copy was received by the Department of Primary Industries and Queensland Health Department.

As no public announcement of the decision was made, producers decided that consumers should be made aware of the situation. This resulted in a large volume of letters of protest being sent to the Minister for Health as the responsible Minister.

A letter was received then from Q Health, dated 24 September 1996, informing producers that the Minister was to nominate a Review Committee.

When the make up of the Committee was announced, **the Queensland Licensed Raw Goat Milk Producers' Association (QLRGMPA) wrote to Q Health requesting the Committee be enlarged to provide for more stakeholders to express their view (Appendix 1).** This request was supported by a number of members of Parliament, and a second member of QLRGMPA was allowed to sit on the Committee.

Our request to Q Health to add two members who were natural therapy practitioners, and two unpasteurised milk consumers, was flatly denied.

After the second meeting of the Review Committee on 16 December 1996, **the two dissenting members of the Committee wrote to the Minister regarding several aspects of procedure to arrive at the decision, and the failure to present research documents to Committee members, and the intransigence of the Chairman in his decision not to record a dissenting opinion.** This problem was resolved after an interview with the Minister on 9 January 1997.

The third and final sitting of the Committee was held on 19 March 1997, however there was insufficient time allowed for full discussion and completion of a cost/benefit analysis. **The analysis produced in the Review Committee Report was not presented to the Committee in its final form,** in fact, was produced, in the main, by the Chairman. The values and weightings to each component were completed by the Chairman and the Senior Project Officer, Business Regulation Review Unit.

LEGISLATION & INDUSTRY COMPLIANCE

Australian Food Standards 1994

In addition to the paragraph quoted on page 3 of the Committee Report, the same FSC provides:-

3(a) *Save where specifically exempted by the relevant State or Territory Authority, goat milk that has not been prepared and packed as specified in clause (4) (5) (6) of this Standard.*

7(b) *There shall be written in the label on or attached to a package containing goat milk that has not been processed as specified in clause (4) of this Standard, in standard type of 6mm the statement -*

WARNING: UNPASTEURISED
immediately preceding or following the words
GOAT MILK

8 *Microbiological standards. When examined by the methods prescribed by clause(9) of this Standard -*

(a) goat milk shall -

**(i) have a standard plate count not exceeding 150 000 micro organisms per millilitre;*

(ii) have a coliform count not exceeding 100 coliforms per millilitre;

* QDA states "50,000 micro organisms per millilitre" (Dairy Industry Standards 1993)

NOTE: The standard plate count is the same as for the FSC Standard for pasteurised goat milk.

(b) pasteurised goat milk shall -

(i) have a standard plate count not exceeding 50 000 micro-organisms per millilitre;

(ii) have a coliform count not exceeding 1 coliform per millilitre.

Queensland Food Act 1981 Exemption (Appendix 4 to Committee Report)

Attention is drawn to paragraph 4, Warning on Label:

Each container of unpasteurised milk must carry the following statement in standard type of at least 3mm:-

"This milk may contain organisms that could be injurious to health".

ANZFA Gazette Notice (14 February 1996) (Appendix 2)

The wording of the Gazette notice and of the relevant sections of the FSC H5 clearly recognise that for unpasteurised goat milk, provided that it *"is produced in accordance with a HACCP based code of practice such as the NSW and WA goat milk schemes"*, could be an effective alternative way of assuring product safety and the potential for exemption by State authority should remain.

LEGISLATION & INDUSTRY COMPLIANCE (Contd.)

Code of Practice

A Code of Practice was put in place by QDA on 10 September 1996.

Compliance

The present labelling regulations in Queensland provide sufficient information to ensure that consumers are provided with information to make an informed choice when purchasing unpasteurised milk intended for human consumption.

Regulations require labels be approved by Q Health before use.

The issue of a Code of Practice by the QDA on 10 September 1996 completed all the requirements listed in the FSC and ANZFA Gazette Notice.

Conclusion

The ANZFA clearly distinguishes between procedures for pasteurised and unpasteurised goat milk in Food Standard Code H5 which demonstrates provision for unpasteurised goat milk.

We believe that the ANZFA, by its **ABANDONING** OF THE PROPOSAL to BAN UNPASTEURISED MILK OF MAMMALIAN ORIGIN INTENDED FOR HUMAN CONSUMPTION in February 1996 (Appendix 2) demonstrates provision for unpasteurised goat milk to exist provided it is given the exemption from pasteurisation by the State Authority and is produced in accordance with a HACCP based system and meets the other criteria in the FSC.

We believe also, that the Codex Committee on Food Hygiene have indicated a degree of recognition to the level of protection given to food products produced under suitable Hazard Awareness Critical Control Point schemes (Appendix 3).

RESEARCH FINDINGS ON RAW GOAT MILK

Raw milk contains, along with the nutritional components of protein, fat, carbohydrate, minerals and vitamins, **antimicrobial protective proteins** which can protect the infant from infections essentially within the gastrointestinal tract (immunoglobulins, lactoferrin, lysozyme, complement components, active leucocytes), and **enzymes** (lactoperoxidase, lipase, xanthine oxidase, etc) which play a role in digestion as well as protection against infection. (1) (2) (3)

Raw goat milk most closely resembles human milk in these components and scientific research has shown raw milk consumption actually **boost the immune system** especially in children and invalids, is easily digested, higher in minerals, higher in beneficial short-chain fatty acids and glycerol ethers (important for infants), higher in riboflavin, niacin and Vitamin A. (4) (5) (6) (7) (8)

"Actual factual proof that goats' milk is the best alternative to breast milk is being gained by biopsies of the lining of the gut of babies: at last doctors are producing indisputable visible proof of something which goat keepers have known instictively for years". (United Kingdom 1977) (9)

A stong synergism (combined total greater than components) exists between lactoferrin, lactoperoxidase, hydrogen peroxide (formed as a byproduct) and thiocyanate (produced from feeds and found by the Department of Primary Industries to be high in goats), lysozyme, immunoglobulins and other protective proteins. So strong is this effect that inoculation of raw milk with Salmonella typhimurium, E. coli, and Campylobacter jejuni and others, results in the pathogens being destroyed and only beneficial lactobacilli and streptococci surviving. Depending on the temperature, this can take several hours, so to find pathogens in raw milk correctly harvested, delivery to the laboratory would have to fall within this time; which is the case in the pathogen Campylobacter being found in 1996. Even Clostridial spores are lysed in raw milk. **This natural system is destroyed by pasteurisation, which alters milk and negates its role in the immune/digestive systems.** Losses in pasteurisation are critical for infants who derive most or all of their nutrition from milk. (10) (11) (12) (13)

So, we have 100 years of pasteurisation and a product as dead as Louis Pasteur, and perhaps another 100 to re-educate people to the growing body of scientific evidence implicating pasteurised/homogenised milk to clinical symptoms.

Xanthine oxidase is one enzyme which survives pasteurisation, but without its natural system, homogenisation allows some into the circulation where it creates havoc by attacking plasmalogen tissue within the artery walls. Further damage is done to the heart muscle. These lesions eventually harden into calcified plaques covered with cholesterol and obstruct blood flow, leading to high blood pressure and angina. The heart disease death rate skyrocketed after the homogenisation of milk became commonplace in the USA. Circulatory disease is rare in countries in which raw milk is consumed. (14) (15) (41)

RESEARCH FINDINGS ON RAW GOAT MILK (Contd.)

A recent study has linked pasteurised cows' milk consumption with incidence of insulin-dependent diabetes mellitus (IDDM) in thirteen OECD countries. Researchers in Sydney in 1996 found that exposure to formula in the first three months of life doubled the risk of diabetes, confirming a similar study in Finland. (16) (17)

B-Lactoglobulin from raw cows' milk has a lower allergy-causing reaction than pasteurised. (18)

Further evidence in the case against heated milk protein was documented in the Atherosclerosis publication in 1972. (19) (41)

Antibodies to heated milk protein in coronary heart disease were found and published in Atherosclerosis in 1980. (20)

No amount of juggling with protein ratios will result in a formula which is close to breast milk ..... the lack of enzymes makes digestion very difficult for young babies whose own enzyme factory, the pancreas, does not become fully operational till three months of age. Those who cannot breastfeed will attest to a scenario of unsettled, crying babies with colic, excess mucus, and even eczema (so-called easily digested heat-denatured milk proteins blocking the capillaries in the skin). Some babies even become sensitised to these proteins while in utero. (21) (22) (23)

Many experiments have been done on these enzymes and it is now known that though they are easily destroyed by heat, they can survive the wide range of pH change from the acid stomach to alkaline small intestine, to continue their role in the complexities of digestion. (24) (25) (26)

Tuberculosis and Brucellosis were the main reasons for pasteurising milk originally, and goats have never been found to have either disease in Australia. (27)

Professor Shepherd enlarged on folate deficiency intimating "spina bifida" was a problem in babies who consumed milk; on correction, he allowed that this was congenital and linked to the mother's diet while in utero. Research has shown raw milk consumption gives a higher haemoglobin as the transferrin/lactoferrin system ensures the correct assimilation of Iron and folate. Raw milk is known to give better brain development in babies; not less as intimated. Anaemia is a known side effect of pasteurised milk if not fortified with iron, and this available iron, without the essential enzyme complex, allows growth of pathogens causing diarrhoeal disease such as Salmonella, E. coli, Campylobacter, Vibrio cholerae, etc. Professor Shepherd admitted Necrotising Enterocolitis and other infections were problems with pasteurised formula. The bacteria he mentions are more likely to be found in pasteurised milk where spore-forming and anaerobic bacilli can proliferate without the aforementioned protection. (28) (29) (30) (31) (32) (37)

Provided goats receive sufficient green feed, three research papers found goat milk contained folic acid, Vitamins B12 and D, at approximately the same as human milk. (38) (39)

RESEARCH FINDINGS ON RAW GOAT MILK (Contd.)

Professor Shepherd did not address the following points:-

* That there are obvious clinical advantages in unpasteurised goat milk
* That pasteurised milk may be linked to Attention Deficit Disorder (40)
* That there is double the morbidity of formula fed babies as against breast fed
* That the risk of Sudden Infant Death Syndrome is related to allergy responses and anaphylaxis especially with pasteurised milk and formulas
* That lactose intolerance is not a problem in raw goat milk as the enzyme lactase is still present
* That whey proteins with a higher nutritional value are affected by heat
* That the Allergy, Sensitivity and Environmental Health Association is lobbying to have such unadulterated foods for the increasing number of children and adults with allergies.

Professor Shepherd did say **"overall it was not unreasonable to put forward the alternative choice (of raw milk) if stringent procedures were in place".**

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COMMUNICABLE DISEASE DATA

This information was not tabled during the Committee meetings; the Chairman was questioned at least three times by Mrs Aland or myself on evidence, if any, of unpasteurised goat milk being the cause of a disease outbreak in Queensland. On each occasion he replied there was "none".

The statement, "similarly there is insufficient evidence to exclude unpasteurised goat milk", is of concern and begs a question on the effectiveness of follow up action on laboratory reports.

In relation to the comment on Campylobacter, the sample was taken on 30 September 1996 and, at that time, in accordance with the Certificate of Exemption (Appendix 4 of the Committee Report), freezing of milk was not allowed.

The producer was notified on the afternoon of 11 October 1996 (Friday) and the dairy was closed. The dairy was inspected by a field officer from QDA on 14 October 1996 (Monday) and a milk sample was taken. The sample was clear, and the dairy re-opened on 18 October 1996 (Friday).

This procedure is in accordance with the Code of Practice.

There is no evidence given of any outbreak of Campylobacter in the district. It is likely that this bears out the research evidence on the presence and activity of anti microbial systems in unpasteurised milk (see Fig.5 P.13 Appendix 7).

We note the first analysis took eleven days and the second sample was completed in 4-5 days. This time lag of eleven days is a concern which bears investigation.

MICROBIOLOGICAL TESTING

"Compliance with the standard for milk quality by all producers has been very acceptable. In the period 1993-94 there was a compliance of 90% for Total Count, 100% for antibiotics, and 96% for Iodine.

Coliform testing was only introduced early in 1994. Testing on a regular schedule has not been fully implemented.

No positives for pesticide or melioidosis have ever been reported to the Authority."

QDA Discussion Paper on Sale of Unpasteurised Milk in Queensland, page 13.

The schedule of milk quality tests now includes coliforms and pathogens.

This negates several research papers on the quality of goat milk in the 1980's and the early 1990's.

Like all safety programmes, the testing regime is continuously being improved and spread over a broader range of tests. This practice is supported by the QLRGMPA.

A summary of tests from the current Code of Practice is attached at Appendix 15.15

QUEENSLAND DAIRY AUTHORITY
and QUEENSLAND DAIRYFARMERS' ORGANISATION

Quotes from Submission to Committee:-

* "A short study of the make-up and funding of the Authority is all that is needed to understand the outcome of the (QDA) Review" - W K Carter, Producer.

* "For the pasteurised milk industry to continue it is essential that supplies of quality milk are available all year and milk is distributed to all areas. This is unlikely to happen while unpasteurised milk sales are permitted".

* "To summarise, pasteurised milk sales will only increase significantly if unpasteurised milk is banned".

* "However if the limitatons on unpasteurised milk are removed this will provide an incentive to the industry and volumes and the sale of unpasteurised milk will increase. The improved viability of the unpasteurised milk producers could be at the expense of the pasteurised milk industry which may find it impossible to compete" .

- QDA Discussion Paper - Sale of Unpasteurised Goat Milk in Queensland, 20/11/95.

In the options submitted to the QDA Board (Appendix 11) the Authority was even more explicit in emphasising the benefits to QUF of banning unpasteurised milk.

Comment:

The findings of the QDA Review of the Sale of Unpasteurised Goat Milk in Queensland have never been released to producers. We are aware that Q Health and Dept of Primary Industries have a copy.

The QDA did not make a submission for discussion by the Committee and the representative did not present any verbal evidence against the sale of unpasteurised goat milk.

We note the letter to the Health Minister (dated 18 April 1997 - one month after the last sitting of the Review Committee) in which the QDA Board have stated their view. We also note they have not provided details to support their view.

It is difficult to believe that anyone on the QDA Board who held a view that was supportive to the sale of unpasteurised milk would retain a seat.

It is known that the QDO and the Shadow Minister for Primary Industries, now Minister, were being approached to support pasteurised producers early in 1995.

In June 1995 the then Shadow Minister wrote, "The goat milk industry is a small but significant industry and producers, like yourself, who have been instrumental in the establishment of the industry must have your investment supported". (Appendix 13)

In April 1995 there was only two producers who sent all milk for pasteurisation, and two who sold milk to the processor and unpasteurised milk from the farm gate.

About this time the Queensland Quality Milk Marketing Association was formed by five unpasteurised producers and two prospective producers. During a visit to the QDO, representatives were told that their major concern was that "if something happened with unpasteurised milk, it would rub off on the pasteurised milk sales".

This is reflected in their letter to the Minister for Health on 12 May 1997 (nearly two months after the Q Health Review). (Appendix 11 to Review Report)

In this they pointed out the high standard of milk produced by the pasteurised industry, yet QDA in their Discussion Paper (Appendix 11) recognises the need for three levels of standards for premises:-

(a) Selling to a processor
(b) Packaging for sale to consumers
(c) Premises for manufacturing products. (Page 5)

The QDA also states (Page 6), "The existing industry regulatory controls over the sale of unpasteurised milk are considered to be outdated and not satisfying the need of the goat milk industry and consumers of goat milk". (Appendix 11 - written in March 1995)

No changes were made by the QDA for standards until the tests were introduced in October 1996.

ISSUES FROM SUBMISSIONS

General

Associate Professor Shepherd arrived at the only meeting he attended at 7 pm (16 December 1996) and left at about 8.45 pm on closure of the meeting.

He stated that he had not been briefed by his proxy, nor had he received any of the paperwork, and he was unaware (until he was briefed by the Chairman) of the background of the Committee.

Copies of all submissions were handed out and advice on Competition Policy and Hilmer Report implications were distributed at the first meeting on 25 November 1996. Copies of the research papers submitted by Mrs Jan Aland (see Annexure A to Research Findings on Raw Goat Milk - Serial 7 & 8) were not included and were not available until the third meeting on 19 March 1997. There was no informed discussion on them at that meeting.

Associate Professor Shepherd had not seen the research papers before the meeting on 16 December 1996.

The Microbiologist at the DPI Testing Laboratory, Hamilton is aware of the natural inhibitors in raw goat milk samples, however there is no evidence of any research in the Southern Hemisphere along the same lines.

We do not accept the premise of Associate Professor Desmarchellier in this instance regarding anecdotal evidence. The only evidence of potential danger produced by the Health Department, after the meeting and at our request, is anecdotal and mainly oriented towards raw bovine milk, and from the Northern Hemisphere.

The statement on page 10 of the Committee Report, "Whilst committee members do not have medical expertise the majority were clearly influenced" is a convenient assumption. The submission from the Australian Natural Therapists Association (reference Appendix 6) provides an opinion that differs from Associate Professor Shepherd, and so do a vast number of the statements made by consumers in their letters.

Freedom of Choice

The analogy of motor cars and electrical appliances and unpasteurised goat milk is hypocrital when one looks at tobacco (19,000 deaths in Australia in 1992 and $800,000,000 costs) and alcohol (3,300 deaths in Queensland 1993-1996). [Source: Alcohol and Other Drugs Service]

A petition was tabled in Queensland Parliament on 15 November 1996, by Mr Len Stephan MLA on our behalf, containing approximately 3,000 signatures (Appendix 9).

A brief analysis of consumer conerns is attached (Appendix 10).

MUTUAL RECOGNITION

The Minister for DPI, when Shadow Minister, wrote to a producer on 25 June 1995 and expressed concern for aspects of mutual recognition.

"I do have concerns about the possible effect of mutual recognition standards which all States have agreed upon. This will allow a product of a lower standard to be sold in Queensland, unless it can be proven that the public is threatened by the sale of such product". (Appendix 13)

A number of consumers have stated, "If unpasteurised goat milk was not available legally, they would have to resort to unlawfully produced goat milk".

Should this occasion arise and the Queensland Government fail to convince NSW, SA, and WA to abandon their unpasteurised goat milk schemes, then an open market would be available in this State and the removal of Queensland producers would be for nought.

Whilst unpasteurised milk can be produced under Government supervised conditions in Queensland, the standard of milk sold can be controlled. If this control is removed, there is every reason to believe that unpasteurised goat milk from other States will be sold in Queensland.

The Queensland Minister for Health in the Parliament in answer to a question on mutual recognition policy said, "inter alia" - "Few jurisdictions allow the sale of unpasteurised goats' milk, and even these *MAY* alter their position should Queensland do so". (refer Q1342 Legislative Assembly)

"There is no certainty at present that all or any of the other States will alter their position at this or any other time". This was stated to the Committee in the briefing by the Senior Policy Officer, Intergovernmental Relations, Department of Premier and Cabinet on mutual recognition.

OVERVIEW - QUEENSLAND GOAT MILK INDUSTRY

"The future of this industry depends on the future or otherwise of unpasteurised milk".

Suncoast - see consumers letters

The overview makes no mention of expenses incurred by pasteurised producers which must be met from the price paid per litre. These include transport to pick up point (in one case 90km return trip), cost of transport to processor, cost of new disposable containers for bulk milk (in 1998/9), promotion levy, licence fees. These expenses are in addition to property overheads and feeds regardless of ex farm or bought in.

Suncoast Milk sales figures of pasteurised goat milk are attached (Appendix 14, Page 2).

An alternative view of the financial effect on unpasteurised milk producers is given in Appendix 14.

The pasteurised producer would require an increase in price to about $1.20 per litre from the processor to retain his present level of turnover. Even at that price the present unpasteurised producer is down $10,995 on present turnover.

Since the inception of pasteurised goat milk in 1982, there have been twenty-two producers "come and go" for a number of reasons, but mainly financial. This problem appears to still exist.

The processor has indicated on a number of occasions that it is uneconomical to process on the present volume.

We do not believe the issue of safety of unpasteurised milk is entirely the concern to the pasteurised milk producers. Market and returns to the producer have always been a major concern, however we do not believe that the freedom of consumer choice should be removed to allow "pasteurised milk to thrive" (QDA Options). This would be against the basic principle of the Hilmer Report.

COST BENEFIT/ANALYSIS

This was not finalised before the conclusion of the last Review Committee Meeting; only the factors were identified at that meeting.

At the briefing for the Committee by the Senior Project Officer, Business Regulation Review Unit, Department of Tourism, Small Business & Industry (TSBI) stated that this was the first time he had done an analysis involving quantative and qualitative of this type, and gave no indication to the Committee of weightings or calculation thereof. Mrs Aland and I have seen the analysis and are at a loss to explain the weightings; e.g., is 101% good or bad?

We believe the following comments on "comments" in the analysis should be noted (page numbers inserted for reference):-

1. Page 3 *Consumers have .... available in stores.*
   No evidence of this in Committee discussions. If one objects one is not likely to be a consumer. The product is now labelled in accordance with Standard and approved by Q Health.

2. Page 3 *Milk consumption overall .... change overall.*
   The meaning of this is not fully understood but looks like bad news for producers and processors.

3. Page 4 *Main issue .... minority group.*
   "Good government for ALL Queenslanders"?
   The Premier, on election result.

We note the Analysis has not been reviewed ..... .

APPENDIX 1

QUEENSLAND
LICENSED RAW GOAT MILK PRODUCERS' ASSOCIATION
PO Box 53
GYMPIE Q 4570
Telephone: (07) 5483 5288

5 November 1996

Queensland Health
Environmental Health Unit
GPO Box 48
BRISBANE Q 4001

Attention: Mr R V Holmes

Your Ref: 96M/342 RVH:MH dated 24 Sep 96

Re: REPRESENTATION ON COMMITTEE OF CONSULTATION ON GOAT MILK

Dear Sir

At a recent discussion on the committee to be formed in accordance with the referenced letter, members have pointed out that the original promise made by the Health Minister's Advisors (Messrs Peter Lacey and Richard Howard), was for a conference attended by all producers, the two government departments, and other interested parties.

To this end, we request that if this Association is to be limited to two representatives, then the remaining producers be able to attend, at the least as observers.

The Association is also concerned that the two major groups who have a considerable interest have not been mentioned; namely, consumers, and practitioners of natural medicine.

We request that they also be invited to take part in the consultation. We would be happy to arrange for representation from these interested parties to address the committee.

Yours faithfully

W K CARTER
for the QUEENSLAND LICENSED RAW GOAT MILK PRODUCERS' ASSOCIATION

ANZFA
Australia New Zealand Food Authority
PO Box 7186
Canberra MC ACT 2600

FACSIMILE MESSAGE

To: MR W. CARTER
FAX NO 07 5482 5711
TELEPHONE:

No. of Pages (including this page): 2
Date: 25/6/97
From: P. Brennan
Phone No:
Fax No:
Message:

ATTACHED IS A COPY OF A GAZETTE NOTICE, PUBLISHED ON 14 FEBRUARY 1996, ADVISING THAT THE FOOD AUTHORITY HAD ABANDONED PROPOSAL P114 — TO BAN THE RETAIL SALE OF UNPASTEURISED MILK...

I CAN'T FIND A RECORD INDICATING THAT LETTERS WERE SENT OUT TO INDIVIDUALS WHO HAD MADE A SUBMISSION (THE LETTER WOULD HAVE CONTAINED THE INFORMATION AS PRESENTED IN THE GAZETTE NOTICE, ANYWAY)..

NOTICES PURSUANT TO SECTION 25

Retail Sale of Unpasteurised Milk and Unpasteurised Cream of Mammalian Origin (P114). The Authority has prepared a proposal (P114) to vary Standard H1 - Milk and Liquid Milk Products, Standard H2 - Cream and Cream Products and Standard H5 - Goat Milk, to ban the retail sale of unpasteurised milk and unpasteurised cream of mammalian origin that is intended for human consumption.

Pursuant to section 23 of the *National Food Authority Act 1991*, the Authority has made a full assessment and has decided to abandon this proposal for the following reasons:

- In the processing of raw milk to packaged milk there are a number of unit operations (including milking, cool storage, filling, packaging, transporting and distribution) which must be controlled to ensure a safe product. Pasteurisation should not be seen as a solution to poor hygienic practices.

- The Code currently requires that milk and cream products be pasteurised except where expressly exempted in individual State and Territory law. The Authority recognises the present need to retain this provision but considers that exemptions should only be granted for:

  - goat (and sheep) milk where this is produced in accordance with a HACCP based code of practice such as the NSW and WA goat milk schemes, or

  - cow milk produced in remote communities where approved by the chief health officer of the State or Territory.

- The present regulations in the Food Standards Code ensure that consumers are provided with sufficient information to make an informed choice when purchasing unpasteurised milk and cream of mammalian origin intended for human consumption.

In abandoning this proposal, the Authority recognises that pasteurisation is one of the most effective means of protecting public health and safety for milk and cream products.

FEDERATION INTERNATIONALE DE LAITERIE – INTERNATIONAL DAIRY FEDERATION

Edward Hopkin
Secrétaire Général / Secretary General
41, Square Vergote,
B–1040 Bruxelles (Belgique/Belgium)
Direct line/Ligne directe: + 32 2 733 16 90

To the Chairmen of
IDF hygiene groups*

Reference:
Please quote:

EH/CB 2954

8 November 1996

Codex Committee on Food Hygiene

We have pleasure in sending you the report prepared by the IDF observers at the meeting of CCFH held in Washington D.C. in October 1996.

We feel they have done an excellent job and offer them our thanks.

Yours sincerely

Caroline Brooks
PA to the Secretary General

* Group	A7	Ewes and goats milk and milk of other species
	A30	Microbiological and safety of raw & unpasteurized farm milk products
	A32	(former B6 Milking machines)
	B21	Influence of technology on the quality of heated milk & fluid milk products
	B36	Hygienic design of equipment used in dairy plants
	B50	Disinfection
	D14	Assurance of hygiene & microbiological criteria for milk & milk products

Enclosure

International Dairy Federation

IDF Observers' Report

on

29th SESSION OF THE CODEX COMMITTEE ON FOOD HYGIENE

Washington, DC
21-25 October 1996

IDF Observers: Dr. O. Cerf (FR) and Dr. R. Byrne (US)

The 29th session of the Codex Committee on Food Hygiene (CCFH) was held in Washington, DC, 21-25 October 1996. Forty-two countries and 13 international organizations were represented. The following are the agenda items discussed, actions taken, and next steps for future actions:

Report by the Secretariat on Matters Referred to the Committee
a) General Matters
Three expert consultations were noted:

1. Joint FAO/WHO Expert Consultation on Biotechnology and Food Safety, in Rome, Italy, 30 September to 4 October 1996.

2. Joint FAO/WHO Expert Consultation on the Application of Risk Management to Food Safety Matters, in Rome, Italy, 29-31 January 1997.

3. FAO Expert Consultation on Animal Feeding and Food Safety, Rome, Italy, 10-14 March 1997.

Matters arising from the 43rd Session of the Executive Committee were noted. The Executive Committee had advanced a number of texts to Step 5 at its previous session along with the CCFH initiative to consider consumer responsibilities in relation to food safety. Technical government comments will be taken into account during this session of CCFH under separate agenda items.

The work being undertaken by the Committee on General Principles in finalizing the Terms and Definitions used in Risk Analysis was noted.

b) Endorsement of Food Hygiene Provisions in Codex Standards
Matters arising from the Codex Regional Coordinating Committee for Asia
The Hygiene Provisions contained in the following standards were endorsed:

- Proposed Draft Standard for Canned Bamboo Shoots
- Proposed Draft Standard for Dried Salted Anchovies
- Proposed Draft Standard for Crackers made from Marine and Freshwater Fish, Crustacean and Molluscan Shellfish.

The Committee did not endorse the proposal to include a microbiological test in the Standard for Canned Bamboo Shoots.

Matters arising from the Codex Committee on Fresh Fruits and Vegetables
The Committee endorsed the use of the standard wording for the Food Hygiene Provisions in the Draft and Proposed Draft Standards for Banana, Mangosteen, Limes, Pummelos, Guavas, and Chayotes.

Matters arising from the Codex Committee on Fish and Fishery Products
The Committee requested that a technical paper on the residual level of chlorine in products such as frozen shrimps and prawns and on recommended levels used in processing be developed by CCFFP.

Matters arising from the Codex Committee on Milk and Milk Products
The Committee noted that the CCMMP had forwarded common hygiene provisions in the standards for seven milk products for endorsement, and had asked for the Committee's consideration of two proposals in addition to the common provisions. On the first proposal, to include microbiological criteria to the Revised Standard for Butter, the Committee agreed that such criteria did not provide health protection additional to that which would be achieved by the implementation of the General Principles of Food Hygiene, including the application of a HACCP plan to the product. It decided that the inclusion of such criteria would be inappropriate.

On the second proposal to modify the common hygiene provisions in these standards by including a specific reference to the use of pasteurization or an equivalent measure, the Committee could not arrive at a consensus. Several delegations strongly resisted the specific reference to pasteurization stating that the application of the revised General Principles of Food Hygiene and the Principles and Guidelines for the Application of the HACCP System negated the need to specify any one processing step as being necessary for health protection. Other delegations strongly supported the proposed inclusion stating that pasteurization provided a well-recognized level of protection against which other measures could be assessed.

There was discussion as to the meaning of the words "equivalent measure" to pasteurization [the exact wording of the proposal was: *Pasteurization, or an equivalent measure approved by the official agency having jurisdiction, shall be used to achieve the appropriate level of public health protection*] and some delegations expressed the need for greater clarification before coming to a decision on this point. Several Delegations emphasized the need to finalize the revisions of the milk and milk products standards, now at Step 8 because in their view, they are not directly linked to the issue of pasteurization. However, other delegations disagreed. The Committee forwarded the common hygiene provisions to the CAC without making any additions to them. The Delegation of France reserved its position in regard to the Committee's decision not to endorse the standards based on the understanding that they provide satisfactory safety without any additions if appropriate reference to the General Principles of Food Hygiene and related texts is made.

The Delegation of the United States reserved its position in regard to the Committee's decision. In its reservation, the US Delegation stated: the public health protection benefits that pasteurization provides had been scientifically established, internationally recognized, and were irrefutable. There were current alternative processes or

technologies which might, under certain conditions, provide equivalent public health protection to pasteurization and more could be expected to evolve. The purpose of the

Codex Alimentarius International Food Standards was to "protect consumers", while facilitating trade. The Delegation urged that the endorsement of standards for international trade in dairy products which did not provide the public health protection benefits of pasteurization or an equivalent process, be weighed carefully to assure that an appropriate balance between "protecting consumers" and "facilitating international trade", consistent with scientific principles, was provided.

"There was much discussion during the review of the CCFH report regarding the reservation by US appearing in the report. The Chair and Secretariat permitted the reservation to remain because it had been submitted as an official reservation during the meeting, following the proper protocol outlined in the Procedural Manual. The Delegate from France then submitted their reservation. This instance stresses the importance of knowledge of the procedures when attending Codex meetings."

Consideration of the Draft Revised Guidelines for the Application of the HACCP System at Step 7

The guidelines were reviewed by an *ad hoc* working group. Several delegations expressed their concern that difficulties might be encountered in applying the HACCP system in smaller businesses and developing countries. The Committee agreed to the revised text with minor modifications, mainly regarding the question of flexibility and application to small businesses and developing countries. The Guidelines were advanced to Step 8 for consideration of the 22nd Session of CAC as an integral part of the Revised General Principles of Food Hygiene.

Consideration of the Draft Revised Principles for the Establishment and Application of Microbiological Criteria for Foods at Step 7

The Committee reviewed the revised draft prepared by a working group. It was agreed that microbiological criteria could be used to determine that processes were consistent with the General Principles of Food Hygiene, but that this need not be referred to explicitly in the definition. An appropriate statement to this effect was included in the sections on Purposes and Application of Microbiological Criteria. The Committee also agreed that criteria could be used to define and check compliance with regulatory provisions concerning hygiene. It was further agreed that microbiological criteria should be used only when no more effective tools were available and where their use would improve the degree of protection offered to the consumer.

The Committee clarified that microbiological criteria should be specific for the product and for the specific stage in the food chain to which they apply. An in-depth discussion occurred related to the use of "presence-absence" tests and their relevance as indicators of public health. It was confirmed that the presence of certain pathogenic organisms did not necessarily indicate a threat to public health.

The Committee decided to provide a more detailed description of the ability of sampling plans to detect organisms in a given food lot, and at the same time confirmed that sampling plans on their own could not ensure the absence of any of the specified organisms.

The Committee advanced the Draft Principles to Step 8 for submission to the 22nd Session of the CAC.

Consideration of Approaches for the Revision of Commodity Codes
The Committee welcomed the opportunity to establish consistency in the revision of the commodity-specific codes and to base these revisions of the newly revised General Principles of Food Hygiene, including the Annex on HACCP, and the revised Principles for the Establishment and Application of Microbiological Criteria for Foods. The Committee noted that the exclusive stress on food safety could limit the usefulness of the Codes and that some flexibility had to be retained to provide guidance on certain non-safety factors (i.e., decomposition or handling practices). The committee noted that the CCFFP had already begun the revision of the Codes of Practice.

The Committee endorsed the following recommendations, referring them to the CCGP for information and submitting them to the CAC for adoption and inclusion in Section H of the Procedural Manual:

a. Codex Codes of Hygienic Practice should serve the primary purpose of providing advice to governments on the application of food hygiene provisions within the framework of national and international requirements.

b. The Revised Recommended International Code of Practice - General Principles of Food Hygiene (including the Guidelines for the Application of the Hazard Analysis Critical Control Point (HACCP) System) and the Revised Principles for the Establishment and Application of Microbiological Specifications for Foods are the base documents in the field of food hygiene.

c. All Codex Codes of Hygienic Practice applicable to specific food items or food groups shall refer to the General Principles of Food Hygiene and shall only contain material additional to the General Principles which is necessary to take into account the particular requirements of the specific food item or food group.

d. Provisions in Codex Codes of Hygienic Practice should be drafted in a sufficiently clear and transparent manner such that extended explanatory material is not required for their interpretation.

e. The above considerations should also apply to Codex Codes of Practice which contain provisions relating to food hygiene.

The committee also agreed that consideration should be given to revision of the standard texts of food hygiene provisions recommended for use in commodity standards contained in the Procedural Manual, Section K.

Consideration of Draft Code of Hygienic Practice for Refrigerated Packaged Foods with Extended Shelf-life at Step 7
An *ad hoc* Working Group which had not yet reached any consensus over a few issues such as refrigeration temperature, necessity for additional hurdles, and the delineation of the Scope presented the document. The Committee agreed that the document should be revised to incorporate the comments received, particularly by making due reference to and avoiding replication with the General Principles of Food Hygiene.

The Committee agreed that the document would be revised by the government of Canada, with assistance provided by France, U.S., & U.K. with potentially controversial parts high-lighted, and be circulated for comments by government at Step 6.

Copy to members

APPENDIX 4

FAX to: Miranda Boyle,
Gatton Post Office
Queensland
FAX 074 622 877

7th., January, 1997.

In response to your telephone enquiry this afternoon, I am glad to be able to inform you that unpasteurised Goats' milk, obtained from a dairy with impeccable hygene near Perth, is observed clinically in my practice treating children with learning difficulties and associated behaviour disorders to be indispensible for the normalisation of metabolic disturbances and allergic responses.

The treatment is based on exercises in movement and balance deriving from Dr. Rudolf Steiner's discoveries in child development. Carefully monitored diet modification is now a part of this work and all cows'milk products are withdrawn.

I have just completed writing up a preliminary research evaluation of the is work. As soon as possible I shall be glad to send you a copy. It clearly shows, on the basis of objective analysis of data, significant improvement in academic performance and in associated behaviour problems. Concurrently, measured metabolic imbalance in subjects before treatment was brought into balance in all cases showing significant improvement.

The project was undertaken with the assistance of Professor A. Harper and research associates. An epidemiologist. Prof. Harper is professor of public health at the HBF-Curtin Research Unit, Curtin University of Technology, Perth.

The part played by goats' milk was not separately evaluated in the project. Clinical observations repeatedly show reduction of middle ear, sinus and upper respirative conditions and allergies when goats' milk is the only milk used. We have had cases where that regime has been broken and cows' milk re-introduced. Always the symptoms returned and reduced again on returning to goats' milk.

I trust that some research is being done or will be done on the positive effects of goats' milk and the reasons for it. There is no doubt that there are some people who cannot tolerate pasteurised milk and not cows' milk in any form.

May I refer you to two studies published in The Lancet: Journal of the B.M.A? 1) Oct., 15, 1983 Is migraine an allergy? and 2) Mar., 9 1985 Controlled trial of oligoantigenic treatment in the hyperkinetic syndrome.

With best wishes.

Alan Grosser, 22 Bryant Ave., Mosman Park, West Australia. 6012.
FAX 09 384 6723

HOMOGONISED MILK COULD KILL YOU

According to statistics 50% of New Zealanders die from heart disease, 25% from cancer and 5% from diabetes.

Research has shown that the epidemic of hardening of the arteries (which leads to more deaths annually than cancer) is directly attributable to homogenized milk.

For more than 20 years Kurt A. Oster, MD, chief of cardiology emeritus at Park City Hospital, Bridgeport, Connecticut, has gathered evidence that provides a conclusive biochemical explanation for atherosclerosis (a form of arteriosclerosis, plaque clogging arteries).

The culprit turns out to be the process of homogenization, introduced to the dairy industry in 1932 as a means of improving marketing for products.

Homogenization merely prevents the cream from separating from the milk. This is primarily cosmetic. It does nothing to enhance the quality of the milk. But according to Dr. Oster's findings, it does a great deal to improve marketability - at the expense of human life.

Fragmented

Homogenization causes the fat globules in whole milk to be fragmented into tiny, tight molecules that will not regroup. Not only do these intense molecules of fat refuse to regroup, they also resist digestion and manage to enter the bloodstream unaltered - which is one reason why so many people are allergic to milk.

"Milk fat contains a substance called xanthine oxidase (XO). When milk is not homogenized both the fat and the (XO) are digested in the stomach and small intestine into smaller molecules, which are either used or excreted from the body. Homogenization allows some of the XO to pass into the bloodstream intact," Oster reported.

XO is found in the liver of many animals, including humans, where it has a specific function: that of breaking purine compounds down into uric acid, a waste product.

"When foreign XO, such as that from cow's milk, enters the bloodstream it creates havoc by attacking specific targets within the artery walls," Oster's report said.

The "specific target" within the arteries is called plasmologen, a tissue making up 30 percent of human heart muscle and artery wall cells. Plasmologen's presence is vital for the integrity of the outer cell membrane analogous to the way mortar holds bricks together in a wall.

Arteries Calcified

The direct attacks by foreign XO, Oster's research indicates, cause lesions within artery walls and the body's marvelous protective mechanisms respond to the damage by scarring and laying down calcified plaques.

"The simple thickening and hardening of the arteries is known as arteriosclerosis, whereas atherosclerosis is characterized by the additional accumulation of cholesterol and fatty deposits laid down adjacent to scars and plaques," Oster said.

"Gradually, the artery wall thickens, obstructing the flow of blood. Arteries lose their elasticity in the later stages of the disease as additional calcium is deposited. Calcification of the arteries can contribute to high blood pressure which is actually not a disease in itself. High blood pressure is merely a symptom," Oster added.

The assault of XO on the artery walls has been further documented by autopsies performed on patients who died from heart and circulatory disease, Oster and Ross reported.

"It has been found that plasmologen was completely missing in artery wall lesions and plaques, and the mystery was solved when researchers found XO in the plaques. The two substances cannot coexist," Oster said.

Eliminating sources of undigested XO from the diet, primarily homogenized milk and milk products, including ice cream and cheese, and taking the vitamin folic acid, which blocks the action of XO and helps rebuild cell membranes in arteries, can stop and in some cases reverse the damage. ☺

REVIEW OF

UNPASTEURISED GOAT MILK

for the

Environmental Health Unit
Queensland Government

submitted by the

Australian Natural Therapists Association
(ANTA)

20 February 1997

Australian Natural Therapists Association Ltd ACN 000 161 142
PO Box 856 Caloundra Q 4551
Contact: Geoff Henry N.D., National President
Phone/Fax: (07) 5492 8206

Australian Natural Therapists Association Ltd.
ANTA - IT'S YOUR CHOICE, NATURALLY A.C.N. 000 [illegible]
National Administration Office: P.O. Box 856, CALOUNDRA Q 4551
1st Floor, 126 Bulcock Street, Caloundra
Phone (07) 5491 9850 *Fax (07) 5491 5079*

20 February 1997

Mr RV Holmes
A/Director, Food Services
Environmental Health Unit
Queensland Health
GPO Box 48
Brisbane Q 4001

Dear Mr Holmes

Re: Unpasteurised Goat Milk

Enclosed is a submission for the Review of Unpasteurised Goat Milk from the Australian Natural Therapists Association.

I note the composition of the review committee does not include a representative from the natural therapies profession. The Australian Natural Therapists Association (ANTA) is one of the associations listed in schedule 1 of the Therapeutic Goods Act. ANTA is a national association with the National Administration Office and National President based in Queensland with Chairpersons in each state. On issues dealing with public health and safety in Queensland and in this instance unpasteurised goat milk, I would have thought it very appropriate to include a representative from ANTA to the committee.

As a representative was not invited from ANTA, and I understand it will be a closed review, I trust ANTA will be advised of the recommendations and the reasons for the recommendations from the review committee.

The onus is on the Queensland Government to prove with documented evidence that unpasteurised goat milk is harmful to human health.

Yours sincerely,

Geoff Henry
National President

ANTA is unaware of any proven documented evidence that unpasteurised goat milk produced in Australia or Queensland is harmful to human health. This is in spite of the fact there are both licensed and unlicensed producers in Australia.

The Queensland Minister for Health has already received numerous submissions, particularly in November 1996, which I trust were forwarded on to the committee who will read them in full before they reach a decision. This submission will not therefore reiterate many of the issues and benefits raised.

To quote from one such submission - "Dr Bernard Jensen, possibly the world's highest regarded naturopathic specialist in tissue therapy and tissue regeneration says, "I believe the best tonic available for children is raw goat milk. Why? Flourin is a chemical element often missing in our system. Flourin is volatile; it is very unstable. Heat destroys it. Pasteurised milk causes tooth decay for this reason. Flourin is the chemical element that keeps teeth from decaying." Dr Jensen also says of goat milk that "....its marvellous germicidal sanity effects prepare the body for the new health tissues." (from Goat Milk Magic, by Dr Bernard Jensen)".

Many of the submissions mention the beneficial enzymes in unpasteurised goat milk. Natural therapists recognise these benefits and often recommend goat milk, preferably unpasteurised, in lieu of cows milk as part of a mucus forming food free diet.

There are many in orthodox medicine who do not accept foods can have a 'mucus forming' effects, there are also many medical practitioners who do accept the natural therapies approach. Groups comprising of medical practitioners such as the Complementary Medical Association, the Australian College of Nutritional and Environmental Medicine have recognised the basis of natural therapy treatments, and recently the Australian Medical Association has announced their acceptance of natural therapy studies for their members.

There is a small percentage of the population that have clinical signs and symptoms of a cows' milk allergy and a larger percentage who have subclinical symptoms. Clinically, Naturopaths find some of the most common adverse reactions to cows milk are constant or recurrent sinus problems, recurrent tonsillitis, eczema, diarrhoea and even asthma. For example; for tonsillitis, which is an infection of the collection of the lymphatic tissues at the back of the tongue; sinus, which is congestion and sinusitis, which is inflammation of the mucous membranes lining one or more of the sinuses connecting with the nose; as part of the treatment of these conditions naturopaths more often than not require the elimination of cows milk products from the diet and may recommend the inclusion of unpasteurised goat milk.

It is believed the protein molecules in cows milk are smaller than that in goat milk and pasteurisation breaks down these molecules even smaller which allows them to cross the cell membrane thereby creating an allergic reaction leading to abnormal mucus production.

It is well documented that pasteurisation affects the protein in milk:

> "The proteins in milk are casein and whey. Caseins are remarkably heat stable but the whey proteins, which are nutritionally superior than caseins, are denatured by heat

treatment. Whey proteins include lactoferrin, alpha-lactoglobulin, beta-lactoglobulin, albumin, lysozyme and immunoglobulins."(1)

Pasteurisation also adversely affects 'friendly' bacteria in milk:

"Pasteurisation is intended to kill harmful bacteria that may be present in milk, however, not all bacteria are destroyed.....pasteurisation will not completely destroy all bacteria, and unfortunately, 'friendly natural bacteria' are also destroyed, leaving possible harmful bacteria."(2)

Many people who consume goats milk do so because of a cows milk intolerance. Unpasteurised goats milk is the preferred choice as it is nutritionally better than pasteurised milk. Therefore, the main advantage of unpasteurised goat milk is the nutritional value.

Due to the absence of documented evidence that unpasteurised goat milk has caused harm to the health and safety to the consumer and the labelling requirements on the product, the disadvantage of unpasteurised goat milk is the limited number of licensed producers.

As ANTA members and their patients are also consumers, the issue of Freedom of Choice is also very relevant for the review. As licensed goat milk producers are required to comply with strict guidelines and no documented evidence of harm from unpasteurised goat milk produced in Australia is available, there can be no health issue for which the government can make unpasteurised goat milk unavailable in Queensland. Therefore as consumers we would expect freedom to access unpasteurised goat milk.

REFERENCES

(1) Dr Frank A. Oski M.D.
Don't Drink Your Milk
Beta Books, Castle Hill NSW, 1983 p 97-100

(2) Manfred urs Koch
Laugh with Health
Renaissance & New Age Creations, Australia 1986

With forwards by: Dr John Tickell, M.B., B.S.
Dr J. Grantley Shelton, F.R.C.O.G., F.A.G.O., F.R.A.C.O.G.
Dr D.P.B Anderson M.B., B.S.

BULLETIN OF THE INTERNATIONAL DAIRY FEDERATION N° 191/1985

ISSN 0250-511B

BULLETIN

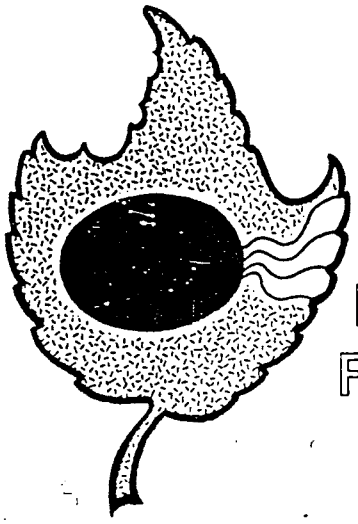
PROTECTIVE PROTEINS IN MILK-

BIOLOGICAL SIGNIFICANCE AND EXPLOITATION

LYSOZYME, LACTOFERRIN, LACTOPEROXIDASE, XANTHINEOXIDASE

APPENDIX 8

Antimicrobial Systems in Milk



IDF
SAB
BSPP
FEMS

PART 2 of Proceedings of Symposium on

NATURAL ANTIMICROBIAL SYSTEMS

University of Bath, England, 10 - 13 September 1985

PETITION

TO: The Honourable the Speaker and Members of the Legislative Assembly of Queensland.

The Petition of residents of the State of Queensland draws to the attention of the House -

Their disappointment and deep concern at the proposed withdrawal by the Minister for Health of the exemption from pasteurisation of GOAT MILK in Queensland. This is a direct denial of freedom of choice in the Market Place.

Your petitioners therefore request the House -

TO Ensure the continuation of the availability of Queensland produced quality UNPASTEURISED GOAT MILK from properly licensed GOAT DAIRIES.

PRINCIPAL PETITIONER

QUEENSLAND LICENSED RAW GOAT MILK PRODUCERS ASSOCIATION - Bill Carter (Chairman)
1633 Bruce Highway (South), KYBONG, VIA GYMPIE 4570

FULL NAME (PRINT CLEARLY)	ADDRESS	SIGNATURE

ANALYSIS OF CONSUMERS' LETTERS

A sample of 50 consumers' letters was taken from copies handed out at the first Committee Meeting in November, 1996. The reasons given for purchasing unpasteurised goat milk are listed below:-

*	Intolerance to other milk	7
*	Child thrives on unpasteurised goat milk	3
*	Freedom of choice	nearly all letters
*	Strong allergy to cows' milk	6
*	Bronchial problems	2
*	Irritable Bowel Syndrome	3
*	Multiple allergies including pasteurised goat milk	1
*	Leukaemia (important adjunct to treatment)	1
*	Digestion difficulties	1
*	Baby eczema	3
*	Serious health concerns (unspecified)	1 (QUF cow milk producer)
*	Asthma	2
*	Coeliac (gluten allergy)	4
*	Raised on raw goat milk (one 80 year old)	3
*	Like to have a supplier of milk they know	1
*	Chronic Fatigue Syndrome	4
*	Taste of pasteurised goat milk	3
*	Avoids processed foodstuff	1
*	Considers raw food better for you	1
*	Cost of pasteurised goat milk	1
*	Quality of life	2
*	Raw goat milk prescribed by a doctor (1992)	2

APPENDIX 11

QDA
QUEENSLAND DAIRY AUTHORITY

OPTIONS PAPER

SALE OF UNPASTEURISED GOAT MILK IN QUEENSLAND

1995

APPENDIX 12

3

E.R Crawford

Pasteurised Goat Milk Representative
"Hillview" M.S 361 Murgon.
Qld. 4605
Ph: 071 684723

25 November 1996

Mr R.V. Holmes
Acting Director Of Food Services
Queensland Department Of Health
GPO Box 48
Brisbane. 4001.
Queensland.

Dear Sir,

Thank you for your invitation to present a submission on the sale of Unpasteurised Goats' Milk in Queensland.

Please find attached copy of our submission which was presented to the Queensland Dairy Authority in 1995 on the same subject.

In addition I have as you requested supplied the following figures on production costs. IE:- Our feed costs at the present moment representing bought in feed equate to approximately 1.25KG to 1.5KG per head per day at $230.00 per tonne landed in silo in bulk on farm. All other feeds are grown ex-farm, therefore production costs can be equated by using above bought in feed plus farm running costs subtracted from per head litreage production, per lactation at one dollar and two cents per litre for summer milk and one dollar and twelve cents per litre winter milk ex farm gate to the factory.

Therefore I do not feel the statement that milk being sold to the factory is economically un-viable.

Hopefully this will be of some use in your discussions on Friday 29th November.

Yours faithfully

Errol R. Crawford.

APPENDIX 13

Trevor J. Perrett, M.L.A.
Member for Barambah
Shadow Minister for Primary Industries and Forestry

LEGISLATIVE ASSEMBLY
QUEENSLAND

Barambah Electorate Office:
Glendon Arcade,
Glendon Street,
Kingaroy, Qld. 4610

Telephone: (071) 62 1381
Fax: (071) 62 4774

22 June 1995

Mr W K Carter
Gaybills Goat Dairy
PO Box 53
GYMPIE Qld 4570

Dear Mr Carter

My Parliamentary Leader, Mr Rob Borbidge MLA, has referred your recent correspondence to him regarding the Dairy Industry Authority review of the goat milk industry in Queensland for my direct reply to you.

As you correctly state, this is a controversial subject and has been for a number of years. I welcome the industry review by the Government and have carefully scrutinised the Discussion Paper.

As Shadow Minister, I have been contacted by producers with differing points of view. It has been the policy of the Coalition to listen to industry when it comes to policy development. Indeed all our primary industry policies, which will be released in the very near future, have been developed with direct industry input, and there is no reason why we should treat the goat milk industry in any other way.

As Shadow Minister, I am prepared to let the review take its course and hopefully a position will be reached which has the support of the majority of producers in Queensland.

The goat milk industry is a small but significant industry and producers, like yourself, who have been instrumental in the establishment of the industry must have your investment supported.

Whatever the outcome of the review and whichever option is agreed upon, I do have concerns about the possible effect of mutual recognition of standards which all States have agreed upon. This will allow a product of a lower standard from another State to be sold in Queensland, unless it can be proven that the public is threatened by the sale of such product. I will be watching this point with interest.

..2/

I remain in receipt of your previous correspondence which was forwarded to me by the Member for Wide Bay, Mr Warren Truss MHR, in November 1993 and have noted the points that were made at that time.

Thank you for your interest in this matter and, if the Coalition is successful in winning government on 15 July, I would be happy to discuss the whole issue with you.

Yours faithfully

Trevor J Perrett

Trevor J Perrett MLA
Shadow Minister for Primary Industries and Forestry
(Member for Barambah)

FINANCIAL IMPLICATIONS OF A BAN ON UNPASTEURISED GOAT MILK

Assumptions

1. Retail price of unpasteurised milk at farm gate is $2.00 per litre.

2. Processor price for pasteurised milk at farm gate is $1.12 per litre (less cost of company pick up vehicle and less promotion levy, less cost of flimsy containers in 1998/9).

3. All farms produce the same quantity of milk, that is pasteurised milk one seventh of total given in Q Health Review Report, and unpasteurised one sixth of total given in Q Health Review Report.

4. Retail price of pasteurised milk: $2.40 per litre.

A. Present Situation

Unpasteurised:				
106,500 x $2.00	=	$213,000 divided by 6	=	$ 35,500 (average turnover)
Pasteurised:				
114,000 x $1.07	=	$160,500 divided by 7	=	$ 22,929 (average turnover)
Suncoast Milk: Including Vendor & Retailer				
Sales - 114,000 x $2.40	=	$273,600 - $160,500	=	$113,100
			=	41% on Sales

B. Pasteurised Only

Producers:				
220,500 x $1.12	=	$246,960 divided by 11	=	$ 22,450
		Annual turnover - Unpasteurised Producer		($ 13,050)
		- Pasteurised		($ 479)
Suncoast Milk: Including Vendor & Retailer				
220,500 x $2.40	=	$529,200 - $246,960	=	$282,240
			=	53% on Sales
		Increase over Situation A.	=	$169,140

FINANCIAL IMPLICATIONS OF A BAN ON UNPASTEURISED GOAT MILK

C. Producer price of $1.20

220,500 x $1.20	=	$264,600 divided by 11	=	$ 24,055
		Unpasteurised Producer:		($ 10,995)
		Pasteurised Producer:		$ 1,605
Suncoast including Vendor & Retailer			=	$264,600
			=	50% of Sales
		Increase over Situation A.	=	$151,500

Suncoast Milk Sales 1982-97

Year	Sales (Litres)
1982/3	40,088
1983/4	66,121
1984/5	107,791
1985/6	117,602
1986/7	140,468
1987/8	158,643
1988/9	173,473
1989/90	145,131
1990/1	156,049
1991/2	99,959
1992/3	97,895
1993/4	112,300
1994/5	97,966
1995/6	109,000
1996/7	150,000 (est.)

10 September 1996

QUALITY TESTING PROGRAM - UNPASTEURISED GOAT MILK

CLASS "D" PRODUCER'S LICENCE

All scheduled testing and clearance testing with the exception of the pathogen testing will be to the account of the licence holder.

TEST	STANDARD	TEST FREQUENCY	RESPONSIBLITY FOR TESTING	ACTION FOR NON-COMPLIANCE TO STANDARDS
Standard Plate Count	Dairy Ind. Standard not exceeding 50,000cfu/ml Food Standards Code not exceeding 150,000cfu/ml	2 weekly	Producer	Above 50,000cfu/ml - Milk sample tested within 7 days if test does not comply sales of milk suspended until a test below 50,000cfu/ml submitted. Above 150,000 - Sales of milk suspended until a test result below 50,000cfu/ml is submitted.
Coliform	not exceeding 100cfu/ml	2 weekly	Producer	Above 100 cfu/ml - milk sales suspended until a test result below 10cfu/ml is submitted.
Salmonella	not detected in 25ml	Monthly for 3 months then every 3 months if all results comply. The above program is repeated for any non-compliance.	Queensland Dairy Authority	Salmonella detected - Milk withdrawn from sale immediately and further sales stopped until cleared by the Authority. Clearance testing carried out: 2 tests a week for 2 weeks 1 test a week for 2 weeks All tests to be negative and no milk sold until all tests are completed. If any tests are positive the testing program will recommence.

TEST	STANDARD	TEST FREQUENCY	RESPONSIBLITY FOR TESTING	ACTION FOR NON-COMPLIANCE TO STANDARDS
Listeria monocytognes E. Coli Coagulase Positive Staphylococci Campylobacter spp. Yersinia enterocolitica	not detected 25ml not exceeding 10cfu/ml not exceeding 100cfu/ml not detected in 25ml. Not detected in 1ml.	Monthly for 3 months then every 3 months if all results comply. The above program is repeated for any non-compliance.	Queensland Dairy Authority	Milk to be withdrawn from sale immediately and clearance testing carried out. Sales of milk can recommence when test results comply to standards.
Antibiotics	<0.003ug/ml	monthly	Producer	The milk that has failed the test is withdrawn from sale. Milk is tested at next 2 weekly test. Detection of antibiotic at this test and all milk must be withdrawn from sale and sales are suspended until a clear test is submitted.
Iodine	<500ug/l	monthly	Producer	Milk is tested at the next 2 weekly test. If detected at this test all sales of milk are suspended until a clear test is submitted.
Pesticides	less than M.R.L.	yearly	Producer	Milk is withdrawn from sale and sales of milk suspended until a negative test is submitted.
Melioidosis	not detected	Seriological yearly Milk 3 monthly	Producer	Milk is withdrawn from sale and sales of milk suspended immediately. Milk sales remain suspended until clear tests are submitted.

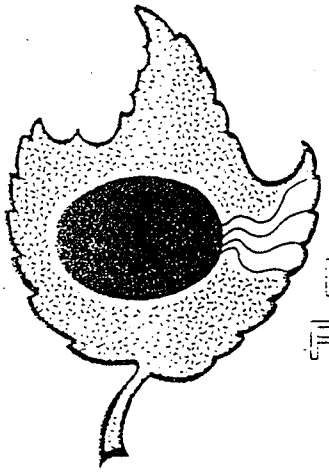
Please Return To:
Miranda Boyle
37 Haslingden Rd.,
Lockyer Waters

APPENDIX B

Antimicrobial Systems in Milk

University of Bath, England, 1985

Antimicrobial Systems in Milk



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PART 2 of Proceedings of Symposium on

NATURAL ANTIMICROBIAL SYSTEMS

University of Bath, England, 10 - 13 September 1985

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CONTENTS

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CONTENTS

FOREWORD

IDF established a group of experts on natural antibacterial systems (reference F19) at its Annual Sessions at Montreux, Switzerland, in 1979. The Group began to prepare a monograph but rapidly came to the conclusion that it would be more useful to organize a Symposium. Interest in the subject had been growing once again but the scientific literature was scattered. The object was to bring specialists together and provide an up-to-date summary of the state of the art. At the same time the plans of the Federation of European Microbiological Societies (FEMS) for a more general event became known and it was agreed to associate the IDF meeting with that of FEMS to provide a broader view. Despite FEMS' plans being far advanced, it was possible to arrange close collaboration in the organization of the event though it has proved necessary to publish the proceedings in two parts, this second part being devoted to the "IDF day" of the Symposium. One paper (L. Björck) and two posters (Dallyn et al. and Sahl et al.) appear in both parts. Details of Part 1 may be found on the inside front cover of this publication.

The Symposium was held at the University of Bath, England, on 10-13 September 1985. A small trade exhibition of companies working in the field added a further facet to the event. In total 160 participants from 19 countries were present, at least half of them staying for both parts of the Symposium.

IDF General Secretariat
January 1986

ANTIMICROBIAL SYSTEMS IN MILK

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INTRODUCTION

This symposium is the first to be devoted entirely to the antibacterial factors in milk. In the past they were included in symposia on microbiology, dairying, nutrition and immunity of the neonate or mastitis.

The clinical significance of colostrum and milk can be retraced to 1892 when Ehrlich discovered that in mice passive immunity was acquired via the placenta and during nursing. He suggested also that antibodies resisted digestion and may be produced in the mammary gland. He used ricin as antigen which promises, coupled to monoclonal antibodies, to became the magic bullet against some forms of cancer.

In 1922 Little demonstrated that calves deprived of colostrum frequently died of septicaemia. Much later it was established that calves (and other ungulates) are born without Igs in their blood; they depend on the ingestion and absorption of colostral antibodies during 24-36 h after birth. The human fetus however acquires Igs via the placenta. The mouse uses both pathways, thus explaining the observations of Ehrlich.

In the fifties, Petersen tried to establish that specific antibodies are synthesized in the bovine udder but received only ridicule. Recognition of this concept had to wait for the discovery of secretory IgA and demonstration that human colostral plasma cells synthesize specific antibodies.

Parallel to this development in the sixties, Soyka demonstrated in piglets the importance of K surface antigens in *E. coli* for the ecology of scouring without septicaemia. K antigens were later recognized to be piliary (e.g. K_{88}) adhesins, promoting attachment in the intestine before the bacteria elaborate the disease-producing enterotoxins. Freter, at about the same time, suggested that local immunity in experimental cholera is correlated with the inhibition of attachment to the intestinal mucosa, but this was also not readily accepted. Eventually medical immunologists began to appreciate the results of veterinary research. This cooperation led to the general concept of Mucosal Associated Lymphoid Tissue (MALT) which includes the gut, lung, mammary, salivary and lacrimal glands and the genital tract. MALT requires considerable traffic of cells between secretory sites, carrying information. It is therefore fitting that the first paper on milk immunoglobulins is presented by Prof. Goldman & Goldblum. They were involved in the identification of the leucocytes in human colostrum and the pathways whereby the antigenic information from the intestine reaches the mammary gland.

Immunoresponses, both in terms of antibody production and graft rejection, can only be mounted by vertebrates. Compared with this late appearance of Igs of a IgM-like immunoglobulin in cyclostomes, the most primitive vertebrates, non-antibody protective proteins or their forerunners appeared early in evolution.

Under aerobic life conditions, protective systems such as superoxide dismutase, catalase and peroxidases evolved to prevent damage by the toxic metabolite H_2O_2. The best studied peroxidases are glutathione, cytochrome, NADH peroxidase etc. From 1963 onwards secretory peroxidases from milk, saliva and tears, and cell peroxidases in polymorphonuclear leucocytes and eosinophils, have been known, in the presence of H_2O_2 to calatyse the formation of antibacterial products from thiocyanate and halogens. A chance observation by Hoogendorn that the LP-system appeared to heal aphthous lesions led to the demonstration by Carlsson in Sweden and Tenovuo in Finland that the lactoperoxidase-thiocyanate system can also protect. Cultured cell lines such as HeLa, CHO cells and gingival fibroblasts were protected against the toxic effect of H_2O_2.

Dr Björck will present the paper on the lactoperoxidase system. The distribution of the enzyme, its structure and oxidation products of thiocyanate which inhibit a wide range of bacteria. I shall discuss the *in vivo* activity of the system and its application to prevent scouring in newborn animals. Dr Schmekel & Härnulv will give the paper on the application of the lactoperoxidase system in the temporary preservation of refrigerated and uncooled milk; the latter application appears to be of considerable value for the dairy industries in developing countries.

Another dichotymy of a "protective" protein is evident in the biological activities of transferrin and lactoferrin. Again their antibacterial activities were described before it was recognized that both proteins have an iron carrying role between blood cells or enterocytes. Such cells possess receptors which are high affinity uptake systems for iron complexes. It is fascinating that virulent bacteria, cultured in media low in iron, synthesize also outer membrane receptors for their iron carrying siderophores. According to Griffith, London & Brock, Glasgow (personal communications) human milk contains antibodies for receptors and siderophores. These findings may finally settle the question of the role of immunoglobulins in the antibacterial activity of lactoferrin. Iron binding proteins are also not restricted to vertebrates because they occur in a spider and were characterized in a crab as a high molecular weight glycoprotein binding two atoms of iron per molecule. Dr Ribadeau-Dumas will present the paper on the biochemical and antimicrobial properties of lactoferrin in neonates, Prof. Hambraeus on the role of lactoferrin for iron absorption.

The paper on lysozyme will be given by Drs Banks & Tranter. Lysozyme is undoubtedly also an ancient protein. There is a definite molecular evolution, as shown by the amino acid residues in the proteins from various sources such as bacteriophage lysin, lysozyme from insects, humans, birds, cows, etc. Nevertheless there is still too little known about the biological role of the different lysozymes. For instance, I was always intrigued that according to Board albumen lysozyme is not so much a protective factor but rather provides the gelatinous properties through its interaction with ovomucin. A gelling role may be also ascribed to lysozyme in the abomasum of the calf. We observed in calves, cannulated in the duodenum, that during the feeding of milk clear whey emerges almost immediately in the duodenum. Considering that rennin clots milk rapidly only in the presence of lysozyme, it may promote also the rapid gelling of milk *in vivo*. In the adult, however, the main function may be to help the digestion of the rumen bacteria which furnish about one third of the N_2 requirement. Human milk in contrast to bovine milk is rich in lysozyme but very little is known about its function. In my paper I shall discuss the Jollès hypothesis that it may act as an immunomodulator or adjuvant in the intestine and that it may be particularly important in suppressing clostridia.

In conclusion, the non-antibody factors occur in all secretions bathing mucous membranes and in that respect the unprotected intestinal tract of the newborn can be regarded as an extension of the dam. These proteins are phylogenetically older than the more specific lymphocyte/plasma cell/immunoglobulin systems and have a role to play in the immunity of the vertebrates and in particular of mammals.

Foot note: Recent comprehensive reviews:

Reiter, B. – The lactoperoxidase system in bovine milk, in (eds)
Pruitt K.M., Reiter, B. – Biochemistry of peroxidase system: antimicrobial effects in the lactoperoxidase system (eds) K.M. Pruitt, Jorma O Tenovuo 1975 Marcel Dekker Inc New York.
Reiter, B. – The biological significance of the non-immunoglobulin protective proteins in milk: lysozyme, lactoferrin, lactoperoxidase in Development in dairy chemistry – Vol 3 (ed) P.F. Fox, Elsevier Applied Science Publishers Ltd, U.K. (in press).

Immunoglobulins in human milk

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Summary

The pattern of immunoglobulins in human milk is different from that found in human serum or cow's milk preparations used in infant nutrition. The major immunoglobulin in human milk, secretory IgA (SIgA), is assembled from proteins produced by plasma cells of the mammary gland and epithelial cells of the mammary gland. The plasma cells that produce dimeric IgA originate from the intestinal tract and respiratory system. The dimeric IgA binds to receptor secretory component (SC) molecules on the mammary gland epithelium. The extracellular segment of SC attached to an IgA dimer is then transported across the cell and secreted as SIgA. These SIgA antibodies are directed against immunogens that trigger the entero-bronchial mammary gland pathways. Because of their antibody repertoire, resistance to enzymatic digestion, and failure to generate inflammation, SIgA is well adapted to defend the mucosal surfaces of the recipient when the infant is unable to mount a complete mucosal immune response. The quantities of SIgA in human milk are closely regulated during lactation and are inversely related to the production of SIgA by the infant. A great deal is yet to be learned concerning the control of the production and ultimate fate and effects of these specialized antibodies in human milk.

The lower frequency and severity of enteric infections in breast-fed infants (Cunningham 1981) particularly in developing countries (Mata et al 1967, Mata & Urrutia 1971), led to the postulate that human milk protects the recipient infant against infectious agents. During the past 30 years, considerable evidence was found that the protection is due to a complex defense system which is adapted to operate in the aeroalimentary tract of the human infant (Reviewed by Goldman & Smith 1973; Goldman & Goldblum 1985; Goldman et al 1985). The system in human milk consists of living cells, growth promoting factors for Lactobacillus bifidus, proteins that bind bacterial nutrients, oligosaccharides and non-antibody peptides that are anti-bacterial, lipids and non-antibody peptides that are antiviral or antiparasitic, enzymes that are antibacterial, and immunoglobulins. Because immunoglobulins are the best characterized components of the natural antimicrobial system in human milk, and because of their suspected importance in defending the infant and perhaps the mammary gland (Goldman & Smith 1973), their specialized features will be reviewed in detail.

Immunoglobulins in Human Milk

The major immunoglobulin isotypes in human blood and milk are quite different (Table 1). Immunoglobulin G (IgG), the principal immunoglobulin in human serum, is present in more modest quantities in human milk (Mata & Wyatt 1971; Peiterson et al 1975; McClelland et al 1978; Ogra & Ogra 1978;

Jatsyk et al 1985). Each of the IgG subclasses have been found in human milk, but the relative proportion of IgG4 is higher in human milk than serum (Keller et al 1983). IgG4 may therefore be produced in or specifically transported to the mammary gland (Keller et al 1983), but it is also possible that the increased proportion of IgG4 is due to a more efficient exclusion of other IgG subclasses from human milk.

The concentrations of immunoglobulin M (IgM) are also much lower in human milk than in serum (Mata & Wyatt 1971; Peiterson et al 1975; Ogra & Ogra 1978; McClelland et al 1978; Jatsyk et al 1985). Immunoglobulin M in blood and milk displays a pentameric structure. However, in contrast to serum IgM, at least some human milk IgM is complexed to an epithelial glycoprotein, the secretory component (SC) (Brandtzaeg 1974). In the few studies that have been published, the antibody specificities of human milk IgM appear to be similar to those of the major immunoglobulin isotype in human milk, immunoglobulin A (IgA). The quantity of immunoglobulin D in human milk is lower than in serum, but the decrease is proportionally less than is reported for IgG and IgM (Keller et al 1984). Immunoglobulin E, the principal type of antibodies responsible for immediate hypersensitivity reactions, is essentially absent in human milk (Underdown & Knight 1976).

The predominant immunoglobulin in human milk is IgA (Mata & Wyatt 1971; Peiterson et al 1975; McClelland et al 1978; Ogra & Ogra 1978; Goldblum et al 1982; Goldman et al 1982; Goldman et al 1983; Butte et al 1984). Immunoglobulin A comprises over 90% of antibody molecules in human milk, but only about 15% of immunoglobulins in adult serum (Table 1). The principal molecular form of IgA in adult serum is a four chain structure consisting of two identical heavy polypeptide chains (either alpha 1 or alpha 2) and two identical light polypeptide chains (either kappa or lambda) linked by disulfide bonds. In contrast, a polymeric form of IgA, secretory IgA (SIgA), comprises over 90% of IgA in human milk. Secretory IgA consists of two four chain units united by a 15 kd polypeptide called the joining or J chain (Schrohenloher et al 1973; Wilde & Koshland 1973; Koshland 1975) and complexed to a 75 kd glycopeptide designated as the SC (Brandtzaeg 1971; Brandtzaeg 1974).

Table 1. Comparative features of IgA in human blood and milk

Features	Human Blood	Human Milk
Total Immunoglobulin Due to IgA	15%	>90%
IgA Due to Subclasses		
IgA1	90%	50-70%
IgA2	10%	30-50%
Molecular Forms of IgA		
IgA Monomers	>85%	<5%
IgA Polymers	10-13%	?
SIgA	1-2%	85-100%

Origins of SIgA

There is no evidence that the SIgA in human milk produced in the postpartum period originates from immunoglobulins in the blood, whereas many studies indicate that the immunoglobulin part of SIgA in human milk is synthesized by plasma cells located in the lamina propia of the mammary gland. Although, it was demonstrated in ruminants that the mammary gland could be sensitized by injecting antigen into mammary gland duct orfices, this did not explain why antibodies in human milk were commonly directed against enteric and respiratory microorganisms. The discovery of an entero-enteric migration pathway for IgA- producing cells led a number of investigators to question whether antibody producing cells in the mammary gland originated in the maternal intestinal and respiratory systems. Their observations (Goldblum et al. 1975; Weiz-Carrington et al 1978; Fishaut et al 1981; Peri et al 1982) support that hypothesis.

The major aspects of lymphocyte traffic to the mammary gland are as follows. Precursors of IgA producing cells found in Peyer's patches in the small intestines and in the lamina propia of the bronchi migrate to regional lymph nodes (mesenteric or bronchial) after they encounter an immunogen to which they are precommitted. Afterwards, those cells leave the lymph nodes via efferent lymphatics to traffic to the superior vena cava and the vascular circulation. Under the influence of prolactin (Weisz-Carrington et al 1978) and perhaps other lactogenic hormones, these specialized lymphocytes home to the mammary gland where they differentiate into plasma cells that produce dimeric IgA antibodies directed against the original immunogens. Thus, mucosal-mammary gland pathways provide antibodies against the infecting agents in the maternal environment. In addition, the intestinal flora of the mother is obtained in part from mucosal flora of the infant. The mother, therefore, appears to function as a surrogate for the baby in that she produces antibodies against her infant's flora when the child is not fully capable of mounting a complete mucosal immune response (Haworth & Dilling 1966; Burgio et al 1980; Hanson et al 1980; Hanson et al 1983).

Most evidence suggests that after IgA dimers are produced by plasma cells at mucosal sites (Brandtzaeg 1973; Koshland 1975), they pass through the overlying epithelial cells by a SC-mediated transport mechanism (Parkhouse & Della Corte 1974; Brown et al 1976; Brandtzaeg 1978; Crago et al 1978; Kuhn & Kraehenbuhl 1979; Mostov & Blobel 1982). Because the synthesis and function of SC is germaine to an understanding of the immunoglobulin system in milk, that process will be described in some detail.

Secretory component is synthesized on the rough endoplasmic reticulum of epithelial cells. The nascent molecules consist of four functional segments (Mostov et al 1980; Mostov & Blobel 1982; Mostov et al 1984). The first segment, an N-terminal signal peptide, is important in directing the protein into membranes. The extracellular (ectoplasmic) region of SC functions as the receptor for dimeric IgA and pentameric IgM and remains with these proteins even after they are released from the epithelial cell as secretory immunoglobulins. A membrane spanning segment anchors SC to the membrane, and a relatively large intracellular (intracytoplasmic) segment is thought to mediate the transport of SC through the cell. The sequence of the intracellular traffic of SC is as follows. The nascent SC molecule is routed to the Golgi apparatus where glycosylation is completed, the basolateral plasma membrane where immunoglobulins are bound, and then to the endocytic vesicle, in which the SC, with or without ligand, is

transported to the apical membrane. Fusion of the vesicle with this membrane appears to be required for SIgA to be secreted. During this process, two chemical modifications of SC occur. Secretory component is covalently linked to dimeric IgA by two disulfide bonds, and then the extracellular region of SC is endoproteolytically cleaved from the membrane spanning and intracellular segments. The fate of the intracytoplasmic segment is unknown, but it is not likely to function again as an SC receptor or transporter (Solari & Kraehenbuhl 1984).

The regulation of the traffic of vesicles containing SIgA through the epithelial cell and their release into the lumen is not well understood, but it appears that the transport is independent of microfilaments but dependent upon microtubules (Nagura et al 1979). Part of SIgA in human milk is found in membrane encased globules that bud off the apical pole of epithelial cells (Moro et al 1983), but it is unclear whether the SC in these milk fat globules consists only of the ectoplasmic portion as in the fluid phase of milk or whether the cytoplasmic and membrane spanning segments still comprise part of those SC molecules.

Quantitative Production of SIgA During Lactation

Although levels of SIgA vary considerably in the milk of different individuals, the pattern of SIgA levels in milk during lactation is very consistent. The highest concentrations of SIgA are found in the earliest milk secretions, eg. colostrum (Peiterson et al 1975; Ogra & Ogra 1978; McClelland et al 1978; Goldblum et al 1982) (Table 2). The concentrations of the protein fall during the next several weeks until a nadir of 0.5-1 mg/ml is reached (Cruz et al 1982; Goldman et al 1982). Those levels remain unchanged throughout lactation (Goldman et al 1982; Goldman 1983) and during gradual weaning (Goldman et al 1983).

Table 2. Concentrations of immunoglobulins (mg/ml) in human milk*

Immunoglobulin Concentrations	Duration of Lactation				
	2-4 days	1 mo.	6 mo.	12 mo.	24 mo.
Total IgA	2.1 ± 2.3	1.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.5	1.1 ± 0.3
SIgA	2.0 ± 2.5	1.0 ± 0.3	0.5 ± 0.1	1.0 ± 0.3	1.1 ± 0.2
IgM	0.12 ± 0.03	0.02	0.02	0.01	**
IgG	0.34 ± 0.007	0.05 ± 0.03	0.03	0.04	**

*From Peiterson et al (1975), McClelland et al (1978), Goldblum et al (1982), Goldman et al (1982), Goldman et al (1983), and Jatsyk et al (1985)

**Insufficient data

The amount of SIgA secreted via the mammary gland per day during complete breast feeding of full-term infants has been studied by determining the volume of milk ingested by test-weighing and the concentration of SIgA in 24 hour specimens of milk obtained within three days after the determination of milk volume (Butte et al 1984). The amount of SIgA ingested per day fell gradually from a mean of 600 mg at 1 month to 500 mg at 4 months. When the data were transformed into the quantity ingested per kg of body weight of the infant per day, it was more evident that the contribution of SIgA from human milk decreased over time (eg. 125 mg/kg/D at 1 month; 75 mg/kg/D at 4 months). Since the production of SIgA by the infant is increasing during that time (Haworth & Dilling 1966; Burgio et al 1980; Hanson et al 1980; Hanson et al 1983), the data suggest that production of SIgA by the infant and the mammary gland of the mother may be linked. There is some evidence from longitudinal studies that the mucosal-mammary gland pathways for SIgA antibody production are active throughout the first year of lactation (Fishaut et al 1981; Goldman et al 1982; Goldman et al 1983; Butte et al 1984), but it is undetermined whether those responses persist beyond the first year of lactation.

Biologic Features of SIgA in Human Milk

Antibody Specificities

The antibody activities of SIgA in human milk reflect the types of foreign agents found in the intestinal and respiratory tracts of the mother. These include antibodies to the cell wall, pili, proteases, toxins, and virulence factors of microorganisms such as Escherichia coli, Shigella species, Salmonella, Clostridium difficile, polioviruses, other enteroviruses, rotaviruses, respiratory syncytial virus, cytomegaloviruses, other herpesviruses, Candida albicans, Streptococcus pneumoniae, Haemophilus influenzae, Entameoba histolytica, Chlamydia trachomatis, and Giardia lambdia (Goldman & Smith 1973; Goldman & Goldblum 1985; Goldman et al 1985 for review). Secretory IgA antibodies to foods including cow's milk and soy proteins are also common in human milk (Cruz et al 1984).

Other Biologic Features of SIgA

Secretory IgA is more resistant to degradation due to trypsin, other proteases, and acid than other immunoglobulins (Lindh 1975). One subclass of IgA (IgA1) is quite susceptible to specific proteases produced by H. influenzae, Streptococcus sanguis, S. pneumoniae, Neisseria gonorrhoeae, Proteus mirabilis, E. coli, and Neisseria meningitidis, whereas other immunoglobulin isotypes including the second IgA subclass (IgA2) are not cleaved by these bacterial proteases (Plaut 1978; Putman et al 1979; Kilian et al 1980; Milazza & Pilisle 1984). In that regard, it is of interest that IgA2 is more prominent in human milk than serum (Table 1). In addition, SIgA antibodies against these bacterial IgA proteases have been found (Gilbert et al 1983). These adaptations appear to permit SIgA antibodies to persist in the hostile environment of the gastrointestinal tract.

Secretory IgA antibodies in human milk have been demonstrated to neutralize bacterial toxins and virulence factors (Porter) and to abrogate the adherence of enteric bacterial pathogen to epithelial cells by binding to their pili (Porter 1979; Davis et al 1982). Secretory IgA antibodies to food antigen may also prevent the gastrointestinal absorption of those

agents and the subsequent systemic sensitization (Hanson et al 1977; Walker & Block 1983).

Secretory IgA antibodies may also protect by synergizing with other factors in human milk including lactoferrin (Stephens et al 1980) and lysozyme and C3 (Aldinolfi et al 1966). In addition, SIgA enhances the activity of lactoperoxidase in the saliva of the newborn by stabilizing the enzyme (Tenovuo et al 1982). Conversely, it does not appear that SIgA instigates inflammatory reactions by activating those revelant pathways. For example, in contrast to IgG or IgM, complexes of antigens with IgA do not activate the classical pathway of complement and thus do not generate chemotaxins-anaphylatoxins. In fact, IgA may prevent some aspects of inflammation by preventing systemic sensitization, competing with IgG and IgM antibodies for antigens, and by suppressing the directed movement of neutrophils (Van Epps & Williams 1976).

In Vivo Fate and Effects of SIgA from Milk

Despite the extensive information regarding the production, structure, and function of SIgA, comparatively little is known about the in vivo fate and biologic effects of SIgA in human milk. It is assumed that SIgA molecules are relegated to the lumen and surface of the alimentary tract and some SIgA remains intact during their sojourn in that system. There is, however, little objective information regarding the in vivo fate and effects of SIgA from human milk in the term infant.

A number of studies have shown that the stool concentrations of IgA are higher in breast-fed than in cow's milk fed babies (Kenny et al 1967; Haneberg 1974; Ogra et al 1977; Jatsyk et al 1985) but there are virtually no quantitative data regarding the traffic and metabolism of SIgA from human milk in the gastrointestinal tract. Recently, Goldblum, et al (In Press Immunolgic outcomes of feeding human milk to very low birth weight infants In Composition and physiologic properties of human milk Amsterdam:Elsevier Science Publishers BV) reported that about 7-8% of the SIgA from a human milk preparation fed to premature infants was recovered in their stool. In that study, stools from those human milk fed infants contained about 10 times the amount of SIgA than was found in the stools from prematures fed a cow's milk preparation. It is unclear, however, whether the increments in the fecal levels of SIgA were due simply to the immunoglobulin fed to the infants or to an induction of intestinal synthesis of SIgA. In that regard, Goldblum et al (1985) found that the frequency of SIgA antibodies to E. coli in urine was higher in those preterm infants fed human milk. Although the serum concentrations of total and secretory IgA in the human milk and cow's milk fed premature infants were similar in that study, others have reported evidence of IgA absorption during the first few days of life (Iyengar & Selvara 1972; Ogra et al 1977; Vukavic 1983). The amount of SIgA from human milk which is absorbed onto the intestinal epithelium, enters the systemic circulation, is redistributed into systemic sites or is metabolized in the intestinal tract or elsewhere is undetermined.

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The Lactoperoxidase System

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ABSTRACT

Various mammalian peroxidases forms, together with certain halides and hydrogen peroxide, unspecific antibacterial systems. One such peroxidase is lactoperoxidase, present in milk and human saliva. Together with the pseudohalide thiocyanate (SCN^-) and hydrogen peroxide it forms a potent antibacterial system, the lactoperoxidase system (LPS), which has been studied extensively in milk and human saliva.

The antibacterial effect of this system is mediated by short-lived oxidation products of SCN^-, i.e. hypothiocyanaite ($OSCN^-$). Indirect evidence also suggest that some higher oxyacids of SCN^-, i.e. O_2SCN^-, may also be involved in the antibacterial effect.

The major oxidation product of SCN^-, $OSCN^-$ reacts predominately with free SH-groups oxidizing them to the corresponding disulfides (-S-S-), sufenylthiocyanate (-S-SCN) or sulfenic acid (-S-OH). Although the antibacterial mechanism of the LPS not has been clarified in detail it is likely that the effect is caused by oxidation of SH-groups in vital metabolic enzymes.

A wide range of bacteria are effected by the LPS. The effect is however quite variable depending on type and strain of bacteria. Reduced nicotinamide-adenine dinucleotides seem to play an important role in the resistance of the bacteria.

The effect of LPS on mammalian cells is also discussed.

1. Introduction

Milk, like most other mucosal secretions contains a number of proteins with antibacterial properties. In milk, these comprise secretory antibodies but also a number of non-antibody proteins whose antimicrobial activity does not depend on an previous exposure to the infective agent. This latter group includes different peroxidases, which themselves do not have any antimicrobial effect but together with appropriate co-factors forms potent antimicrobial systems. One such peroxidase is lactoperoxidase (LP), which together with a certain halide i.e. the pseudohalide thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2) form a potent antibacterial system (LPS), which not only is present in milk but also in saliva.

This paper will summarize the most important facts on the biochemical and antibacterial mechanisms of this system, with special reference to milk. The biological significance and some practical implications of the LPS will be summarized in other contributions during the symposium.

2. Mammalian peroxidases

Peroxidases are a class of enzymes that are widely distributed in nature, both in plants and in animals. The chemical reaction catalysed by peroxidases can be written:

$$AH_2 + H_2O_2 \rightarrow A + 2\ H_2O$$

where AH_2 and A are reduced and oxidised forms of a suitable electron donor. A wide variety of organic and inorganic compounds can serve as electron donors but substrate specificity varies inbetween different peroxidases. Many mammalian peroxidases can, in contrast to plant peroxidases, utilize halides and the pseudohalide thiocyanate as electron donors.

The group of mammalian peroxidases includes gluthatione peroxidase, thyroid peroxidase, eosinophil peroxidase, myeloperoxidase and lactoperoxidase. Of these myeloperoxidase in polymorphonuclear leukocytes and lactoperoxidase, which has been shown to be present apart from milk also in other secretions, are known to catalyze antimicrobial activity.

2.1 Occurrance of lactoperoxidase

More than half a century has now passed since Hansen (1924) first could correlate peroxidase activity in bovine milk with antibacterial activity against Salmonella. About twenty years later Theorell & Åkesson (1942) purified this enzyme and named it lactoperoxidase. This name has later on turned out to be somewhat misleading since this enzyme subsequently has shown to be present in other secreations. Morrisson and Allen (1963) could by use of immunochemical methods demonstrate presence of LP in the salivary gland. Allen & Morrison (1966) also showed that bovine LP was immunochemically indistinguishable from the peroxidase in goat and sheep milk. Studies of human secretions have subsequently demonstrated peroxidase activity in saliva (Slowey et al., 1968) cervical mucus (Shindler et al., 1976) and nasal glands (Watanabe, 1978). Peroxidase activity has also been found in human milk (Goethefors & Marklund, 1975). Wether this peroxidase is LP or myeloperoxidase is disputed. Moldoveanu et al. (1982) reported that the peroxidase activity in human milk is derived from milk leukocytes i.e. myeloperoxidase. Langbakk & Flatmark (1984) have, on the other hand, reported that they isolated a peroxidase from human colostrum with chromatographic and immunoreactive properties similar to that of bovine lactoperoxidase.

Although, the peroxidases present in saliva and cervical mucus are very similar to LP Pruitt & Tenovuo (1985) have suggested that they should be named salivary peroxidase and uterine peroxidase until they have been further characterised.

2.2 Chemical structure of lactoperoxidase

Lactoperoxidase is a glucoprotein with a molecular weight of approximately 78.000 and containing one heme group (Theorell & Pedersen, 1944). The iron content has been found to be between 0.0680–0.0709 % and the carbohydrate content 9.9–10.2 % (Carlström, 1969a). The amino acid composition is given Table 1.

Lactoperoxidase can relatively easily be isolated in quantities from bovine milk. A rapid method, in which the enzyme first is adsorbed into a cation exchanger CG-50-NH_4 and than further purified on phenyl-Sepharose has been published by Paul et al. (1980).

Several different fractions of LP have been demonstrated in milk. Polis & Shmukler (1953) separated milk LP into two fractions A and B, fraction A being more acidic. Carlström (1969b)subsequently managed to demonstrate a total of ten different fractions of LP, named A 1–4, B 1, B 2-I, B 2-II and B 3–5. No significant difference in activity between these fractions could be observed. Paul & Ohlsson (1985) have proposed the following relations between the various LP fractions:

B 1 → B 2-I → B 2-II → B 3 → B 4 → B 5
A I → A 2 I → A 2 II → A_3

Table 1. Amino acid and carbohydrate composition of lactoperoxidase B 1, expressed as the nearest integral number per 78.000 g of protein (Carlström, 1969a)

Aspartic acid	71	Cysteine	0
Glutamic acid	61	Cystine	8
Amide nitrogen	62	Tyrosine	15
Threonine	32	Phenylalanine	30
Serine	33	Tryptophan	15
Proline	42	Lysine	34
Glycine	40	Histidine	14
Alanine	37	Arginine	37
Valine	28	Mannose	26
Isoleucine	27	N-Acetylglucosamine	14
Leucine	68	N-Ac-galactosamine	4
Methionine	11	Sialic acid	0

The horizontal changes occur under conditions known to favour deamination of asparagine or glutamine residues while the vertical shift (B → A) is due to a loss of 4 glucosamine, 6 mannose and 1 galactosamine residues. According to this pattern B 1 represents the intact or least degraded form of LP with a molecular weight of 78.431 and an iron content of 0.071 percent (Paul & Ohlsson, 1985).

The identity and type of binding of the heme moiety in lactoperoxidase has been discussed. The inability of acid acetone to extract the heme was taken for evidence for a covalent heme-protein bond, which tentatively was identified as an ester or amide bond (Hultqvist & Morrison, 1963; Morell & Clezy, 1963). More recent investigations have however demonstrated that no covalent heme-protein bond exist and that the prosthetic group is protoheme IX (Sievers, 1979).

Dumontet & Rousset (1983) have isolated a peak on ion exchange chromatography they supposed to be a non-heme lactoperoxidase from bovine milk. This molecule was said to have the same apparent molecular weight but unlike LP it had no absorbance at 412 nm and no enzyme activity. Otherwise it showed partial immunochemical identity with LP. Ekstrand et al. (unpublished) have found similarities in amino acid composition and spectral properties, i.e. the 2nd derivative of the UV-spectrum, between this substance and LP but also similarities with lactoferrin. Whether this is an "apo-form" of LP or is idendical to lactoferrin can be further proven by comparisons in physico-chemical properties and analytical FPLC-chromatography.

3.1 Oxidation of halides by lactoperoxidase

Lactoperoxidase catalyses the oxidation by hydrogen peroxide of certain halides and the pseudohalide thiocyanate. In most cases peroxidation occurs in 2 distinct 1 e^- transfers (Maguire et al., 1971)

LP + H_2O_2 → Compound I
Compound I + AH_2 → Compound II + AH'
Compound II + AH_2 → LP + AH'
2 AH → A + AH_2

The oxidation of halides by LP however proceeds in contrast to the oxidation of many organic compounds by way of a single 2 e^- transfer. Compound II has not been possible to detect during oxidation of halides (Thomas, 1985)

$H_2O_2 + X^- \rightarrow H_2O_2 + OX^-$

The oxidized form of the halide can upon being released from the enzyme equilibrate with the corresponding halide (X_2), hypohalous acid HOX and possibly also with complex ions such as X_3^-.

The ability of peroxidases to oxidize different halides varies and is related to the halide oxidation potential. Myeloperoxidase catalyses the oxidation of Cl^-, Br^-, SCN^- and I^- whereas LP catalyses the oxidation of Br^-, SCN^- and I^-. Eosinophile peroxidase have the same specificity as myeloperoxidase and thyroid peroxidase the same as LP.

Kanner & Kinsella (1983) have reported that LP also could oxidize Cl^-. Ekstrand & Björck (1985) have however not been able to confirm their results. It seems likely that the results of Kanner & Kinsella could have been caused by some artefact, i.e. a contamination of Br^- in the preparation of sodium chloride used.

3.2 Lactoperoxidase catalysed oxidation of SCN^-

Reiter and co-workers (1964) were able to demonstrate that oxidation of SCN^- was involved in the antibacterial mechanism of the LP catalysed antibacterial effect in bovine milk. Thiocyanate can be oxidized non-enzymatically by hydrogen peroxide. Wilson and Harris (1960, 1961) postulated on basis of kinetic studies that above pH 4 the oxidation takes place over a serie of oxyacids of thiocyanate, the net reaction being the following:

$$4\ H_2O_2 + SCN^- \rightarrow HSO_4^- + HOCN + 3\ H_2O$$

The lactoperoxidase catalysed reaction is believed to follow the same reaction. The complete oxidation of SCN^- however only yields products with no antibacterial activity. It was therefore postulated by Oram & Reiter (1966) that the active agents had to be an intermediate oxidation product. They concluded that sulphurdicyanide ($S(CN)_2$) could be the antibacterial agent. Hogg & Jago (1970) showed that the inhibitor existed in an acid-base equilibrium (pK_a 5.1 ± 0.1). As they found that the polarographic properties of the active antibacterial agent did not resemble that of water solutions of $S(CN)_2$ or thiocyanogen ($(SCN)_2$), they suggested that the active agent was cyanosulphurous acid (HO_2SCN) and/or cyanosulphuric acid (HO_3SCN). Björck et al. (1976) were able to demonstrate that the antibacterial effect was dialysable, thus supporting the hypothesis that the antibacterial agent was a small molecule and that LP itself did not have to be in contact with the exposed bacteria. Aune & Thomas (1977) and Hoogendoorn et al. (1977) independently concluded that hypothiocyanite ($OSCN^-$) was the antibacterial agent of the system. Aune & Thomas (1977) proposed the following mechanism of the peroxidase catalysed oxidation of thiocyanate:

$$2\ SCN^- + H_2O_2 + H^+ \rightarrow (SCN)_2 + 2\ H_2O_2$$

$$(SCN)_2 + H_2O \rightarrow HOSCN^- + SCN^- + H^+$$

$$HOSCN \rightarrow OSCN^- + H^+$$

Alternatively SCN^- may be oxidized directly to $OSCN^-$:

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O$$

The oxidation product could be quantified by oxidation of 5 thio-2-nitrobensoic acid:

$$OSCN^- + 2\ R\text{-}SH \rightarrow R\text{-}S\text{-}S\text{-}R + H_2O + SCN^-$$

The evidence for the accumulation of $OSCN^-$ is the loss of one SCN^- per H_2O_2 and the recovery of one SCN^- upon oxidation of two sulphdryls to the disulfide.

Hoogendorn et al. (1977) prepared $OSCN^-$ non-enzymatically by the hydrolysis of thiocyanogen $(SCN)_2$ in strong alkali solutions. These solutions were inhibitory towards Streptococcus mutans in the same way as LPS. They were also able to demonstrate that at alkaline pH $OSCN^-$ is fairly stable due to different resonance forms of the molecule in the ionic form but not in the acid form.

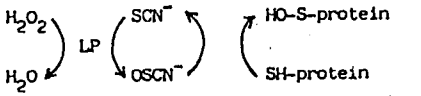
More recent investigations indicate that $OSCN^-$ may not be the only antibacterial agents formed by the LPS. Björck and Claesson (1980) showed that non-enzymatically prepared $OSCN^-$ did not give the same antibacterial effect towards gram-negative bacteria as Escherichia coli as did the complete LPS. They also found no stoichiometric one-one relation between loss of SCN^- and formation of $OSCN^-$ at ratios of SCN/H_2O_2 which gave a substantial antibacterial effect. Tenovuo (1979) and Tenovuo et al. (1981) also showed that hypothiocyanite ions give less inhibition of bacterial acid production than the products of the complete LPS. These results indicate that in addition to $OSCN^-$, some other short-lived product(s) are formed by the LPS as originally suggested by Hogg & Jago (1970). This is further supported by the polarografic study of the oxidation products of SCN^- made by Pruitt et al. (1982). Their results indicate that higher oxyacids are formed. In conclusion, although $OSCN^-$ unquestionable is formed in the LP catalysed oxidation of SCN^- and has antibacterial properties there are today plenty of evidence that some higher oxyacids of thiocyanate such as HO_2SCN and/or HO_3SCN are involved in the antibacterial effect of the LPS.

It has also been discussed whether singlet oxygen or other radicals are involved in the effect of LPS (Reiter, 1979) in analogy with the myeloperoxidase Cl^-/H_2O_2 system (Rosen & Klebanoff, 1977). The only evidence for this is the report that a carotenoid pigmented strain of Sarcina lutea was not killed by the LPS while a non-pigmented mutant was. However, other differences than lack of pigment in the mutant strain may have caused the difference in sensitivity since superoxid dismutase and radical scavengers such as mannitol do not interfere with the antibacterial effect of LPS (Björck, unpublished results).

4.1 Antibacterial mechanism of LPS

The major oxidation product of LP catalysed oxidation thiocyanat $OSCN^-$, which is in equilibrium with the corresponding acid HOSCN, pKa 5.3 (Thomas, 1981), reacts with free sulphydryl groups oxidizing them to the corresponding disulfides

(S-S), sulfenyl thiocyanates (S-SCN) or sulfenic acid (S-OH). Aune & Thomas (1978) have summarised the reaction as follows:



Hypothiocyanate may also react with reduced nicotinamide-adenine dinucleotides i.e. NADH and NADPH oxidizing them to NAD^+ and $NAPH^+$ (Hoogendoorn, 1974). Both these reactions are reversible in presence of an excess of reducing agents such as cysteine, glutathione, mercaptoethanol and sodium hydrosulfite. These agents can also interfere with the antibacterial effects of LPS by reacting directly with the oxidation products of SCN^-.

It has also been reported that hypothiocyanite reacts with histidine but only at high concentrations of histidine (Aune, Thomas & Morrison, 1977). Aromatic amino acids are modified when combined with the complete LPS, which also may indicate that highly active transient intermediates other than $OSCN^-$ are formed as discussed previously.

Oxidized forms of thiocyanate do however not react with ß-carotene as is the case with LP-peroxidation of iodide (Ekstrand & Björck, 1985)

4.2 Effects on microorganisms

Although the detailed antibacterial mechanism of the LPS is complex and has not yet is been clearified in detail it seems likely that the effect against bacteria is caused by oxidation of SH-groups (Thomas & Aune, 1978) in vital metabolic enzymes and/or depletion of reduced nicotinamide adenine dinucleotides. This will give rise to both direct and indirect effects. Oram and Reiter (1966) showed that in one strain of S. cremoris, hexokinase and aldolase were substantially inhibited. Hoogendoorn (1974) found similar effects in S. mutans. Subsequently hexokinase from yeast cells (Adamsson & Pruitt, 1980) and glyceraldehyde-3-phosphate dehydrogenase from various strains of oral Streptococci were found to be inhibited by LPS (Carlsson et al. 1983).

Active transport mechanisms of amino acids in Lactobacillus acidophilus (Slowey et al., 1968) and the glucose transport in S. agalactiae (Mickelson, 1977) are blocked by the LPS. Björck (1977) showed that oxygen uptake in E. coli stops immediately upon exposure to LPS. Leakage of cellcomponents such as amino acids and potassium have also been reported to occur in E. coli, indicating a damage to the cytoplasmic membrane (Marshall & Reiter, 1980).

The effect of the LPS can be overcome by some types of bacteria, the effects are reversible and the bacteria are inhibited for a certain period of time. For other bacteria, the effects are irreversible and a loss of viability of these bacteria takes place.

A wide range of bacteria are effected by the LPS. The effect is however quite variable. Many gram-positive organisms such as lactic acid Streptococci and Lactobacilli are tempoary inhibited by the LPS. Against bacteria such as E. coli, Salmonella and Pseudomonads spp the system has in most cases a bactericidal effect (Björck et al., 1975; Reiter et al., 1976).

The effect may however vary between strains of the same species and the conditions of exposure. Adamson & Carlsson (1982) have shown that E. coli cells exposed to the LPS recover better under anaerobic conditions and on rich media e.g. blood agar. Reduced nicotinamide-adenine dinucleotides seem to play an important role in the resistance of the bacteria. Oram & Reiter (1966) found one strain of S. cremoris, which showed little suceptibility against LPS. They suggested that this was due to that it had the capacity to reduce $OSCN^-$ by NADPH. Carlsson et al. (1983) have later reported similar mechanisms in S. mitis and S. sanguis. Hoogendoorn (1976) has suggested that inhibited cells recover by utilising NADPH to reduce $OSCN^-$. When the supply of NADPH is depleted the cells are unable to recover from the effects of LPS. This is similar to what has been suggested by Thomas et al. (1983).

5.1 Effects of LPS on mammalian cells

White et al. (1983) investigated any mutagenic effect of LPS using several mutagen-sensitive strains of Salmonella typhimurium and a Saccharomyces cerevisiae (yeast) strain. They also studied UV-spectra of calf thymus DNA exposed to $OSCN^-$. They found that $OSCN^-$ did not produce any mutagenic effects in any of the strains, nor did it give any oxidation of the calf thymus DNA. Härnström et al. (1983) have investigated the effects of LPS against cultured mammalian cells, i.e. Chinese hamster ovary cells, Hela cells and human gingival fibroblasts. They found that oxidation products of SCN^- had no toxic effects in contrast to 0.1 mM H_2O_2, which gave a substantial toxic effect. Tenovuo & Larjava (1984) have also reported a protective effect of lactoperoxidase and SCN^- against hydrogen peroxide toxicity for gingival fibroblasts cultured in vitro. They also found that elevated concentrations of $OSCN^-$, that give an antibacterial effect, did not damage the eukaryoti cells measured as (^3H) - thymidine uptake. This lower sensitivity of mammalian cells to LPS is not fully understood but may be due to an impermeability of the mammalian cell membrane to $OSCN^-$ (Härnström et al., 1983).

These findings have also resulted in a hypothesis of a dual role for the oral peroxidase system, viz. not only to regulate the metabolism of the oral flora and antimicrobial action against oral pathogens had but also protect human cells from any toxic effects of bacterially produced hydrogen peroxide (Tenovuo & Larjava, 1984).

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Biochemical and antimicrobial properties of lactoferrin

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SUMMARY

The primary structure of human lactoferrin (Lf) was published recently (1). Some data concerning its shape have been obtained by X-ray crystallography at low resolution. Lf is closely related in sequence, shape and properties to serum transferrin (Tf) and egg ovotransferrin (Of, conalbumin). These 3 proteins are formed of a single peptide chain, ~ 700 residue long, carrying 2 N-glycosidically linked carbohydrate moieties. They possess 2 homologous domains, formed by the 2 half molecules, which each carry a Fe (III) and bicarbonate binding site. Tight binding of iron or bicarbonate alone cannot occur. The 1 st and 2 d domains have 6 or 8, 9 to 11 disulphide bridges respectively. The sequence homologies human Lf/human Tf, human Lf/hen Of, human Tf/hen Of are 59, 49 and 51% respectively. When non-iron saturated they all display bacteriostatic properties due to chelation of iron needed for bacterial growth, which dissapear by iron saturation. Among the 3 proteins, only Tf has a well known biological function : the transport of iron from ferritin stores to its utilization sites, mainly to erythroblasts. The main differences between Lf and the other 2 proteins are : a tighter binding of iron, especially at low pH, the location and structure of its carbohydrate moieties, its occurrence in all exocrine secretions and in neutrophil granulocytes. Its main postulated biological functions are : a bacteriostatic action which is enhanced by immunoglobulins and lysozyme, a role in the iron metabolism, a role in the regulation of myelopoiesis. Receptors for Lf have been found on different cells, including enterocytes .Lf occurs, at various levels, in the milk of all species. Its main source is human milk.

1. Metz-Boutigue M.H. et al. 1984 Eur. J. Biochem. 145 : 659-676.

Transferrin (Tf) is a soluble glycoprotein of Mr ~ 80,000 which can bind tightly two iron (III) ions per molecule. It occurs in the blood serum of vertebrates where it plays an important role in transporting iron from ferritin stores, mainly located in liver and spleen, to reticulocytes and many other cells. In these cells iron is used for manufacturing proteins such as hemoglobin, myoglobin, iron enzymes etc ...

A closely related protein, lactoferrin (lactotransferrin, Lf), occurs in the exocrine secretions and neutrophil leucocytes of the mammals. As we will see its biological function is not so clear cut as that of transferrin.

A transferrin is present in the blood serum of birds. The ovotransferrin (conalbumin , Of)which has been characterized in the egg white of the hen and the transferrin of this bird are the products of the same single gene. They differ only by their carbohydrate moieties and the cells in which they are synthesized (oviduct tubular gland cells and hepatocytes respectively) (Lee et al., 1980).

Although these three proteins, Tf, Lf and Of, have quite similar structures and biochemical properties, which indicate a common phylogenic origin, they do not share a common biological function at first sight.

This review is concerned with the description, occurrence and mode of action of lactoferrin in vitro. It also deals with its in vivo activity. However the data which will be given on this last aspect are based regrettably on a very limited range of experiments.

I. Tissual and intracellular localization of lactoferrin

A number of data have been obtained or reviewed by Masson (1970). By using monospecific antibodies Lf was detected and determined in the milk of a dozen species. These milks contained Tf as well. The contents in both proteins as well as their relative proportions were quite different from species to species. Human milk, which is poor in Tf, is the richest source of Lf (ca. 4.9 and 1.6 mg/ml in colostrum and milk respectively ; Nagasawa et al., 1972). Cow milk is poor in both proteins. Bovine colostrum and milk contain approximately 6 and 0.2 mg/ml Lf respectively (in Blanc, 1981). The concentration is markedly increased during infections of the mammary gland (Harmon & Newbould, 1980) and during its involution (Welty et al., 1975). On the opposite both Lf and Tf occur at a fairly high level in guinea-pig milk, while Tf is predominant in rat milk.

Human milk Lf was isolated and characterized in the sixties by several investigators independently (see in Masson, 1970). Law & Reiter, Cheron et al. reported the isolation of the bovine protein in 1977. Rabbit and mare Lf were isolated recently (Dayal et al., 1982 ; Jollès et al., 1984).

In the human, Lf also occurs in most exocrine secretions : bronchial mucus, saliva, nasal secretions, tears, gastric juice, duodenal mucus, pancreatic secretion, bile, cervical mucus, seminal plasma, urine etc ..., as well as in neutrophil granulocytes (Masson, 1970).

The intracellular localization of Lf has been studied by Masson (1970) using immunofluescence and incorporation of radiolabelled amino acids. This author detected its biosynthesis in secretory epithelial cells of the mammary, salivary and bronchial glands. In the mammary cells the protein was located in the apical region, likely in Golgi vesicles. He also showed that Lf was a component of the secondary (specific) granules of the human neutrophil granulocytes. This granules also contained alkaline phosphatase and lysozyme. The granulocytes release apolactoferrin, the iron-free lactoferrin, during phagocytosis in vitro and it is thought that circulating Lf (ca. 0.30 µg/ml) derives from this source (Van Snick at al., 1976 ; Hetherington et al., 1983). The Lf content of human neutrophil granulocytes is about 3.4 µg/10^6 cells (Masson, 1970).

II. Structural features of lactoferrin

The three transferrins, Tf, Lf and Of, have quite similar structures. They consist of a single peptide chain of Mr ~ 80,000 (ca. 700 aminoacid residues) to which one or two carbohydrate chains are attached through N-glycosidic linkages. The molecules are formed of 2 symmetrical homologous parts which originate likely from the duplication of an ancestral gene as evidenced by sequence studies. Each part carries an iron binding site.

For each ferric ion bound to such a site, one bicarbonate anion is concomitantly bound, and approximately 3 protons are released. Iron or carbonate cannot bind alone tightly. Low-resolution X ray crystallographic studies have shown that the molecules have maximal dimensions of 95 x 60 x 50 Å and are indeed formed of 2 quite similar lobes whose main axes are inclined at about 30° to one another (Tf : Gorinsky et al., 1979). An iron binding protein of approximately 41,000 Mr was recently described in prochordates. It was shown that the gene coding for this protein in the prochordate Pyura havstor hybridizes with human Tf cDNA. This suggests the occurrence in this species of a gene related to the single lobed ancestor protein mentioned above (Lum et al., 1985).

The primary structure of human Tf (Mac Gillivray et al., 1982 ; Yang et al., 1984), hen Of (Jeltsch & Chambon, 1982 ; Williams et al., 1982) and human Lf (Metz-Boutigue et al., 1984) are known. Since the sequences of the former two have been obtained both from the proteins and their cDNAs, it has been shown that they are synthesized as preproteins, like all secretory proteins, with N-terminal-leader sequences that are removed in the endoplasmic reticulum. In both cases the leader sequences are 19-residue long and they display 56% homology at the nucleotide level. A similar sequence likely occurs for Lf which has only been sequenced at the protein level.

The peptide chain of this protein is 703-residue long and contains 16 disulphide bridges. Most of them are located in positions homologous with those of Tf and Of. The two halves of the Lf chain display 37% homology, a figure close to those obtained for the other two proteins. The homologies Lf vs. Tf, Lf vs. Of, Tf vs. Of are 59%, 49% and 51% respectively. Glycosylation of lactoferrin occurs on Asn 137 and 490, that is in positions that are not homologous with those of the glycosylation sites of the other two proteins. Earlier studies on the different transferrins suggest that Fe (III) in each site is bound to several tyrosine and histidine residues, while the bicarbonate anion could be attached through an arginine residue (Metz-Boutigue et al., 1984). A microheterogeneity occurs at the level of the two carbohydrate chains. Five types have been isolated and the structures of three of them have been elucidated. They are quite similar, all branched in 2 positions, differing only in the 2 longest branches by the presence or absence of a fucose and/or of terminal N-acetylneuraminic acid. These structures display some differences with those of the carbohydrate chains of Tf (Spik et al., 1982).

III. Main biochemical characteristics of lactoferrin

1. The iron status

In the main source of Lf, human milk, the iron concentration is low (0.3 to 0.5 µg/ml). Still only 2 to 4% of the iron is bound to lactoferrin, which then occurs at a low degree of saturation. Approximately 30% of the iron in human milk is bound to milk fat, mainly as a component of xanthine oxidase, the rest occurring as low molecular weight complexes (eg with citrate) (Lönnerdal et al., 1980 ; Franson & Lönnerdal, 1984). Lactoferrin in human neutrophil granulocytes appears to be devoid of iron (Bennet & Kokocinski, 1979).

However it was shown long ago that, like Tf and Of, human and cow Lf can be saturated with 1 moles of Fe (III) per mole of protein in the presence of bicarbonate anion by incubation with ferrous sulphate and ascorbic acid. Two Cu (II) ions can also be fixed (see Masson, 1970). While the apoprotein is colorless, the saturated iron- and copper-lactoferrins are red (hence the name "red protein") and yellow respectively. The iron-saturated

protein shows a broad absorbance maximum at 460 nm, with $E^{1\%}_{1cm} = 0.55$ at this wavelength (Masson, 1970). A comparison has been made of the optical and EPR spectroscopic parameters which characterize the two metal binding sites of Lf and Tf. They are strikingly similar and show in each case that the 2 binding sites are equivalent. Using dialysis equilibrium it was shown that, as for the other transferrins, the binding constants of Lf for Fe (III) are equivalent for the 2 sites, with a value of 2.6×10^5 at pH 6.4, that is 260 times the corresponding constant for Tf. This means that the former binds iron much more tightly than the latter (Aisen & Leibman, 1972). As reported earlier Lf, as well as its 2 parent proteins, binds concomitantly 2 bicarbonate (or some other) anions, each with one Fe (III) or Cu (II).

A fundamental difference occurs between Lf and Tf in the behaviour of the iron-protein complexes as a function of pH. When the pH is lowered, the latter begins to lose iron below pH 6.5, while this occurs below pH 4.5 for Lf. At pH 4 the saturations are approximately 10% for Tf and 75% for Lf (Masson, 1970). For both proteins the 2 iron binding sites behave in a non-equivalent manner as the pH is lowered (occurrence of an "acid labile" site). Complete desaturation of Lf can be achieved by dissolving the protein in sodium acetate buffer (I = 0.2), 40 mM EDTA, 0.M sodium phosphate, pH 4, keeping the solution overnight and then dialyzing 3 days against water. It has been shown that phosphate ions weaken the association iron-transferrin (Mazurier & Spik, 1980). In a recent study, Legrand et al. (1984) have been able to cleave iron-saturated Lf with trypsin into 2 large fragments, N-tryptic (fragment 3-281) and C-tryptic (the C-terminal part, of Mr $\sim$ 50,000). Each isolated fragment carried iron binding site and 1 glycan moiety. Further digestion of the smallest fragment gave a fragment of Mr 18500 (fragment 91-257) still binding (irreversibly) some iron and containing 1 glycan moiety. Let us note that iron-saturated Lf is more resistant to proteolytic enzymes than the apoprotein (Spik & Montreuil, 1966).

2. Interaction of lactoferrin with other proteins

It is well known that Lf is positively charged at neutral pH (see in Masson, 1970), a property which is commonly used for its preparation, and that its electrophoretic mobility varies according to its environment. Thus the isolated protein migrates slower than any of the naturally occurring forms. This behavior has been ascribed to non-specific ionic as well as hydrophobic interactions with other proteins such as serum albumin, caseins etc ... (Hekman, 1971). A recent report shows that some of the human milk Lf is covalently bound to sIgAs through disulphide bridge(s) (Watanabe et al., 1984). Finally a calcium-dependent polymerization of human Lf, leading to a tetramer as the predominant species, was reported by Bennett et al. (1981) to occur in vitro as well as in vivo.

IV. Biological significance of lactoferrin

Although a lot of investigations have concerned the biological role of Lf, the situation is not clear since the different activities which have been found in vitro for this protein have never been clearly shown to occur and play a definite role in living mammals.

Three main putative functions have been assign to Lf :

- as said above this protein occurs is all exocrine secretions. In these,

IgAs and lysozyme are also present. Together with them Lf could act as an antibacterial agent. Indeed it has been known for a long time that non-saturated Lf, Tf and Of could prevent in vitro the growth of a number of microorganisms by chelating the iron.

- In the case of milk such a role could be useful for the mother's organism mainly at certain stages such as the prepartum period, the period of involution, or in case of mastitis. Furthermore, as far as the offspring is concerned, Lf could play a role similar to that of Tf in blood by making iron more available to absorption in the gut.

- Finally Lf of neutrophil granulocytes could be involved in phagocytosis and regulation of myelopoiesis

1. Antibacterial effects of lactoferrin

The studies concerning these effects have been mainly conducted on human and bovine milks, in which it is believed that Lf, IgAs , peroxidase and lysozyme, are the main macromolecular components responsible for the antibacterial property that has been demonstrated long ago in vitro. Indirectly milk oligosaccharides could also protect the gut against indesirable microorganisms. Finally, fatty acids have been also considered as host-resistance factors in human milk (Kabara, 1980).

First of all, let us recall the following points :

- the antibacterial action of Lf in vitro requires it be at a low degree of iron saturation. Most studies show that iron saturation inhibits this action. Fundamental discrepancies occur among authors regarding the native state of lactoferrin in milk. For some of them the native protein is iron-free or saturated at a low level, iron being taken up during manipulations and isolation (Lönnerdal et al., 1980). For others Lf in the native state is supposed to be completely saturated, iron being lost during the purification stages (Reiter, 1983). A degree of saturation of 14% to 24% was reporter by Masson (1970) for pure human Lf. A figure of 21% was found in our laboratory for preparations of bovine Lf (Daniel, 1980).

- the inhibition of bacterial growth only occurs in the presence of bicarbonate. In many in vitro experiments bicarbonate was added in milk prior testing its antibacterial activity. On the other hand the presence of citrate in milk can make iron available for bacteria . However pancreatic bicarbonate could overcome this effect in the gut (Reiter, 1983).

- whereas human milk is rich in Lf, we have seen that this is not true for all species. In some cases Tf or Tf + Lf reach a fairly high level. In others, such as bitch milk or even cow milk, both occur at low level (Masson, 1970). Then the antimicrobial effect of milk Lf (or Tf) in vivo in the gut of the newborn, if any, is likely not a general phenomenon.

- although immunologically reactive human and bovine "lactoferrins" have been found by Spik et al. (1982) in the faeces of newborn infants and that this protein was still able to bind iron, the proportion of "active" protein which was found in the stools of human milk-fed infants was low (10 mg/24 h). It is well known that Lf, expecially in its iron-free form, is highly susceptible to gastric and gut enzymes. On axenic mice, Daniel (1980) showed a

complete degradation of bovine apo-lactoferrin given per os, with maximal proteolysis occurring in the small intestine. Only an intragastric supplementation with sodium bicarbonate and soja trypsin inhibitor allowed a partial protection of apo Lf along the digestive tract.

All these observations do not rule out a bacteriostatic role of Lf in milk. First of all, this role likely occurs prepartum within the mammary gland and also during involution as well as in case of mastitis. Under these conditions the level of Lf in milk is quite high and a concomitant action of IgAs can be anticipated. Indeed it has been shown that the in vitro bacteriostasis induced by human or bovine Lf is enhanced by either human IgAs or bovine IgGs (Spik et al., 1978 ; Stephens et al., 1980 ; Moreau et al., 1983), although there is no clear explanation for this observation. With the system bovine apolactoferrin-bovine IgGs the inhibition only affects the lag phase of E. coli S17 and EMO1 which is lengthened by apo Lf alone, the lengthening being considerably increased by IgGs and abolished by iron. On the other hand neither the slope of the growth exponential phase, which can occur more than 15 hrs after inoculation, nor the maximal optical density were modified (Moreau et al., 1983). This phenomenon could be attributed to the delayed biosynthesis and excretion of siderophores by E. coli. Following this experiment an in vivo study was carried out in human newborns receving either humanized milk or the same milk supplemented with LF + IgGs during 48 h of life. One group was given E. coli E MO1 per os at birth while another one was not. Between the ages of 1 and 5 days, the kinetics of extablishment of the E. coli strains spontaneouly occurring in the gut of non-inoculated babies was not significantly different between the group which received milk supplemented with LF + Ig and the one which did not. Similarly the faecal levels of E. coli EMO1 were similar in both groups and from day 1 the population level was high. This findings show that the in vitro bacteriostatic effect of LF+ IgG on the growth of E. coli EMO1 is not found in vivo (Moreau et al., 1983).

Finally is must be pointed out that Arnold et al. (1977) observed a dose-dependent bactericidal action of human Lf on Streptococcus mutans, Vibrio cholerae and E. coli in vitro which was abolished by saturation of Lf with iron.

2. Lactoferrin could be an iron carrier

The close similarity between Lf and Tf and the known function of the latter as an iron carrier led to numerous investigations concerning a putative role of Lf in the iron transport. Receptor-like binding of Lf to a variety of cells (normal and leukaemic human blood cells, enterocytes) has been observed. It has been shown in particular that iron-saturated human, and to a lesser extent, bovine Lfs are able to donate their iron to pieces of human duedenal mucosa while Tf and Of were unable to do it (Cox et al., 1979). The intact protein was excluded from entry into the enterocytes. Quite recently (Davidson & Lönnerdal, 1985), monkey and human milk Lfs were shown to behave similarly with infant monkey brush border membranes. The Lf-receptor complex was isolated and a Mr of $\sim$ 170,000 was calculated for the receptor.

There is no doubt that iron must be supplied to the enterocytes in a soluble and available form, but the precise role of milk Lf in the human newborn gut will not be known as long as the precise iron-saturation state of Lf in the small intestine is not known. The newborn has important stores of iron at birth . The iron content of milk is low and it seems that most

of this iron is not bound to Lf (Lönnerdal et al., 1980). A number of other molecules (eg. caseins) are good iron carriers. It may thus well be that Lf from milk or, in the adult, from the intestinal mucosa plays its main role by recovering iron which escapes from this mucosa.

3. The function of granulocyte Lf

Its seems quite probable that Lf participates in the process of the phagocytosis by granulocytes by cooperatively acting with myeloperoxidase and a mechanism has been proposed to explain the inflammatory hyposideraemia through involvment of Lf in the transfer of iron to the reticulo-endothelial system (Van Snick et al., 1975).

Finally complex interacting roles of Lf, Tf and acidic isoferritins have been shown to occur in the regulation of myelopoiesis (Broxmeyer et al., 1985).

As a matter of conclusion we can say that the available data concerning the putative roles of Lf, although sometimes conflicting, suggest for it an "iron scavenger" function in the organism. It has been shown that human milk Lf is rapidly cleared from the circulation by being avidly sequestered by the liver and spleen. Its iron is then slowly transferred to bone marrow and finally to erythrocytes (Bennet & Kokocinski, 1979).

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Lysozyme

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ABSTRACT

Lysozymes (1,4-8-N-acetylmuraminidases) have a very wide distribution in nature. The physiochemical properties of particular niches and the type of microbial challenge have had a profound influence on the chemical composition and substrate specificity of these basic polypeptides. Lysozymes of plants have a marked chitinase activity whereas those of animals have an avidity for the peptidoglycans of eubacteria. Lysozyme of hens' eggs albumen has been studied in great detail; its amino acid composition, crystallography, mode of cation are well documented. These attributes will be compared with those of lysozyme of mammalian milk. The sources and amounts of the latter in bovine and human milk will be considered and their biological roles discussed.

The *in vitro* activity of lysozymes is influenced by concentration, chemical composition, accessibility of substrate, ionic strength and nature of the anions in the reaction mixture.

With Gram-positive bacteria substitution of peptidoglycan and cell wall accessory material may inhibit lysozyme action. The lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria bars the diffusion of lysozyme to its substrate. This barrier can be disrupted by rapid shifts in temperature or pH, the removal of Ca^{2+} + mg^{2+} from the LPS, treatment with complement etc. Lysozymes are associated also with the release of toxicants, immunostimulation and superoxide stimulation in macrophages. The potential application of this class of enzyme in food preservation will be considered in the context of the molecular biology of lysozymes and their substrates in target cells.

1. Introduction

Although much has been written about the ubiquitous occurrence, chemical structure and biological properties of lysozyme, the role of this protein in host defence systems is still as much a mystery now as it was 60 years ago when Fleming (1922) first discovered the "extraordinary bacteriolytic phenomonen" of nasal mucus. It was Fleming who gave the enzyme the name by which we know it today - lysozyme- and the wide distribution of lysozyme in secretions such as milk, saliva, egg white and tissue extracts led him to believe, understandably so, that it constituted the major component of these secretions that was responsible for the destruction of bacteria. We now know that although lysozyme probably plays some role in the prevention of excessive microbial contamination of these biological fluids, the presence of other antimicrobial factors, such as for example, lactoferrin and the lactoperoxidase system may mean that this role in milk is but a minor one.

This article aims to summarise the excellent work that led to lysozyme being established as the first protein to be sequenced containing all 20 amino acids and for which a detailed mechanism was known. It also discusses the biological role of lysozyme as seen at the present time and finally presents some examples of its commercial exploitation. Although this paper was presented during a programme about milk proteins, much of what is discussed applies to lysozymes in general.

2. Definition and Occurrence

Lysozymes are defined as 1,4-β-N-acetylmuramidases and according to enzyme nomenclature are classified as E.C. 3.2.1.17. They occur widely throughout nature in various secretions ranging from plants to animals and micro organisms (Table 1). Most of these lysozymes have been purified by a variety of techniques (Jollès & Jollès 1984) ranging from the classical crystallisation procedures through gel filtration

Table 1 Occurrence of Lysozyme

Source	Type	Reference
Milk	c	Jollès & Jollès 1971
Blood	c	Taylor 1983
Phagosomes	c	Elsbach & Weiss 1983
Mouth	c	Iacono et al. 1980
Avian eggs	c/g	Tranter & Board 1982
Fish	c/g/?	Murray & Fletcher 1976
Insects	c	Jollès et al. 1979
Plants	chitinase	Bernier et al. 1971
Phage	c	Owen et al. 1983
Fungi:		
Chalaropsis	CH	Fouche & Hash 1978
Bacteria:		
Streptomyces	SE	Sarma et al. 1979

and ion exchange chromatography to the rather more recent procedures of affinity chromatography using sheep anti-human lysozyme IgG coupled to epoxy-activated Sepharose-6B. Several different types of the enzyme exist which differ on the basis of structure, catalysis and immunogenicity. Indeed, one amino acid substitution can alter the immunological characteristics of the lysozyme. Throughout the avian world there appear to be 2 immunologically distinct types; that of hen egg white (lysozyme c) and the one isolated originally from Embden goose egg white, lysozyme g (Tranter & Board 1982). Although both enzymes consist of a single polypeptide chain, goose lysozyme has approximately 185 amino acid residues compared with 129-130 in lysozyme c and hence has a higher molecular weight (19,000-21,000 cf. 14,000). The g type enzyme has an unusual preponderance of paired amino acid residues and all its half-cystine residues are situated in the N terminal half of the chain. As will be mentioned later, the active residues, GLU35 and ASP52, in the c type correspond to GLU73 and ASP86 in the g form.

Human milk and hen egg white lysozymes are two homologous proteins (Dubois et al. 1982). Compared with the egg white type c, the milk enzyme has an extra amino acid residue - a glycine - inserted in the region of the hinge of the β-pleated sheet at position 48 (Canfield et al. 1974).

The complete primary amino acid sequence is known for 18 lysozymes and the partial sequences have been determined for several others. Most of the ones known are type c enzymes (e.g. Jollès et al. 1979); only 3 complete g type sequences, in egg whites from the Black Swan, Ostrich and Embden Goose, have been elucidated.

X-ray techniques have established the 3-D structure of crystalline lysozyme down to a resolution of 2Å (Blake & Swan 1971). The molecule is roughly ellipsoidal (45x30x30Å) with a deep cleft running up one side. As would be expected the polar side chains of the molecule are distributed on the surface in contact with water, while the interior of the molecule contains the majority of the non-polar hydrophobic residues. The cleft is also partially lined with hydrophobic residues. The molecule appears to contain 3 lengths of a-helix (5-15; 24-34; 88-96) and two lengths of anti-parallel pleated sheets which occur between residues 41-45 and 50-54. There is a loop region composed of residues 64-80 with a disulphide bond joining residues 64 and 80. This loop region may be partly responsible for the antigenic specificity of the enzyme. Six units of substrate are able to fit into the cleft in a certain way and are held by numerous forces from the amino acid side chains in that region. Residues GLU35 and ASP52 in lysozyme c are the main ones involved in the hydrolysis of the substrate.

3. Substrate specificity

The proper name for lysozyme, 1,4-β-N-acetylmuramidase, suggests that it cleaves the glycosidic bond between the C1 of N-acetylmuramic acid (NAM) and the C4 of N-acetylglucosamine (NAG) in bacterial peptidoglycan. The cell wall of "Micrococcus lysodeikticus" (M. luteus) is extremely sensitive to lysis by lysozyme and the decrease in absorbance of suspensions of this organism still remains the major assay for its enzymic activity. Some lysozymes display also a chitinase activity corresponding to a random hydrolysis of 1,4 β NAG

linkages in chitin. Indeed the variation in the type of substrate demonstrated by certain types of enzyme may have had a direct influence on the specificity of these lysozymes in a type of "substrate-induced" evolution (Schindler et al. 1977; Jollès & Jollès 1984). Type c lysozymes, for example, act equally well on peptide substituted or unsubstituted peptidoglycan or chitin oligosaccharides. Type g lysozymes have a similar specificity for linear peptidoglycan but do not effectively degrade chitin oligosaccharides. Thus c and g lysozymes are more active against peptidoglycan from bacterial cell walls. The papaya and fig plants proliferate in climates that are favourable for fungal growth and invasion of plant tissue. Hence the papaya lysozyme acts mainly as a chitinase in a specific plant defence system. The phage lysozyme, which requires peptide-substituted substrates, would also appear to be uniquely adapted to its host, Esch. coli in which all the NAM residues are peptide substituted (Table 2)

In the case of c type lysozyme, common to serum, human and bovine milk and the majority of avian egg whites, the susceptibility of bacteria to lysozyme depends on the accessibility of the substrate. Thus Gram-positive bacteria are generally more susceptible to lysozyme because they tend to have a much simpler cell wall containing up to 90% peptidoglycan (Witholt et al. 1976). Some Gram-positive bacteria, notably the staphylococci, may contain teichoic acids and other materials that bind lysozyme and impede the diffusion to its substrate. Furthermore it has been reported (Reinicke et al. 1983) that additional cell wall material accumulated by staphylococci in the presence of chloramphenicol is more resistant to the action of lysozyme. In some Gram-positive bacteria e.g. Bacillus cereus, resistance to lysozyme can be attributed to the absence of N-acetyl groups on the glucosamine residues. A limited sensitivity to the enzyme can be induced after N-substitution with acetyl, propionyl, butyryl or formyl groups (Amano et al. 1980). The peptidoglycan component in Gram-negative bacteria is much less (5-10%) and is buried beneath an array of lipoproteins and lipopolysaccharides (LPS) which prevent the enzyme freely diffusing to its substrate. Hence, these bacteria tend to be more resistant to lysozyme. These barriers can be

Table 2 Substrate specificity*

Lysozyme type	Substrate type 1	2	3
c	++	+++	++
g	+++	++	+
Papaya	+	+	++
T4 phage	+++	-	-

* Adapted from Jollès & Jollès 1984

1 = Peptide-substituted glycan
2 = Unsubstituted glycan
3 = Chitin oligosaccharides
Digestibility of substrate:
+++, well-digested; ++, fair; +, poor; -, not digested

disrupted in vitro by a variety of chemical and physical treatments which predispose the bacteria to the action of the enzyme (Table 3). TRIS and EDTA (Leive 1968) act by binding divalent cations, mainly Ca^{2+} and Mg^{2+}, which are essential for the integrity of the LPS layers. Antibiotics such as polymyxin B can affect the integrity of the outer membrane (OM) probably as a result of their ability to complex with LPS (Fierer & Finley 1979). Other polycationic antibiotics such as aminoglycosides can cross the OM of Pseudomonas aeruginosa by first binding with the LPS thereby disorganising the membrane. Polylysine may act like EDTA by removing LPS and creating phospholipid bilayer regions in the OM. Physical disruptions such as a change from neutral to alkaline pH, osmotic shock (Birdsel & Cota-Robles 1967), drying (Webb 1969) and freeze-thaw cycles (Ray et al. 1984) can also predispose the bacterial cell to lysozyme action (Table 3). The validity of the work on pH shift is open to question because the original experiments of Zinder & Arndt (1956) were performed in TRIS buffer which, as noted above, can affect the OM permeability per se. With freezing, the OM undergoes a configurational change as demonstrated by the inability of frozen Esch. coli to adsorb LPS-specific phages (Ray et al. 1976; Kempler & Ray 1978).

4. Biological role

Although lysozymes are ubiquitous in occurrence, their function in higher vertebrates is still open to question. Much emphasis has been placed on the role of lysozyme as an antibacterial agent in the natural defences of milk, serum and especially antibody-free avian eggs. As this series of articles is concerned with antimicrobial systems in milk, it should be noted that the concentration of lysozyme in bovine milk is very low (13 µg/100ml) compared with that in human milk (10 mg/100ml). There is however, the possibility that salivary, gastric and intestinal secretions augment these levels (Reiter 1985). Although lysozyme probably does have some contribution to make against bacteria it must be remembered that serum, milk and egg white each contain several active agents which undoubtably work in concert. In addition, it is notable that lysozyme per se is highly effective only

Table 3 Agents enhancing lysozyme action*

Agent	Organisms affected
EDTA	many
Ascorbate	Ps. aeruginosa
TRIS	Esch. coli
	Ps. aeruginosa
Polymyxin B	many
Aminoglycosides	Ps. aeruginosa
Ca^{2+} pretreatment	Esch. coli
Polylysine	many
Serum/complement	Esch. coli
pH shift	many
Temperature shift	many

* Adapted from Taylor 1983

against Gram-positive species; the majority of pathogens - particularly those of eggs - being Gram-negative. It remains to be seen whether or not any of the factors described in Table 3 or similar influences are important *in vivo*. It needs to be stressed that other factors accompany lysozyme in biological secretions. Iron-chelating proteins, for example, such as the transferrins occur widely in biological fluids such as serum, milk and egg white. Although some workers (e.g. Tranter & Board 1982) have been unable to show lysozyme sensitivity of *Esch. coli* in the presence of ovotransferrin, alterations in the bacterial cell envelope by these proteins are well documented and may alter the resistance of bacterial outer membranes allowing lysozyme penetration.

The role of complement and immunoglobulins is well characterised and will not be discussed here. Although Gram-negative bacteria are killed by antibody-complement (AB-C) systems, they lyse to a significant extent only in the presence of lysozyme (Aldinolfi *et al*. 1966; Taylor 1983). The bacteriolytic action of serum is negated when lysozyme is removed by absorption onto bentonite (Caroll 1979) or neutralised by anti-human lysozyme antibodies (Glynn & Milne 1967). In addition, the rate and extent of lysis is related directly to the lysozyme concentration. Furthermore, pretreatment of cells with lysozyme has no effect on the lysis of serum-exposed cells which suggests that the enzyme only gains access to the peptidoglycan after disruption of the OM by the AB-C system.Other cationic proteins such as b-lysins found in serum or the basic protein isolated from polymorphonuclear leukocytes (PMNL's) have been shown to affect bacterial cell permeability and may predispose the cell to lysozyme action. Although there is some doubt over the ability of complement to survive in the gastrointestinal tract due to the action of trypsin and chymotrypsin, it is noteworthy that some of the components of milk, e.g. lactoferrin, lysozyme and antibody, all pass through the gastrointestinal tract of the infant without significant damage and are able to exert an effect in the intestine. Most avian egg whites have a high alkaline pH (7.5-9.5) which may have a two-fold influence. Firstly, the alkaline medium *per se* may disrupt the OM of bacteria and secondly may provide a suitable pH for lysozyme action. In this context it should be noted that lysozyme has a broad pH spectrum (4-1Ø) but an alkaline pH optimum.

Lysozyme may also have an indirect effect on defence systems as an immunomodulator (Table 4). This may be due to the presence of breakdown products of lysozyme action, e.g. N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), the water-soluble

Table 4 Biological properties of peptidoglycan-cell wall fragments*

1. Induction of humoral and cellular immune response
2. Activation of lymphocytes (e.g. release of lymphokines)
3. Activation of macrophages (e.g. release of monokines, lysosomal enzymes, prostaglandins)
4. Phagocytosis, transport and storage by macrophages
5. Resistance to degradation (persistence in tissue)
6. Induction of chronic inflammation

* Taken from Heymer *et al*. 1985

component of Freund's adjuvent (Stewart-Tull 1985). Although mammalian lysozyme splits isolated peptidoglycan, it possesses a limited bacteriolytic activity (Ginsberg 1979). It is noteworthy, however, that there is evidence that lysozyme may not be effective as a muralytic enzyme but rather as an activator of pre-existing bacterial autolytic wall enzymes (Wecke *et al*. 1982). Thus, the fate of micro organisms would be mediated in part by the availability of agents - such as lysozyme - that were capable of activation of cell autolysis and also by the presence of inhibitory substances (e.g. polyelectrolytes) which can block bacteriolysis in tissues (Ginsberg & Lahav 1983).

Further evidence in favour of the view that lysozyme acts as an immunostimulator has been obtained in experiments where the levels of secretory IgA were increased in faeces of infants fed on a lysozyme-containing milk formula (Lodinové & Jouja 1977). Cell wall breakdown products have also been shown to enhance the activation of macrophages, demonstrated by an elevation in the levels of superoxide radicals (Heymer *et al*. 1985). These products may also induce rapid clearance of bacteria by PMNL's (Di Luzio & Morrow 1971). It is also worth considering the fact that lysis of bacteria by lysozyme may generate components of the cell wall that are toxic to the host. During embryogenesis in avian eggs, for example, the albumen is transferred via the amnion to the embryo. Pharmacologically-active LPS and lipoprotein produced by the action of lysozyme in egg white may be toxic to the cells of the developing embryo. If this were the case, it would lend further support to the theory that lysozyme does not play such an important enzymatic role *in vivo*.

Other interactions with the host may arise because of the basic reaction of the lysozyme protein. Combination with acidic mucus glycoproteins has been noted which presumably influences the gel structure and consequently the rheological and transport properties of the tracheobronchial mucus. In addition, lysozyme can interact with ovomucin in egg white to give the gelatinous structure observed in fresh eggs (Tranter & Board 1982). In these ways the resultant gels may impede the movement of bacteria from the initial points of infection. A lysozyme-albumen complex has been shown to aggregate the pili of *Esch. coli* K12 *in vitro* (McMichael & Ou 1979). Piliated bacteria were more readily aggregated than their non-piliated counterparts. Thus lysozyme may prevent or hinder attachment and colonisation of potential pathogens in the intestinal tract.

5. Commercial applications

It has been shown that lysozyme can inhibit the outgrowth of vegetative cells from spores of *Clostridium tyrobutyricum* irrespective of whether or not the mother cells are resistant to lysozyme (Wasserfall & Teuber 1979). Spores of clostridial species in cheese milk can survive pasteurisation temperatures and are responsible for the gas blowing in the ripening of "sweet-curd" cheeses such as Edam and Gouda. Egg white and purified egg white lysozyme have been shown to prevent completely this late gas defect (Goudkov & Sharpe 1965) in

Edam cheese (Wasserfall & Prokopek 1978) and in Italian hard cheeses (Lodi & Carini 198Ø). Nctably dried egg white is ineffective in such prevention (Wasserfall et al. 1976). Clostridial spores have been reported to cause spoilage of Grana cheese also and germination of the spores in milk may be inhibited by low (1Ø-5Ø ppm) concentrations of lysozyme. Higher levels, however, are required to inhibit Clostridium tyrobutyricum. Lysozyme was found to have a negligible inhibitory action on starter bacteria e.g. Lactobacillus bulgaricus and L. lactis at a level of 25 ppm in Grana and Montasio cheese which prevented defects during the ripening stage. Support for the substitution of lysozyme for nitrate in order to prevent fermentation by butyric acid bacteria in Samso cheese has been published also (Nielson 198Ø).

Recent developments appear to have concentrated on modification of existing lysozymes. For example, the disulphide bond in the active cleft region of T4 phage lysozyme has been genetically engineered (Hawkes et al. 1984) to increase the thermostability of the enzyme as a whole, making it more effective in cheesemaking (Anon. 1985).

It is now widely accepted that colostrum (Larguia et al. 1974) and human milk are beneficial to the developing newborn infant, in that they can protect against colonisation of the intestinal tract by pathogens until the infant can build up its own defence system (Finkelstein et al. 1983; Kovar et al. 1984; Reiter 1984). As milk contains an array of non-antibody antimicrobial proteins, the effect of any single factor is difficult to determine. There is, however, an increasing interest in formulated feeds supplemented with these protective factors with a view to the production of a formula that approximates to mothers milk. As infectious diarrhoea is a prime cause of human infant morbidity and mortality in under-privileged countries (Snyder & Merson 1982), protection of a sibling against gut organisms associated with enteritis is of interest to those who attempt to supplement the diets of malnourished infants. Unsupplemented commercial infant formulae have been shown to support the unrestricted growth of pure cultures of Vibrio cholerae, Esch. coli and Salmonella typhimurium whereas human milk was bacteriostatic or bacteriocidal. A combination of secretory IgA and apolactoferrin was shown to be of greatest importance in this killing action; direct involvement of lysozyme, however, was not proven. Human milk itself generates a predominantly bifidobacterial flora with some streptococci, bacteroides and clostridia. Some formula feeds achieve a reasonably desirable balance in the faecal flora but fail to suppress clostridia which are potential pathogens in babies and other newborn animals. What little evidence is available on the addition of lysozyme to feeds is confusing and has generally been carried out with hen egg albumen lysozyme. The results of these trials may be misleading because milk lysozyme is more effective against both Gram-positive and negative bacteria than egg white lysozyme (Reiter 1984). Clearly the effect of lysozyme in formulated feeds needs more research particularly in view of the observation that these enzymes may inhibit the outgrowth from clostridial spores in milk and cheese.

This article has attempted to give an overall view of lysozymes in general and to discuss some of the problems associated with the resolution of the biological role of this enzyme. Much work needs to be done before the biological role is elucidated but as it has remained a mystery for 6Ø years there is still a doubt as to whether its role will ever be properly defined.

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The composition of the milk has so far essentially be considered from the nutritional point of view (Gaull et al 1982). This is for obvious reasons as one of the major characteristics of the nutritional situation during the neonatal period is by all means the fact that the offspring has to rely upon one single source of nutrients, the milk.

During the last decade there has, however, been an increased interest to the more specific physiological roles of certain milk components. Thus the role of the immunoglobulins, but also of certain other components which play a role in the defence mechanism against gastrointestinal infections, i.e. lactoferrin, and of hormones, which function as growth modulators, has been studied more in detail.

The components in milk can be divided into the following categories:

1. Nutritional components. These comprise the classical nutrients, e.g. protein, fat, carbohydrate, minerals and vitamins.

2. Antimicrobial components. These include a number of biologically active macromolecules which can protect the infant from infections essentially within the gastrointestinal tract, e.g. immunoglobulins, lactoferrin, lysozyme, complement components, active leucocytes.

3. Others. These include a number of enzymes, e.g. lactoperoxidase, lipase, xantin oxidase, which may play a role for the digestion as well as protection against infections. Growth modulators including epidermal growth factor (EGF) and taurine are other examples of microcomponents to which much more interest has been devoted during the last few years.

Although most biologically active components are proteins this does not necessarily mean that they should only be considered as protein sources of nutritional significance and therefore referred to the first category.

Iron-binding proteins in milk

Milk contains two kinds of iron-binding proteins, transferrin and lactoferrin. All mammals seem to be able to excrete iron-binding proteins in their milk but there is a striking species difference with respect to the concentrations of these two proteins (table 1). Thus human milk is unusually rich in lactoferrin, which is 10-100 times the content in cow's milk (Masson & Heremans 1971). 10 X IN GOAT

The transferrin found in milk seems to be almost identical to the transferrin found in serum. Lactoferrin is synthesized in the mammary gland. It was earlier called the red protein, and it is also secreted in saliva, semen and tears (Masson 1970). Lactoferrin is one of the major whey protein components in human milk constituting 10-30% of the total protein content. Although earlier studies on the effect of maternal malnutrition has not shown any effect on the lactoferrin content in the milk (Lönnerdal et al 1976), it has recently been reported that the lactoferrin concentration should be reduced in milk from malnourished mothers (Houghton et al 1984).

Table 1 Content of iron, lactoferrin and transferrin in milk from different species

ug/ml	Iron	
0.3-0.6	human, cow, goat	
1.4-2.4	sow	
2-4	rabbit	
3-14	rat, dog	
ug/ml	**Lactoferrin**	**Transferrin**
<50	rat, rabbit, dog	dog, human
20-200	cow, goat, sow	sow, cow, goat, mare
200-2000	guinea-pig, mouse, mare	guinea-pig, mouse
>2000	human	rabbit rat

Lactoferrin like transferrin is a glycoprotein and binds two ferric atoms with the incorporation of two molecules of bicarbonate. However, neither lactoferrin nor transferrin are fully saturated with iron. Lactoferrin has since long been considered as the iron-binding protein in milk. However there are also a number of other components in milk that bind iron, i.e. outer fat globule membrane, casein and a low molecular weight complex. Interestingly, only 20-30% of the total iron content in breastmilk is bound to lactoferrin, making it saturated only to 3-5% (Fransson & Lönnerdal 1980, Lönnerdal 1984).

Transferrin is known as an iron carrier in the body and it is suggested that lactoferrin plays a similar role for the absorption of iron of the suckling (Brock 1980). Thus iron bound to lactoferrin is absorbed to a very high degree (50-75%) when compared to other forms of iron (hemoglobin-iron and inorganic iron). Lactoferrin has similar physico-chemical properties to transferrin, but the association constant for iron is about 300 times higher. The iron-lactoferrin complex is stable from pH 4 and it does not release its iron until pH 2 or lower is reached (Masson 1970). It is also remarkably resistant to proteolytic digestion and denaturation especially when it is saturated with iron (Brock et al 1976).

Lactoferrin is also considered to play an essential role in the resistance against intestinal infections caused by Escherichia coli (Bullen 1972). It has been considered to exert its protective factor as it occurs in a highly unsaturated form in the milk and consequently binds iron so strongly that it is not available for the bacteria in the intestinal tract (Bullen 1972).

A number of specific questions regarding the physiological role of lactoferrin in milk could be raised:

1. Is there a conflict between the nutritional and the antimicrobial role of lactoferrin?
2. Is there a difference between in vitro and in vivo antimicrobial effect by lactoferrin?

3. What is the relation between the total iron distribution in milk and the iron saturation of lactoferrin?
4. What is the effect of maternal iron status on lactoferrin-iron and iron distribution in milk? Is there a leakage or physiological "trapping"?
5. Does lactoferrin play a role for the absorption of iron in the gut?
6. Does lactoferrin have any effect on the immune response, and if so, has its iron saturation any significance on this effect?.

Nutritional availability of Lactoferrin as protein source

Alfa-lactalbumin, secretory IgA and lactoferrin are the three dominating components in the whey protein fraction in human milk. Alfalactalbumin, which is a part of lactose synthetase has a high nutritive value due to an almost ideal mixture of the essential amino acids (Hambraeus 1982). Two of them, secretory IgA and lactoferrin, however, as well as lysozyme seem to play a physiological role which requires that they are functionally intact within the gastrointestinal tract. They may consequently not be nutritionally available as protein source. Lactoferrin constitutes between 10-25% of the total protein content in mature human milk, and secretory IgA another 5-10%. In addition lysozyme constitutes another 5% of the total protein content. Consequently as much as 40% of the total protein in human milk may not be nutritionally available and should not be included in calculations of nutritionally available protein in human breast milk (Hambraeus et al 1984). Recommendations about protein intake during the neonatal period which do not take this into consideration may consequently be too high. It can however be disputed whether or not lactoferrin is nutritionally available as source of amino acid, nitrogen or iron at a later stage in the body after it has played its specific physiological role. Significant amounts of immunologically intact lactoferrin and secretory IgA have been found in the faeces of breastfed infants (Spik et al 1982). However, there is a need to get a better quantitative estimation of the extent to which lactoferrin is absorbed and consequently whether or not it should be included in the calculation of nutritionally available protein in breastmilk. Furthermore it would be of interest to study this in preterm as well as fullterm infants as there may be a difference in the intestinal permeability for proteins and iron-protein complexes.

The role of lactoferrin in iron metabolism

The role of lactoferrin in iron absorption is controversial. Iron deficiency anemia is a common nutritional problem in infancy but is less frequently observed in breastfed infants. Although human milk has a low iron content the iron amount provided by breastmilk thus seems to be adequate during the first 4-6 months of life to prevent iron deficiency anemia in the breastfed infant (MacKay 1928, Saarinen 1978). This is possibly due to a high bioavailability of iron (Feuillen & Plumier 1952, Saarinen et al 1977). Lactoferrin has been assumed to play an active role in this respect. Animal studies have given support to this assumption (Fransson 1983, Fransson et al 1983 a b c). On the same time it is anticipated that its high iron affinity is a means in the host defence against gastrointestinal infections by keeping iron away from the microflora ion the intestines.

The assumption that iron absorption from human milk is very high is based on absorption studies using isotope methods. However, when an iron isotope (^{59} Fe) is added to human milk it is not evenly distributed between the various iron compartments as earlier presumed. Thus 80% of the added amount of radioactive iron was found to be associated or occurred in the lactoferrin compartment of the whey protein fraction (Lönnerdal 1984). This suggests that earlier data on the high bioavailability of iron in human milk may essentially refer to the lactoferrin-bound iron.

Different studies have shown that iron in human milk is much more available than iron in cow's milk or in infant formula, where iron is added as ferrous sulphate. The mechanism behind this high bioavailability is not known but it has been proposed that lactoferrin has a role to play. Few studies are still available regarding the effect of lactoferrin on iron absorption in vivo and conclusions from in vitro studies are contradictory (Lönnerdal 1984).

Various hypothesis exist regarding the action of lactoferrin on iron absorption in the gut. One classical hypothesis is based on the assumption that lactoferrin protects against excess iron absorption by binding iron in the duodenum, thus preventing it from being absorbed (DeLaey et al 1968, DeVet & van Gool 1974). According to another hypothesis lactoferrin facilitates iron absorption by donating iron to specific receptors at the brush border membrane in the small intestine (Cox et al 1979). The higher level of lactoferrin in the early milk, together with a low gastrointestinal activity, has also been suggested to prevent iron from being absorbed in the newborn. As the infant matures and the need for exogenous iron increases, the level of lactoferrin in milk decreases, the proteolytic activity increases and more iron is released from lactoferrin. Once released absorption of this iron may be facilitated by other factors in the milk (Brock 1980).

The role of lactoferrin as some sort of homeostatic regulator of the bioavailability of iron from breast milk has been discussed. The lactoferrin content in breast milk seems to be influenced by the iron balance of the mother. Thus the lactoferrin content is increased in mothers which have a very high iron intake in their diet. Interestingly however an increased lactoferrin content has also been described in anemic mothers (Fransson et al 1984). The nutritional importance and significance of the lactoferrin content in various milk products may therefore have great nutritional interest.

The effect of the iron status of the mother of the iron intake in the maternal diet on the total iron content in human milk and its distribution is still not fully understood. Thus Murray et al (1978) did not find any effect of iron deficiency (Hb <10 g/dl) nor of iron overload (Hb >12 g/dl) on breast milk iron concentration. It is however possible that the iron status and iron intake may influence the lactoferrin content in the milk. Severe anemia in lactating mothers (Hb <8 g/dl) in India has been reported to result in increased lactoferrin content in the milk when compared to control mothers in the same ethnic group (Hb >11 g/dl) (Fransson et al 1984). The reason for this increased lactoferrin concentration in the milk could be a possible preferential binding of iron to lactoferrin by the mammary gland in order to make iron available to the

infant. Another explanation could be a non-specific leakage of iron and lactoferrin in the mammary gland secondarily to a subclinical mastitis. Increased lactoferrin content has been reported in both malnourished and wellnourished Ethiopian mothers who all have a high dietary intake of iron (Lönnerdal et al 1976). These observations might indicate some sort of impact on lactoferrin synthesis which may represent a mechanism to maintain the bacteriostatic and transport role despite differences in iron balance. It has been suggested that iron bound to lactoferrin may be a valuable form of iron supplementation (Fransson et al 1983b).

Lactoferrin and the immune response

The interaction of minerals and trace elements on the immune system has been studied during the last years (Chandra 1980). As iron is an essential nutrient for normal development and functional integrity of the lymphoid tissue it could be expected that there is a relation between iron status and the cellular immune response mediated by lymphocytes and granulocytes (Chandra 1976). The relationship between the iron status if the individual and the incidense och infections and the immune response has been reviewed during the last years (Beisel 1982, Bullen et al 1978, Chandra et al 1977, Vyas & Chandra 1984).

It is known that iron deficiency leads to a mild reduction in cell-mediated immunity and a reduction in lymphocyte proliferation response to mitogens and antigens. Interestingly the latter response is reported to be affected also in latent iron deficiency without anemia (Chandra & Saraya 1975, Chandra 1980).

Iron deficiency has also been reported to result in a disturbance in the response to recall antigens (Vyas & Chandra 1984). On the other hand iron deficiency does not seem to affect the serum immunoglobulin levels, neither their response to infections. The primary cause of altered immunity so far reported seem to be essentially due to a reduced tissue and cellular iron content (Chandra 1973).

It has been found in studies of interaction between nutrients and immunological response that also an abundance of some trace minerals, e.g. zinc, may lead to an impaired immune response (Chandra 1984). This accentuates to need to analyze any similar situation with respect to iron as well as to the relation of total iron content versus iron saturation of lactoferrin in the milk.

Antimicrobial effect of Lactoferrin

It has been shown to inhibit the growth of certain microorganisms in vitro, and saturation of lactoferrin has also been shown to reverse this bacteriostatic effect (Reiter 1978). However, no satisfying in vivo study on the bacteriostatic effect of lactoferrin has so far been carried out.

As iron is an essential nutrient not only for the mammalian cells and for the immune response but also for microorganisms, it has been discussed to what extent there is a competition between the microbes and the host organism, and if this is disturbed during iron supplementation. Bacteria are known to have the ability to produce "siderophores" to facilitate their assimilation of iron. The bacteria have also been classified in four

categories based on their dependency on iron for virulence and nature of infection (Payne et al 1978). It is on the other hand known that unsaturated lactoferrin has a bacteriostatic effect in vitro (Bullen et al 1972, Bullen & Armstrong 1979), which is decreased when iron is added in excess (Reiter 1978, Bullen & Armstrong 1979). As lactoferrin is an exceptionally strong binder of iron it can successfully compete with the siderophores of the bacteria. The bacteriostatic function of lactoferrin furthermore seems to be enhanced by interaction with secretory IgA, another major component in the human whey protein fraction (Arnold et al 1977). The bacteriostatic function is also potentiated by specific antibodies.

It has however been discussed whether or not supplementation with iron orally or parenterally may be hazardous (Lönnerdal et al 1980, Keen et al 1982). This is partly based on the findings that lactoferrin plays a bacteriostatic role in vitro as long as it is not saturated with iron (Arnold et al 1977, Bullen et al 1972). The fact that bovine milk lacks lactoferrin and that many infant formulas have been iron-enriched using various forms of iron salts has been questionned.

It has been suggested that iron deficiency protects against infection (Masawe et al 1974, Murray et al 1978). Interestingly, it has also been reported that an increased intake of iron through iron supplementation could result in increased frequency of infections (Massawe et al 1974, Mc Farlane et al 1970, Murray & Murray 1978, Murray et al 1975). The conclusions drawn in these studies have however been critisized (Vyas & Chandra 1984) as chronic iron overload is not characterized by frequent infections. Furthermore, in vivo saturation of serum with iron does not affect its bacteriostatic properties. Some studies in animals, however, indicate that parenteral administration of iron may reduce the number of bacteria necessary to produce disease or death (Vyas & Chandra 1984). Iron-dextran injections have also been reported to result in a high incidence of gram-negative septicemia in newborn (Barry & Reeve 1977). Similarly it has been reported that large doses of iron in initial stages of nutritional management may be harmful in PEM and predispose to bacteriemia and increased mortality (Vyas & Chandra 1984). However it has also been shown that this is transient and that only a few days delay will compensate this. The negative effect of per oral administraton of iron has however not been shown. Although this might mean that iron administration per se will not affect the immune system or be hazardous for the host defense system for infections it may still affect the bacteriostatic capability of lactoferrin. If the lactoferrin given then is only partially saturated, it may function as an iron source and still have a protective capacity against gastrointestinal infections (Lönnerdal 1984). Furthermore it could be of interest to study the interactions of various minerals and trace elements on iron supplementation and the role of lactoferrin.

Conclusion. Although lactoferrin thus seems to have a significant nutritional as well as antimicrobial role to play for the neonate its biological significance is still incompletely known. It is thus of great interest to further study this from various aspects. This has a great practical implication from the nutritional as well as health point of view as the optimal composition of breast milk substitutes still has to be solved. Furthermore during recent years it has been possible to separate lactoferrin from bovine whey in substantial amounts at a reasonable cost. This makes it

realistic to add lactoferrin to infant formula in the near future. If this then is of physiological significance it could be an essential step in the development of infant feeding practices.

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Biological Significance of the Antibacterial Factors in Milk: Immunoglobulins, Lysozyme, Lactoferrin, Lactoperoxidase

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During the symposium the main antibacterial factors in colostrum and milk have been presented in individual papers (see Introduction to the symposium). The task of this paper is therefore restricted to supplementing and correlating the biological significance of these factors. It contains some references to old papers which may bear fresh interpretation in the light of present knowledge or, in more honest parlance, "with hindsight". It is for the reader to evaluate the interpretations offered. For a considerable time comprehensive reviews have appeared which deal in greater detail with this subject.

The Complement-Mediated Bacteriocidal Effect of Antibodies

Michael et al. (1971) attempted to establish a direct correlation between the level of Igs in colostra, their agglutinating and bacteriocidal activities and the bacterial flora of the faeces. Comparing the counts of coliforms and lactobacilli in the faeces of breast and bottlefed infants the beneficial effect of feeding colostrum was clearly shown - coliforms were suppressed. The decline of the total and individual classes of Igs however is difficult to relate to the fall in agglutinating and cidal activity of the colostrum and the faecal extract tested against an enteropathogenic serotype of E. coli 0127. Unless we accept that sIgA binds C in the presence of lysozyme and becomes cidal (Adinolfi et al. 1966) we must assume that the cidal activity of the colostral and faecal extracts was due to IgM which is the most efficient cidal Ig. (It has been calculated that 30 molecules of IgM per bacterium suffice to activate C (Sterzl et al. 1962)) Bovine colostrum was known for some time to contain C, at least the first 4 components according to the conglutinin test (Reiter, Oram 1967). Colostrum became bacteriocidal for E. coli after dilution, presumably avoiding the Neisser-Wechsberg effect; high concentrations of IgG in sera were known to prevent cidal activity (Muschel et al. 1969). (Poutrel, Reinard (1985) reported that an unidentified component of milk can also reverse haemolytic and cidal effects). Heating colostrum at 56°C for 30 min or adding C_3-inhibitor N-acetyl-tyrosine abolished the cidal activity (Reiter, Brock 1975). Since human milk has now been reported to contain all C components (Neut et al. 1980), it would be interesting to know whether human colostrum with little IgG is cidal without dilution or also requires dilution. Although C is extremely labile during storage, according to Gordon, Thompson (1935) the haemolytic activity is not destroyed between pH 2.4-11.9; nothing seems to be known about the survival of C in gastric juice (proteolysis). It appears that the primitive approach of Michael et al. (1971) yielded valuable information which could be exploited, using the sophisticated methods available now.

Human and bovine serotypes of E. coli share antigens because the cidal tests were made with a human serotype of E. coli (NCTC 9703:K_{52} (B_4) H-non motile) (Reiter, Brock 1975). Also, 24 out of 50 enteropathogenic (EPEC) and 10 out of 50 non-enteropathogenic E. coli (NEPC) isolated from infant's faeces were found to be killed in bovine colostrum (Reiter 1981). Moreover, quite unrelated bacteria can share antigens (Sharpe, Latham, Reiter 1969). Strict anaerobes, isolated from bovine rumen (Butyrovibrio), for instance possess common antigen with Salmonella typhimurium (Sharpe, Reiter 1972). Antibodies in serum or secretions need not depend on the antigenic experience of a specific organism but can arise from unrelated organisms, which adds to the importance of a "healthy" or "normal" intestinal flora initiating what used to be called "natural antibodies".

LYSOZYME (L) (CAT Milk 25 ug/100 ml

Sources: The concentration of L in bovine milk is very low (av. 13 ug/100 ml) compared with human milk (av. 10 mg/100 ml) (Vakil et al. 1969).

The structure and biochemical activity is discussed elsewhere by Dr. Board and colleagues.

Milk-L has a higher bacteriocidal and lytic activity against extrememly susceptible assay organisms (e.g. Micrococcus lysodeicticus) and a wider antibacterial spectrum (Petersen, Hartnell 1955; Vakil et al. 1969). Its activity depends not only on the concentration and accessibility of its substrate - peptidoglycan - but also on the ionic strength of the medium and nature of the anions: lysis of Streptococcus mutans (Goodman et al. 1981) and Veillonella alcalescens (Tortosa et al. 1981) is increased according to the general pattern of anionic selectivity in biological systems $Cl^- < SCN^- < HCO_3$ (Wright, Diamond 1977). SCN^- is derived from milk, saliva and by secretion in the stomach while HCO_3^- is the main intestinal buffer; these anions could therefore promote the L-activity in the IT. Hitherto the lipoproteins in the outer membrane in Gram-negative organisms were thought to protect against lytic activity unless the organisms are first exposed to the complement mediated activity of specific antibody producing lesions in the outer membrane, treatment with chloroform or ethylene diamine tetraacetic acid. Besides the anionic enhancing effects, the transfer of Gram-negative organisms from a medium of low to high pH (Petersen, Hartnell 1955) increases the lytic activity of L and widens the antibacterial spectrum; in vivo effects have not yet been investigated.

Intestinal flora: Many papers have been published on the intestinal flora but the data invariably describe the faecal flora - at best a reflection on the flora of the large intestine. This may be excusable in the case of human babies but not for experimental animals. It was known since 1931 that L appears in the faeces of breast but not bottle fed infants (Rosenthal L, Lieberman H cited by Fleming 1932) and faecal bacteria were shown to be killed by human and bovine milk after inactivation of complements! At that time all the inhibition was attributed to L reducing drastically "Gram-negative bacilli". It would be interesting to repeat these experiments with L purified from milk. Data available on the effect of L added to formula feeds are not only confusing but only refer to albumen L.

The aim of 'humanizing' the nutrient composition of cow's milk is to achieve a similar balance in the faecal flora as with human milk. In breast fed babies the faecal flora consists predominantly of bifidobacteria in comparison with streptococci, enterobacteriaceae, bacteroides; (in a ratio of up to 10 000:1) (e.g. Yoshioka et al. 1983). Clostridia are always very low or even absent. Some of the formula feeds achieve a reasonably desirable balance in the faecal flora (Warton 1982) but fail to suppress clostridia, which are potential pathogens because of their toxin production in babies and newborn animals.

Originally it had been reported that lactoferrin inhibits the germination of spores of Bacillus stearothermophilus (Oram, Reiter 1968) but this was subsequently traced to contamination with L (Reiter 1976) which complexes with lactoferrin. It has now been shown that albumen L can prevent the outgrowth of spores of Clostridium tyrobutyricum which causes undesirable gas formation in cheese (Wasserfall, Teuber 1979). Certain species of clostridia and their toxins are known to cause diarrhoea in infants (and newborn animals) and may even cause some cases of sudden infant death syndrome (cot death) (Sonnabend et al. 1985). It would therefore be of interest to supplement formula feeds for infants with L isolated from milk and not egg white.

Adjuvant effect: N-acetylmuramyl-L-alanyl-D-isoglutamine, abbreviated as muramyldipeptide (MDP), the minimal water-soluble structure of Freund's adjuvant, was shown to enhance sIgA production, (the activation of macrophages increased release of superoxide O_2^-) and rapid clearance of bacteria in vivo by polymorphonuclear leucocytes and suppression of IgE antibody responses

(Butler et al. 1983). It is therefore not surprising that Jolles (1976, 1984) suggested that L may have an indirect effect acting as a immunomodulator through the release of cell wall components. Prior to this Lodinova, Jouja (1977) observed that the level of sIgA increased in the faeces of babies given a formula feed containing albumen L. Namba et al. (1981) fed L (and pronase) to guineapigs which increased the cellular response and circulating antibodies to hepatitis B surface antigen. Cell wall digests of Bifidobacterium longum also enhanced the host immune responses. Experiments with germ free rats (Morisaki et al. 1983) illustrate the general system of mucosal associated lymphoid tissue (MALT): oral administration of a live, cariogenic strain of S. mutans plus its peptidoglycan (or MDP) enhanced salivary IgA responses and gave appreciable protection against caries when challenged with S. mutans. Previously it has been shown that saliva and human milk can contain natural S. mutans antibodies (without immunization) (Arnold et al. 1976) and we can assume, therefore, that the oral immunization of the germ free rats, although not tested, would have also increased the IgA in their milk. The presence of L in leucocytes (with the exception of bovine polymorphs) is well documented. Recently it was shown that the peptidoglycan of Bacillus subtilis is degraded in a macrophage cell line (Vermeulen, Gray 1984) yielding several glycopeptides. Human L externally can also stimulate phagocytosis (ingestion) by PMN. Albumen-L had no effect. The L effect was not the result of opsonization but appeared to affect the membrane of the cell itself (Klockars, Roberts 1976). It appears therefore that L in the blood (Selsted, Martinez 1978) could promote phagocytosis and through the digestion of cell walls enhance immuno-responses. In the IT the level of L secreted by the Paneth cells (Isaacson 1982) may be increased by diffusion from the blood and/or derived from the infiltration of leucocytes containing L (McClelland, Furth 1975).

L may also prevent or hinder attachment and colonization of potential pathogens: in vitro albumen-L was shown to aggregate the pili of E. coli K_{12} and piliated bacteria were more readily aggregated than non-piliated bacteria (McMichael 1979). It is surprising that this effect has not yet been tested with strains known to attach in the IT. In this context, the reported agglutination by the L-lactoferrin complex (Perraudin et al. 1974, Perraudin, Prieels 1982) may also hinder attachment.

In conclusion, it would be of considerable interest to extend the research on L, particularly its effect on the intestinal flora in animals and investigate whether the supplementation of milk L to infants feed has a protective effect.

LACTOFERRIN (LF)

The biochemical and antimicrobial properties of LF and its role in neonatal feeding have been dealt with by Prof. B. Ribadeau-Dumas and Prof. L. Hambraeus in this symposium. This section discusses therefore only selected aspects.

Iron absorption. It can now be accepted that in vitro human brush-border membranes possess receptors specific for human LF because bovine LF was less able to yield its iron for absorption (Cox et al. 1979). Eventually these receptors will be characterized, like the transferrin receptors on various cell types (Newman et al. 1982). Hambraeus and co-workers proved in mice and piglets that the uptake of iron from iron-saturated LF, added to artificial feeds, was equal to sulphate iron.

If LF inhibits bacteria in vivo it must be assumed that any iron freed during the digestive process is chelated having the same function as transferrin in blood which restricts free iron to trace levels. In contrast to human milk, porcine milk for instance is rich in iron (1.4-2.4 µg/ml) but poor in LF (20-200 µg/ml). Is the LF therefore able to chelate all the iron in porcine milk? Bovine milk is low both in iron and LF; it contains also high levels of citrate (4-6 mg/ml) which has a higher affinity for iron than LF (Reiter et al. 1975). It is therefore feasible that citrate acts as a carrier for iron, either absorbed passively or through citrate special receptors in analogy to bacterial membrane receptor proteins (Wagegg, Braun 1981).

Feeding iron saturated LF does not really prove that LF is involved under natural conditions in iron absorption. In contrast to piglets, breast fed infants rarely become anaemic. The high bioavailability of iron has been attributed (e.g. Rassin 1984; Greene, Courtney 1984) to the content of vitamin C and E, polyunsaturated fats, lactose, phosphorus and protein. LF as an iron carrier is not even mentioned but we need to know whether the high LF content in human milk is essential for the good bioavailability of iron. Now that we have LF available for large scale experiments it ought to be possible to define the role of LF and follow the route of native iron in the different milks to LF (citrate) and eventually blood.

Bacteriostasis in vitro. Masson et al. (1966) sought to establish whether LF in human secretions possessed similar antibacterial properties to serum transferrin and demonstrated the inhibition of "Staphylococcus albus". At the same time Oram, Reiter (1966/1968) purified LF from bovine milk and demonstrated the interaction of Bacillus stearothermophilus. Bacteriostasis was reversed by Fe^{2+} and enhanced by Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}. The enhancement was attributed to a possible imbalance of cations, as well as the lack of Fe^{2+}, as suggested by Feeney (1951) for conalbumin. Some iron binding agents such as 8-hydroxy quinoline, 1,10-phenanthroline were also found to be inhibitors but not transferrin. These results have been overshadowed by the discussion on the role of specific antibodies but may warrant reexamination and reinterpretation.

Bullen et al. (1972) in a much quoted paper appeared to have demonstrated that LF requires specific antibodies for bacteriostasis. The data show rather that E. coli O111 was inhibited by LF ~99% compared with the control and increasing in the presence of specific antibodies to ~99.99%. Some workers confirmed the need of antibodies (e.g. Spik et al. 1978, Stephens et al. 1980) while others did not (e.g. Law, Reiter 1977, Samson et al. 1979). Although E. coli O111 was the assay organism, nobody but Bullen took the precaution to passage E. coli O111 repeatedly through guineapigs to maintain its virulence. Under iron restricted conditions "virulent" E. coli synthesizes, amongst other factors, siderophores and outer membrane proteins which act as receptors for the iron of the siderophores (Neiland 1982, Griffiths 1983), but nobody seemed to have taken the precaution to test whether their "laboratory strain" synthesized siderophores (enterocholin), or followed the inhibition long enough; Brock et al. (1983) observed a reversal of the bacteriostasis after incubation for 24 hours instead of the usual 5-6. Human sera and human milk are now known to contain naturally occurring antibodies to the receptor proteins (Griffiths 1985, Griffiths Pers. comm.) and enterocholin (Moore et al. 1980, Brock Pers. comm).

Moreau et al. (1983) used a non-pathogenic isolate of E. coli from the faeces of a healthy human adult to demonstrate that IgG purified from bovine colostrum increased the bacteriostatic activity of 0.125 mg LF/ml but there was no increase at the higher level of 0.5 mg LF/ml. Law, Reiter (1977) used 2.5 mg of bovine LF/ml and also failed to increase bacteriostasis for a serum sensitive and the serum resistant strain of E. coli (O111, O101) by the addition of bovine colostrum. Since colostrum was cidal for E. coli O111, it was concluded that antibodies are not involved in bacteriostasis, at least in the case of bovine LF. This interpretation was strengthened when it was found that all the strains of E. coli isolated from babies' faeces, representing enteropathogenic and non-enteropathogenic serotypes (n:100), were inhibited by bovine colostrum, addition of iron reversed the inhibition (Reiter 1978). However Brock et al. (1983) suggested that the bacteriostatic-enhancing effect of antibodies may be due to a specificity broader than that delineated by O antigens such as enterobactin.

Bacteriocidal effect in vitro. In a series of papers, from 1977 onwards, Arnold et al. (1980, 1982) claimed that human LF killed a wide range of microorganisms - Gram-positive, Gram-negative, aerobic and anaerobic bacteria, as well as yeast. This effect appeared to be strain specific because enterobacteriaceae, shown by other workers only to be inhibited, were not killed; also lactic acid bacteria remained unaffected. The assay conditions were rather artificial because killing was only observed with organisms suspended in distilled water or saline. Apparently (Arnold et al. 1984) the LF preparation was contaminated with sIgA and activity

was only observed in its presence. Also, only a serum sensitive but not serum resistant strains of E. coli was killed. Salmonella typhimurium mutants with progressive deletions in their carbohydrate side chains became increasingly susceptible to killing by LF plus sIgA. These results have now been confirmed, (without knowledge of the latest findings of Arnold et al.) by Boesman-Finkelstein, Finkelstein (1985). E. coli, S. typhimurium and particularly Vibrio cholerae were killed by apo-LF plus purified sIgA from human milk or antibodies against V. cholerae outer membrane proteins. Interestingly, lysozyme together with apo-LF and bicarbonate (2 mol/mol LF) also increased the cidal activity of skimmed human milk. Since the citrate level counteracts the effect of bicarbonate (Reiter et al. 1975) and up to two thirds of sIgA can be attached to fat globule membranes (Honkamen-Buzalski, Sandholm 1981) these experiments ought to be repeated in whole human milk.

Antibacterial activity in vivo. LF was shown to be resistant to proteolysis in vitro (Brock et al. 1978) and was recovered from faeces of breast fed infants, still capable of binding iron (Spik et al. 1978). It is possible that the in vitro inactivation of LF at low pH and reactivation of LF at high pH happens also in vivo. At pH 5.0 and pH 3 LF lost 25 or 90% respectively of its iron binding capacity but recovered nearly all activity after 4 h at pH 7.4 (Law, Reiter 1977). Bullen et al. (1972) demonstrated that E. coli O111 given orally to suckling guineapigs was suppressed, allowing lactobacilli to become dominant in the small and large intestine. This happened within 3-5 days after oral inoculation. In guineapigs on an artificial diet E. coli remained dominant up to 6 days. The suppression of E. coli was attributed to the high LF content of guineapig milk. When suckling guineapigs were also dosed twice daily with haematin for 3 days the count of E. coli O111 was enhanced 10 000 fold in the small and 100 fold in the large intestine compared with the control animals not receiving haematin (lactobacilli counts were not determined). This experiment proved that haematin was an alternative source of iron available for E. coli and LF could not, of course prevent its utilization. Guineapig milk is not only rich in LF but also in LP (10-20 fold compared to bovine milk, Stephens et al. 1979) which makes it therefore unsuitable for an investigation aimed to test the effect of LF as a protective factor. In vitro inhibitory activity of guineapig milk against E. coli for instance was reversed only in samples at 3 days post partum and required reducing agents later in lactation, indicating that the lactoperoxidase system was active (see later).

The multiplicity of protective factors was avoided by Moreau et al. (1983) who inoculated germ free mice with E. coli and fed them a semisynthetic diet with or without LF and/or bovine IgG. After 7 hours the faecal counts were not different. This is not surprising because in suckling piglets the E. coli counts in the large intestine were reduced only 3 days after inoculation (Bullen et al. 1972). Also, intestinal walls of germ free animals differ from those of conventional ones (for instance a germ free lamb inoculated with a non-invasive E. coli of the human serotype O111 became septicaemic and died of a massive invasion by the non-invasive organisms) (unpublished). Their attempt to investigate the effect of LF and IgG in a formula feed failed; however, breast feeding also failed to suppress E. coli, and it is therefore doubtful whether their claim that LF/IgG inhibits E. coli in vitro but not in vivo is justified.

THE LACTOPEROXIDASE SYSTEM (LP-s)

The chemical characteristics and distribution of LP as well as the oxidation products of SCN^- have been dealt with in this symposium elsewhere by Dr. Björck. This section aims to assess its natural occurrence as a defence mechanism in the intestinal tract (IT) and mammary gland, highlighting its possible exploitation in vivo. The application for the preservation of milk will be presented by Dr. Schmekel, Dr. Härnulv.

LP-s effect on lactic acid bacteria (l.a.b.)

Under aerobic conditions l.a.b. generate H_2O_2 which converts LP to the compound I state that oxidizes thiocyanate (SCN^-) to intermediary oxidation product(s) which inhibit sensitive l.a.b. temporarily in milk. It was suggested that the same inhibitory system occurred in saliva (Reiter et al. 1963) because SCN^- was already known to be part of a salivary inhibiting system (Zeldow 1963). The intermediary product inhibited growth, lactic acid production, O_2 uptake and oxidized NADH. However, some strains of the same species (Streptococcus cremoris) were resistant to the LP-s, which had actually led to the identification of SCN^- as the oxidizable product because oxidation of I^- or indican proved to be non-specific (Reiter et al. 1964); LP-H_2O_2-I or indican inhibited sensitive and resistant strains. This became important in ecological systems such as in the intestinal tract (IT) and saliva, where resistant l.a.b. can provide H_2O_2 for the inhibition of catalase-positive organisms such as coliforms (see later). Resistant l.a.b. were shown to possess an $NADH_2$-oxidizing enzyme which reverses inhibition (Oram, Reiter 1966), later renamed NADH-$OSCN^-$-oxidoreductase (Carlsson et al. 1983) after the intermediary oxidation product of SCN^- was identified (and chemically synthesized) by Hoogendorn et al. 1977. Individual enzymes of the glycolytic pathway, in particular hexokinase, were shown to be inhibited (Oram, Reiter 1966), which was later confirmed to be due to the reaction with sulphydryl groups in the enzyme (Adamson, Pruitt 1981). The antibacterial activity of the LP-s has been directly related to the oxidation of bacterial sulphydryls (Aune et al. 1977). Anaerobiosis and reducing agents reverse the inhibition of l.a.b.

LP-s effect on Gram-negative organisms

Coliforms, pseudomonads, salmonellae, shigellae etc. are killed by the LP-s if H_2O_2 is provided exogenously. The killing depends on pH, number of organisms, bacterial species and strains within a species (Reiter et al. 1973/74, Bjorck et al. 1975, Reiter et al. 1976). The latter depends (Purdy et al. 1983) on the growth phase, outer membrane structure determining permeability such as deletion in O antigens (smooth to rough) and sulphydryl levels in the outer membrane (Thomas, Aune 1978). Treatment of E. coli (and S. lactis) with the LP-s causes leakage of K^+ and amino acids indicating damage to the inner membrane (Marshall, Reiter 1980) and inhibits energy dependent glucose uptake (Michelson 1977), amino acid, purine, pyrimidine uptake and hence synthesis of protein DNA and RNA (Marshall PhD thesis 1978, cited in Reiter 1978). Law, John (1981) showed that a non-SH depending enzyme - D-lactate-dehydrogenase - bound to the inner membrane of E. coli or in solution is also inhibited by the LP-s thus preventing the generation of proton motive force across the inner membrane. None of these observations explain the damage to the inner membrane causing leakage (see before), which could not be detected by electron microscopy. The involvement of free radicals appeared to be an attractive approach explaining the membrane damage.

LP-s and free radicals. There are several incentives to investigate whether the killing of bacteria by the LP-s is or is not mediated by free radicals. Adams et al. (1972) reported for instance that the specific free radical $(SCN)_2^-$ can be generated by the reaction of hydroxyl radical ($OH^\cdot$). $(SCN)_2$ formation is one of the proposed schemes for the LP-catalyzed oxidation of SCN^- to $HOCN^-$, $OSCN^-$ O_2 SCN^-, O_3 SCN^- (Hogg, Jago 1970; Aune et al. 1977). Superoxide (O_2^-), $OH^\cdot$ and singlet oxygen ($^1O_2$) are the free radicals involved in the intracellular killing by macrophages and polymorphonuclear leucocytes (PMN). The myeloperoxidase (MPO)-H_2O_2-Cl^- system has been extensively documented as evolving $^1O_2$ via the oxidation of $^-OCl^-$ by H_2O_2 (see review of Clark 1983). It is however difficult to understand why SCN^- oxidation has never been implicated since it is unlikely that such a highly permeable anion does not diffuse from the blood into PMN. Also, macrophages are devoid of MPO but known to ingest avidly horseradish peroxidase used as a marker (Bigger, Sturges 1976). It is therefore very likely that milk macrophages ingest LP - at least in bovine milk). Krinsky (1974) demonstrated that a colourless mutant of Sarcina lutea was much more readily killed by PMN than the pigmented wild S. lutea. He ascribed the

resistance to the carotenoid pigment which acts as a quencher for $^1O_2$ generated by the MPO-H_2O_2- Cl^- system. Piatt, O'Brien (1979) brought evidence that LP-H_2O_2 oxidized Br^- via O Br^- to $^1O_2$ using the same ratio of Br^-:H_2O_2 as had been used for SCN^- (Oram, Reiter 1966). Unfortunately, since then it has been demonstrated that most $^1O_2$ trapping agents are not $^1O_2$ specific and can react with a variety of oxidants including e.g. 2,5 diphenylfuran conversion to cis-dibenzoylethylene, and even chemiluminescence is not specific to $^1O_2$. Kanner, Kinsella (1983) devised a model system whereby LP-H_2O_2-halide (Br^- Cl^- I^-) decomposed β-carotene (based on its discolouration, sensitivity 1 nM). The optimum concentration of H_2O_2 for the activation of the LP-s varied for each halide and for the first time it appears that LP can also oxidize Cl^- but requires the highest conc. of H_2O_2 compared with those for I^- or Br^-. The optimum pH was between 4 and 5. Tryptophan, serine stimulated the Cl^- reaction while cysteine, glutathione, methionine or histidine inhibited it. Antioxidants such as BHT or BHA did not inhibit the system. The authors demonstrated that LP-I or Br^- but not Cl^- at relative high levels of H_2O_2 generate O_2 and β-carotene inhibits or even eliminates the evolution of O_2; they suggest that if β-carotene acted (in their model system) as a quencher then the evolution of O_2 should not be inhibited but increased; but instead β-carotene is destroyed. They propose that β-carotene behaves as a halide quencher or trapper and by its activity prevents the interaction of halide radicals with a second molecule of H_2O_2, thus generating triplet unexcited O_2. It is obvious that the possible destruction of β-carotene ought to be tested now with the LP-H_2O_2-SCN^- system.

Until recently, it was thought that the LP-s does not yield O_2, therefore $^1O_2$ could not contribute to its antimicrobial activity (Thomas 1985). Now for the first time Carlsson (1983) has demonstrated that O_2 is formed but the pH optimum is high at 8.5 as compared with 4.5 for Br^-. While Piatt, O'Brien (1979) showed that chemically synthesized O Br^- evolved $^1O_2$ after the addition of H_2O_2, Carlsson failed to demonstrate O_2 formation from $OSCN^-$. He assumes that the SCN^--dependent catalytic activity of LP depends on enzyme-$OSCN^-$ complexes.

In our own experiments (Reiter, Marshall, Philips, largely unpublished but partly cited in Reiter 1979) were used. Xanthine oxidase-xanthine, recognized to generate O_2^-, the system killed E. coli but the rate of killing was reduced when the superoxide dismutase (SOD) level was increased by culturing in carbohydrate low medium (Hassan, Fridovich 1977); Catalase completely reversed the cidal activity. Increased levels of SOD did not however reduce the rate of killing by the LP-s; it was known that catalase only reverses the LP-s at unphysiological levels, otherwise the LP-s could not operate in milk which always contains catalase (Reiter et al. 1976). This and the virtual failure of the specific conversion of 2,5-diphenylfuran seemed to eliminate the evolvement of O_2^- or $^1O_2$. There remained the clearcut results of the failure to kill pigmented S. lutea by the LP-s, while the white mutant was killed. There is an obvious need to reexamine the LP-s because of the proven evolvement of O_2 and possible β-carotene destruction which is far more sensitive than the malonaldehyde formation which was found to be negative. The pigmented and white mutants of S. lutea, or rather their outer and inner membranes, may well be important in future research on the LP-s.

Practical application of the LP-s.

Under prevailing practices domestic animals are prone to scour because of bacterial and/or viral infections. The incidence and length of scouring varies greatly but is definitely aggravated by formula feeds instead of the host specific milk or suckling the dam. The antibacterial factors in milk are heat labile to varying degrees (Ford et al. 1977) and therefore deprive the animals of natural protection. The human infant is also exposed to intestinal upsets under good hygienic conditions but is endangered under poor conditions prevalent in developing countries. It is therefore necessary to either preserve the proteins, using low temperature-time combinations during manufacture or replace them and readjust their relative concentrations.

Since LP is the principal protective non-antibody protein in bovine milk, the calf was chosen to investigate the in vivo effect of the LP-s on E. coli (0111) (Reiter et al. 1980). The LP-s was activated in raw milk by the addition of glucose-oxidase-glucose, as a source of H_2O_2, and SCN^- to increase the low levels prevailing under present feeding regimes (0.01-0.02 mM increased to 0.125 mM). Calves cannulated either in the abomasum or upper duodenum were given E. coli in heated milk, followed by the LP-s activated raw milk. Samples were withdrawn at intervals and found to be reduced within 30 min of feeding. Addition of a reducing agent completely reversed the cidal effect, proving the LP-s was active in vivo. Before feeding the abomasum contained high concentrations of SCN^- (av. 0.45 mM), obviously secreted but only traces of LP which survived from the raw milk; the LP levels in the abomasum were above 1 u/ml for several hours. Glucose oxidase-glucose could be replaced by MgO_2 releasing H_2O_2 of pH < 6.0 or H_2O_2 producing lactobacilli (e.g. L. lactis). The number of H_2O_2 positive lactobacilli was up to 10^7/ml, fluctuating between 40 and 60% of the total counts of lactobacilli. A more detailed survey (Marshall et al. 1982) of H_2O_2 positive lactobacilli confirmed that L. lactis was the highest H_2O_2 producer in vitro; such lactobacilli colonized the oesophagus, oesophageal groove, abomasum, omasum and duodenum. Isolated lactobacilli differed in their capacity to adhere to tissues in the IT. The secretion of SCN^- in the abomasum and colonization by H_2O_2 producing lactic acid bacteria ought to activate the LP-s when raw milk is fed without any additions. This was indeed confirmed using the same experimental conditions. The count of E. coli was reduced by 99% but by 99.99% in the presence of glucose oxidase-glucose or MgO_2. Feeding heated milk with no LP completely failed to reduce levels of E. coli. Feeding raw milk unfortunately does not guarantee that the LP-s is always activated: we observed great variations in the SCN^- levels in the abomasum and H_2O_2 producing lactobacilli; the latter also depending on the age after birth. The addition of a source of H_2O_2 and SCN^- assures therefore the activation of the LP-s. It also shows that even raw milk can be improved upon. Recently Dellaglio, Sarra (1984) reported that L. lactis is the dominant lactobacillus in the faeces of adults.

Subsequently from 1977 onwards (Reiter et al. 1981) the effect of feeding activated raw milk to calves was assessed by the incidence and length of scouring and increase of weight compared with control animals, receiving only raw milk. The combined trials confirmed over 4 seasons that the LP-s significantly increased the liveweight gains of calves. The higher the overall level of mortality and incidence of scouring of the control calves, the greater was the difference between the calves fed with activated and non-activated raw milk. A total of 251 calves were involved in these trials. Since then a great number of calves (> 1000) have been fed calfstarters based on powdered skim milk in which the LP had been preserved during manufacture (Ewos AB, Sweden); the LP is activated by a source of H_2O_2 and increased levels of SCN^-. The successful trials were conducted by T. Waterhouse, West of Scotland Agricultural College, Ayr and B. Gudmundsson, Ewos AB, Sweden (pers com).

A different approach was developed by Sud-Lait sc, Belgium: LP and LF are isolated from bovine milk and purified. The preparations can then be added to artificial feeds. It has now been demonstrated (N. Kaekenbeck, Institut Veterinaire de Cureghem, Belgium, N. Delahault, Centre d'Economie Rurale, Marloie, Belgium - (pers com) that calves infected with E. coli K_{99} were protected against scouring as judged by the consistency of their faeces and degree of dehydration.

Mastitis. The non-lactating udder can harbour pathogens, hence the wide spread treatment with antibiotics as part of the NIRD mastitis control. There are however many objections against the indiscriminate use of antibiotics. On the basis of a review (Reiter, Bramley 1975) on the defence mechanisms in the bovine udder (colonization in the teatduct, role of phagocytosis and vaccination, lactoferrin and LP-s) the final conclusions at the IDF Seminar on Mastitis, Reading (1975) recommended that ... "research should be accelerated in the important area of specific and non-specific defence mechanisms in the bovine udder". There is little progress to report on the ability of LF to inhibit E. coli mediated by the high concentration of LF and the optimal conditions for the iron binding because of the decline of citrate and increase in bicarbonate

(Reiter et al. 1975; Reiter, Bramley 1975). A number of workers, however, have now confirmed the original suggestion (Reiter et al. 1970) that the LP-s can inhibit Streptococcus uberis in the early dry period (Reiter, Bramley 1975; Roguinsky 1977; Brown, Michelson 1979; McOonald, Anderson 1981, 1983). Mickelson Brown (1983) suggest that the different capacity of S. uberis to recover from the effect of the LP-s after addition of a reducing agent in vitro depends on their lesser ability, compared with S. dysgalactiae, to restore sulphydryl components oxidized by the system. They also confirmed the requirement of cystine, cysteine or glutathione in a synthetic medium but excess of these compounds reversed the LP-s inhibitory effect. This balance between growth promotion and inhibition was previously stressed by Brown, Baetz (1976) for S. agalactiae cultured in whey but not followed up by other workers.

The concept of an active LP-s in the udder was suggested to depend on the presence of O_2 for the generation of H_2O_2 either by the organisms themselves or by the phagocyting leucocytes ingesting fat globules and casein (Russel, Reiter 1975; Russel et al 1977). It is therefore necessary to measure directly oxygen tension on both in the lactating and non lactating udder. Korkonen, Reiter (unpublished cited in Korkonen 1981) detected H_2O_2 in milk at nM concentrations released by phagocyting PMN but only after inactivating catalase and LP by Na-azide. It may be possible to detect H_2O_2 in milk cannulated directly from the udder under conditions which exclude atmospheric O_2. (Previous unpublished determinations indicated 15% O_2 saturation, sufficient to inhibit S. uberis by the LP-s). The best proof of the natural occurrence of oxidation of SCN^- has been established by Pruitt et al. (1983) who detected $OSCN^-$, the oxidation product of SCN^-, in saliva obtained under aseptic conditions by cannulation of the stenson duct of the human salivary gland (parotid). This is up to date the best proof that metabolizing tissue can furnish H_2O_2 for the LP-s and needs to be investigated in the lactating and dry udder.

In the lactating udder the LP-s may yet have another function. The movement of PMN towards a site of infection is directed by a chemical gradient produced at the site i.e. chemotaxis. Lipids derived from the oxidation of arachidonic acid has been shown to be a true chemotaxin for PMN in vitro (e.g. Stephen et al. 1975). More recently it has been shown (Stossel et al. 1974) human blood leucocytes can peroxidize lipids and arachidonic acid is iodinated by the peroxidase - H_2O_2 - halide systems (Boeynaems et al. 1981). It is therefore possible that macrophages in the mammary gland ingesting fat globule and casein as well as LP (see before) mediate the incorporation of SCN^- and/or iodide into the arachidonic acid thus producing chemotaxin for the mobilization of the PMN to infiltrate into the gland.

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Activation of the Lactoperoxidase System as a Means of Saving Milk in Tropical Countries from Early Spoilage

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An approach to the stabilization of the quality of milk, studied closely by Alfa-Laval, is to potentiate the activity of its naturally occurring antimicrobial systems. There are several such systems in milk that help keeping the number of bacteria at a low level. One of these is the lactoperoxidase (LP) system. Pioneering research on the mode of action of this system was done by Dr. Bruno Reiter and co-workers at the National Institute for Research in Dairying, England. Later, the idea of utilizing it for milk quality preservation was tested at Alfa-Laval. The results of these experiments clearly demonstrated the feasibility of retarding the multiplication of bacteria in milk by activation of its lactoperoxidase system.

The lactoperoxidase system consists of the enzyme lactoperoxidase (LP), which catalyzes the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2) to compounds showing specific antibacterial activity. It has now been established that the major oxidation product is hypothiocyanite ($OSCN^-$).

The hydrogen peroxide is rapidly used up in the oxidation of thiocyanate and thus disappears from the milk. During storage of the stabilized milk, the hypothiocyanite is either reduced to thiocyanate or further oxidized to innocuous end products such as carbon dioxide, sulfate and ammonia. The higher the storage temperature, the more rapid this conversion. Upon pasteurization in the dairy, any residue of the antibacterial compound is eliminated.

Thiocyanate is a natural component of milk. The concentration (normally 3 to 5 ppm) is, however, too low for optimum activity of the LP system. Therefore, about 10-12 ppm of thiocyanate has to be added.

Hydrogen peroxide at low concentrations (up to 2 ppm) has been found in freshly drawn milk. After leaving the udder, however, it rapidly breaks down. Therefore, in order to activate the LP system, about 8-10 ppm of hydrogen peroxide has to be added with the aim of making the initial concentration of the two reactants approximately equimolar (0.25 mM).

It should be noted that the concentration of hydrogen peroxide needed to activate the LP system is much lower than the 300-800 ppm used in the "traditional" method of preserving milk with hydrogen peroxide. These two methods must not be mixed up with each other. The mechanism of action is completely different: The use of hydrogen peroxide in high concentrations results in a general oxidation of, for instance, milk proteins, whereas activation of the LP system leads to the formation of compounds with specific antibacterial activity.

Initially, the possibility of extending the refrigerated storage of milk by this method was studied. The results showed that it is quite possible to keep the milk for at least 5 days at 5°C, provided the initial bacteriological quality is reasonably good.

A decrease in the standard plate count (SPC) is often observed after activation of the LP system. The reason for this is that, in a mixed population, some sensitive organisms are killed whereas the other more resistant ones are only inhibited.

Later, the possibility of increasing the keeping quality of raw milk at high ambient temperature was also tested. The results from field experiments in Kenya, Mexico, Sri Lanka and Pakistan show that activation of the LP system is an efficient method to retard the onset of bacterial multiplication.

The experiments carried out so far have shown the following approximate relation between milk temperature and length of the lag-phase before bacterial multiplication starts:

Temperature (°C)	Lag-phase (hours)
30	7 - 8
25	11 - 12
20	15 - 16
15	24 - 26
5	≥ 120

Moreover, these data show that a combination of moderate cooling and activation of the LP system is an alternative that, for instance, could make possible overnight storage of evening milk.

The LP method may thus be utilized in the following ways:

- o Storage and transport of milk at 30°C up to 7-8 hours.
- o Overnight storage in combination with moderate cooling (for instance cooling with water to temperatures below 15°C).
- o Storage up to 5 days in combination with cooling to 4°C.

It is, however, not only the temperature that will affect the extension of the keeping quality. The result is also greatly influenced by the initial level of contamination, the effect decreasing with increasing number of bacteria. Activation of the LP system is thus a method to preserve the quality of good (or reasonably good) quality milk but not a means of concealing or "improving" bad quality milk.

The LP method of milk quality preservation thus offers several advantages over the "traditional" method of using high concentrations of hydrogen peroxide:

- The LP system shows a specific antibacterial effect contrary to the general oxidative effect of hydrogen peroxide at high concentrations.

- The effect of the LP system is largely bacteriostatic implying that it is not possible to "improve" bad quality milk.
- The LP method requires much smaller amounts of activators (8 + 12 ppm) as compared to 300 to 800 ppm for the hydrogen peroxide method. The LP activators are easy to handle and any overdosage can be revealed by simple analysis of residual thiocyanate.

The LP system seems to have a wide distribution in nature. In man and animals, peroxidase enzymes occur not only in milk but also in, for instance, saliva, tear fluid, intestine mucosa, thyroid gland and eosinophils. Bovine milk is rich in LP and it can be diluted up to 100 times without affecting the antibacterial activity of the LP system.

Hydrogen peroxide is probably a rather common metabolite. The concentration is, however, difficult to determine accurately since any hydrogen peroxide formed is rapidly reduced by catalase or peroxidase. To be able to measure hydrogen peroxide production, these enzymes therefore have to be inactivated. In this way, it was established that, for instance, bovine polymorphonuclear leucucytes are capable of producing small amounts of hydrogen peroxide.

Many lactic acid bacteria can also produce quantities of hydrogen peroxide sufficient to activate the LP system. Apart from the starter cultures being used in the manufacture of many fermented milk products, hydrogen peroxide producing lactic acid bacteria have also been isolated from, for instance, the human oral cavity and the calf abomasum.

COCH + STARGRASS PASTURE

The thiocyanate anion occurs naturally in the human body. It is mainly liberated after ingestion of either glucosinolates or cyanogenic glucosides. Vegetables such as cabbage, cauliflower, brussel sprouts and turnips are rich in glucosinolates, which upon hydrolysis release thiocyanate. Concentration of SCN^- up to 100 ppm are not uncommon and even higher values have been reported.

The cyanogenic glucosides primarily release cyanide, which is then detoxified in the human body by conversion into thiocyanate. Cassava, sweet potatoes, peas and beans are examples of important sources of cyanogenic glucosides. In this connection, it should also be mentioned that smokers show considerably increased levels of thiocyanate, particularly in the saliva. This thiocyanate originates from the cyanide in the tobacco smoke.

The components of the LP system can thus all be found in the human body. It is therefore not surprising that the antibacterial oxidation product, hypothiocyanite, has been detected in human saliva.

To sum up, it has been established that:

1) It is possible to increase considerably the keeping quality of milk even at high ambient temperature by activation of its LP system.
2) The components of the LP system are widely distributed in nature.
3) Proof of the <u>in vivo</u> activity of the LP system is accumulating.

Before this method can be used in, for instance, the collection of raw uncooled milk in tropical countries, however, it has to be approved by the relevant national authorities. In Sweden, the National Food Administration has decided that the method can be used in emergency situations when raw milk can not be properly cooled and kept under refrigeration in the usual way. Such a situation will arise if, for instance, there is a breakdown in the electrical power supply. Moreover, the Joint Expert Committee on Food Additives (JECFA) is considering the use of thiocyanate in relation to this method. The use of hydrogen peroxide in concentrations much higher than what is required to activate the LP system has already been accepted by WHO/FAO "when technical and/or economic reasons do not allow the adoption of cooling facilities for maintaining the quality of raw milk".

Finally, the method is also being discussed within the IDF. At the Annual Sessions in 1984, a draft IDF statement (D-Doc 128, 1984) was presented. It is concluded that: "The use of the LP system as an alternative method for preserving raw milk seems to offer several advantages compared to the use of concentrated hydrogen peroxide. This is a completely new approach to preserve raw milk which could offer distinct advantages under certain conditions primarely in developing countries and it is recommended that the FAO/WHO should further investigate the possibilities of the practical use of the method".

At present, the National Committees have given their views and a final IDF statement can be expected in connection with the Annual Sessions in October 1985.

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POSTER 1

The Sporicidal Properties of Vegetable and Fish Oils

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Vegetable oils have been used in preparing food packs for many years. Some fish in oil packs are known where very low heat processes have conferred microbiological stabilities to the packaged product. Dallyn & Everton (1970) reported one such product, Italian canned tuna in oil. Further investigation of this phenomenon has elucidated the probable bactericidal system in this canned product.

Vegetable and fish oils have been examined for their antimicrobial properties using spores of *Clostridium sporogenes* strain PA 3679 as a test organism. The test methods used were (1) spores heated in aqueous extracts of oil at 100°C and decimal reduction times determined and (2) spores on wet antibiotic assay paper discs heated in oil at 100°C for varying lengths of time and cultured for survivors in broth. Both methods showed that many oils had antimicrobial properties in heated water/oil systems.

Exposure of oils to air generally resulted in an increase in sporicidal properties.

Chemical analysis of aqueous extracts using HPLC and other methods showed that hydrogen peroxide was the principal antimicrobial agent. Fish oil (sild), the most unsaturated of the oils examined, produced the highest amounts of hydrogen peroxide. The hydrogen peroxide appeared to be formed from the dismutation of superoxide. Whilst the system was effective for Italian, canned tuna in oil other commercial applications would need careful monitoring to avoid any *Clostridium botulinum* hazards.

Reference

Dallyn, H. & Everton, J.R. (1970). Observations on the Sporicodal Action of Vegetable Oils Used in Fish Canning. Journal of Applied Bacteriology, 33, 603-608.

POSTER 2

Mechanism of Bactericidal Action of the Cationic Peptides Nisin and Pep 5

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The staphylococcin-like agent Pep 5 is a mixture of closely related peptides ($M_r \sim$ 3,500; IEP ~ 10.5). Like other basic peptides of similar molecular properties (e.g. melittin, cecropins, peptides from granulocytes and macrophages) it displays a marked bactericidal activity. The detection of lantionine in Pep 5 hydrolysates demonstrated a strong relationship to the peptide – antibiotic nisin. Both peptides are shown to possess an almost identical mechanism of action.

The first event after Pep 5 or nisin addition to susceptible cells was a rapid efflux of cytoplasmic low M_r compounds such as ATP, amino acids, and K^+. A concomitant drop of the transmembrane potential ($\blacktriangle \Psi$) was observed. This energy depletion along with the efflux of precursors resulted in a complete cessation of biosynthesis. Both peptides required a high membrane potential for proper interaction with the cytoplasmic membrane; deenergised cells were less susceptible.

The *in vivo* resistance of gram-negative bacteria is due to the inability of both peptides to penetrate the outer membrane. Cytoplasmic membrane vesicles derived from *E. coli* as well as lung fibroblast cells were disrupted, whereas leakage of artificial liposomes could not be induced.

Besides membrane disruption, which should be regarded as the primary cause of cell death, an induction of autolysis was observed. Both peptides rapidly liberated murein hydrolases (muramidase/glucosaminidase and endopeptidase/amidase) from staphylococcal cells. The velocity of hydrolysis of purified cell walls by crude enzyme preparations was doubled in the presence of Pep 5.

POSTER 3

Technological Significance of the Endogenous Antimicrobial Substances of Bovine Milk

by W.M.A. Mullan

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Bovine milk contains indogenous antimicrobial agents which can influence the manufacture, or quality, of dairy products. These agents include immunoglobins, the lactoperoxidase, thiocyanate-hydrogen peroxide (LP) system and lysozyme.

Immunoglobins can induce the agglutination of certain strains of lactic acid bacteria which are used as starters in cottage cheese manufacture. Agglutination is a serious production fault. The greater storage life pasteurized of whole milk, and the removal of clostridial spores, by creaming, are beneficial effects of immunoglobin activity in milk. Immunoglobins also have the potential, in some methods, to interfere with antibiotic detection.

LP system activation, by the addition or generation of H_2O_2, can be used to increase the shelf life of raw uncooled milk or to improve the quality of certain milk products produced from milk contaminated with psychrotrophic bacteria. Because some lactic acid bacteria are inhibited by this system, starters which are used to produce fermented products from raw or pasteurized milk must be resistant to the LP system or not produce H_2O_2.

The technological significance of native milk lysozyme is not well understood. Lysozyme has been implicated as the inhibitor responsible for false positive results for antibiotics in mastitis milk.

Further research is required to develop reliable tests for preventing agglutination of cottage cheese starters, to evaluate the potential of the LP system for improving the quality of dairy products and to identify the inhibitor(s) present in mastitic milk.

POSTER 4

Inhibitors in Abnormal Milks

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Samples of colostrum, mastitic milk and dry secretions, taken from cows not previously treated with antibiotics, were tested by an agar diffusion technique using *Bacillus stearothermophilus, Bacillus subtilis* and *Micrococcus lysodeikticus* as test-organisms. Zones of inhibition were observed with the 3 organisms after 2 successive periods of incubation at different temperatures.

At least 2 inhibitory substances were detected. One of them, thermostable, frequent in colostrum and dry secretions, gave a zone of inhibition after the first period of incubation, while another one, thermolabile, produced a zone of lysis after the second period of incubation. The latter was found particularly in mastitic milk but also in colostrum and dry secretions. The first substance differs from the known inhibitors of normal milk, lactoperoxidase and lactoferrin. The substance causing lysis resembles lysozyme but differs markedly from egg white lysozyme (by its thermostability, pH stability, action on various micro-organisms). It probably originates from blood, as the same lytic substance seems to be present in cow's blood serum.

When testing milk for antibiotics by the agar diffusion technique with *B. stearothermophylus* or *B. subtilis* as test-organisms, samples of milk containing colostrum or dry secretions may show zones of inhibition due to the thermostable substance.

In vitro a bacteriostatic effect against the *S. aureus* strain used occurred only at an apolactoferrin concentration higher than 50 mg/ml. Such dosage could never be observed during lactation, dry period and after experimental infections.

POSTER 5

Interactions Between Phagocytotic Activities of Polymorphonuclear Leucocytes and other Antibacterial Factors in Bovine Milk

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The phagocytic activity of polymorphonuclear leucocytes is one of the important factors in the natural defence system of the mammary gland. Investigations on changes in phagocytotic activities and in other related defence factors were performed after experimental infections/irritations. In addition the dependency of phagocytotic activity from different defence factors and the cell count pre-irritation were studied.

Experimental irritations of the mammary gland resulted in a significant increase of somatic cell count, bovine serum albumin, IgG, IgG_2 and IgM in milk. The cell count and lactoferrin concentrations were significantly influenced by the individual cow. In comparison to milk, collected pre- and post irritation, blood serum was the only bactericidal acting incubation fluid. In milk, collected after irritation, a significant correlation between growth of *Staphylococcus aureus* and content of bovine serum albumin, IgG_1 and IgG_2 was found. The bactericidal activity of blood and milk neutrophils, isolated after irritation, increased significantly from milk pre- to milk after irritation. Significant relations between bactericidal effects of milk neutrophils and proteins related to the defence system were only found for IgA. Increasing levels of lactoferrin reduced killing of *S. aureus* by milk neutrophils.

The phagocytotic activity of the milk neutrophils, isolated from cows with a higher cell count pre-irritation, was significantly stronger when compared to neutrophils isolated from cows showing low cell counts.

POSTER 6

The Lactoperoxidase System in Milk Replacers for Calves

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Young dairy calves in the UK, whether dairy replacement or for beef production, are most commonly reared for 5 to 12 weeks on milk replacer with cereal concentrate supplementation. During this period mortality is high, frequently due to entiritis and associated diseases. Reduced animal performance is also typical. The lactoperoxidase system has been investigated as a means of increasing performance in young calves. Commercial calf milk replacers only rarely contain low or detectable levels of lactoperoxidase. Practical means of incorporating the lactoperoxidase system within calf milk replacers have therefore also been developed. Fourteen calf trials have been carried out plus a large scale study on commercial dairy farms.

In initial trials fresh whey or an ultrafiltrated whey concentrate was used as a source of lactoperoxidase. Subsequent trials used a low heat-treated skim milk powder at inclusion rates of up to 30% of milk replacer. Thiocyanate, as sodium thiocyanate, was included at 0.25 mM. Magnesium peroxide was used as a source of hydrogen peroxide. A mineral/SCN/MgO_2 premix was commercially produced and included in a dry mixed milk replacer which also contained the lactoperoxidase from inclusion of low heat-treated skim milk powder. Control milk replacers containing skim milk with higher (medium) heat treatment and with control levels of magnesium were also produced. All replacers were tested for lactoperoxidase, thiocyanate level and additionally the activity of the system was tested *in vitro* against *E. coli.* (NCDO 904). Of the 14 trials, in 12 there were positive effects of the active lactoperoxidase system treatment upon calf growth and concentrate intake and in most trials calf diarrhoea was reduced. In the commercial trial with 396 calves the active lactoperoxidase system led to an overall positive benefit in growth rate to 4 weeks of age.

POSTER 7

Antibacterial Action of the Lactoperoxidase System on *Campylobacter jejuni* in Cow's Milk

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From the literature it can be learned that *Campylobacter jejuni* rapidly decreases in viable numbers when inoculated in raw cow's milk. The present study established that this may be caused by the antibacterial action of the lactoperoxidase system (LPS) in the milk. Inactivation of the system by heat treatment of milk, by adjustment of the pH of milk to 7.5, or by addition of cysteine to milk allowed *C. jejuni* to survive, whereas stimulation of the system by adjustment of the pH of milk to 5.5 or by addition of thiocyanate and hydrogen peroxide caused an increased reduction in viable numbers. This reduction also became distinct when lactoperoxidase (LP) was added to heat-sterilized milk, whereas survival of the organism was much enhanced in UF permeate of milk. The rate of reduction in milk was greater at 30°C than at 7°C.

In consequence of these findings, the isolation of *C. jejuni* from milk is more likely to succeed if the milk is analysed rapidly after drawing, or when LPS is inactivated directly after sampling by increasing the pH or by addition of cysteine.

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POSTER 8

Str. uberis Mastitis and the Lactoperoxidase System

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The lactoperoxidase enzyme present in milk, together with thiocyanate anions and hydrogen peroxide, provides a non-specific antibacterial system against enteric pathogens, psychrotrophs and species of streptococci. As up to 40% of new dry-period udder infections are caused by *Str. uberis*, the efficacy of the LP system has been examined in mammary secretions collected from the lactating and the dry gland.

LP, SCN^-, pH and somatic cell counts were measured in secretions collected 14 d before drying-off, 7 and 21 d dry and 3-18 d post calving. Inhibitory activity against *Str. uberis* was measured and susceptibility of the udder to infection tested by intramammary infusion of 250 cfu at the above stages. LP, SCN^-, pH and somatic cell count increase during involution and fell post-calving. Secretions were inhibitory towards *Str. uberis* before drying off, 7 d dry and post calving, but not at 21 d dry. Heating secretions to 75°C for 30 min destroyed the inhibitory activities which could then be restored by addition of LP and a source of H_2O_2. Growth of *Str. uberis* in the secretions correlated with intra-mammary susceptibility. Challenges at 14 d before drying-off, 7 d and 21 d dry and post calving resulted in 43.8, 25.0, 81.3 and 37.5% of quarters becoming infected.

POSTER 9

FPLC—Analysis of Antibacterial Components of Bovine Milk: Lactoperoxidase, Lactoferrin

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Three non-specific antibacterial agents in bovine milk: lactoperoxidase, lactoferrin and lysozyme, are basic proteins (pI = 9-10). The conventional preparation methods for these proteins therefore often include initial ion exchange chromatography steps on cation exchangers.

In order to study the appearance of these proteins in samples of bovine milk, a rapid and reliable separation of all three in one step is desirable.

By use of the new FPLC-technique and Mono S^R cation exchange column (Pharmacia AB, Uppsala, Sweden) it was possible both to quantify and qualitatively characterize these three protein components in one single chromatographic run.

The identification of each component was done by determination of enzyme activity, analysis of iron content and spectral properties. Reference substances were run under identical conditions.

Lactoperoxidase and lactoferrin appear close to each other on SDS-PAGE due to their similar molecular weights. No component was found, either on a large-scale separation on CM-cellulose or with FPLC-analysis, which could be identified as a lactoperoxidase-molecule without heme-group.

Bovine lysozyme was prepared on CM-cellulose and its activity was analysed with standard techniques using *Micrococcus lysodeikticus* cells. The purified lysozyme was analysed for antibacterial properties and run as reference substance on FPLC. Its position was compared with that of the human enzyme.

POSTER 10

High Performance Chromatographic Separation of Bovine Lactoferrin & Lactoperoxidase

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Chromatographic separations of lactoferrin (LF) and lactoperoxidase (LP), isolated from bovine milk were studied on high performance reversed-phase and high performance cation-exchange columns. A preliminary ion-exchange step using the cation exchange resin, Bio-Rex 70 (Bio Rad Lab, California), at pH 7 was necessary to both concentrate these minor proteins and to separate them from the major acidic proteins of milks and wheys. The basic proteins, eluted by sodium chloride from Bio-Rex 70, were then separated on a RP-300 column (Brownlee Aquapore, Santa Clara) using 6% formic acid with a gradient of a 6% formic acid in 2-propanol Protein recoveries for LF were greater than 95%, however, no LP activity was recovered. Some LP activity could be recovered if the separation was conducted at pHs greater than 4.5 but peak shapes were poor and LF recoveries were low.

Milk samples were analysed by passing them through the Bio-Rex 70 at their natural pHs. Values obtained for LF were about 100 to 150 mg per litre. Wheys prepared from the same milks contained about half the concentration of LF, suggesting some binding to casein.

Chromatography of LF and LP on the cation-exchange column, Mono S (HR 5/5, Pharmacia) was achieved using phosphate buffer at pH 7 containing 10% 2-propanol with a sodium chloride gradient. Protein recoveries were good but the specific activity of LP recovered was low (15%).

POSTER 11

The use of Lactic Organisms in the Control of Pseudomonads in Chilled Dessert Products

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As part of a broader program to investigate ways of extending the shelf life of chilled dairy products, the possibility of whether the antimicrobial activities of lactic organisms against the growth of psychrotrophic pseudomonads could be applied to the protection from spoilage of chilled dessert products, and thereby contribute to shelf life extension, was examined.

Dessert products, whose formulation containing cream, skimmed milk powder, gelatin, corn flour starch, sucrose (or dextrose), and, in some desserts cocoa, was based on a commercial recipe, were inoculated with spoilage psychrotrophs and lactic organisms. The organisms used were strains of *Pseudomonas fluorescens,* psychrotrophic *Micrococcus* species, and Flavobacteria; and *Leuconostoc mesenteroides, Lactobacillus plantarum, Streptococcus lactis,* "lactic ferment" (Hansen CH-01 mixture) and *Leuconostoc cremoris* (Hansen strain). The population and pH changes in the inoculated dessert products were followed during static storage at 5°C.

Leuconostoc mesenteroides and *Lactobacillus plantarum* could not control the pseudomonads in desserts, although this possibility was indicated in screening tests on agar media. *Streptococcus lactis* retarded the growth of *Ps. fluorescens* in dessert product without pH decrease; mixed "lactic ferment" (CH-01 Hansen) was a potent inhibitor while at the same time rapidly lowering the pH of the dessert. The most promising of those tested was *Leuconostoc cremoris* (Hansen) which as an initial inoculum 9% v/v could prevent the *Pseudomonas* population attaining more than 10^4/ml after incubation of the inoculated dessert under refrigeration for 13 days. It was also shown that *L. cremoris* (Hansen) was more effective in chocolate rather than other desserts.

POSTER 12

Bacteriological Quality of Raw Milk Stabilized by Activation of its Lactoperoxidase System

by M. Zajac

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Microbiological quality of raw milk depends on the initial contamination, temperature and time of its storage. In practice, the microbiological quality of raw milk is maintained by its cooling and keeping at low temperature, optimally at 4°C. Recently studies have been carried out on the utilization of LP system for microbiological stabilization of raw milk. The laboratory experiments (1, 3, 5) and field trials (2, 4, 6) confirmed the suitability of LP system for microbiological stabilization of raw milk.

The purpose of the present work was to analyse the quantitative changes in the selected groups of bacteria in LP-stabilized milk during its storage at 10°C.

Materials and Methods

The material for the experiment was raw milk. Microflora of each milk sample was determined 3 times, i.e. the first day (t_o) and after 24 (t_1) and 48 h (t_2). After the first analysis, to each 1 l of milk, in the experimental lot 0.0162 g Na SCN and 0.0325 g Na_2CO_3 x 1,5 H_2O_2 were added. The milk in the experimental and control lots was stored at 10°C. Experiment was repeated 20 times. In each repetition the following counts were carried out: standard plate, coliform, psychrotroph, thermoduric, lipolytic and proteolytic.

Changes of count of particular groups of bacteria in milk, estimated in accordance with the following formulas:

$t_1 / t_o = a$, $t_2 / t_o = b$ and $t_2 / t_1 = c$, depending on the presence or absence of active LP system, were subjected to the statistical analysis. The significance of differences in changes of bacterial count between both examined lots were compared by Fischer - Snedecor test.

Results and Discussion

The results of the analyses of hygienic milk quality of both examined lots, as mean values from 20 samples, have been presented in Table 1.

The hygienic quality of particular samples of fresh milk, evaluated by the examined parameters was very different. Milk samples of the best quality contained about 10^4 bacteria/ml and the samples of the lowest quality possessed as many as 10^7 bacteria/ml.

Standard plate count (SPC) in the control milk, during 48 h observations, was subjected to almost 12-fold growth. In the stabilized milk, the efficient inhibition of growth of SPC was observed only during 24 h. The calculated "a" coefficient was 0.59. The "b" coefficient for total contamination of this milk amounted to 5.11.

Coliform count in the control milk revealed fast increase during 48 h observations, "b" coefficient was defined by the value of 19.25. In the stabilized milk, drop in coliform count, expressed by "a" coefficient is 0.14 and "b" coefficient is 0.55. These values are an evidence of strong and long-lasting activity of LP system in relation to coliforms.

Psychrotroph count in the control milk, during 48 h increased almost by 20 times. In spite of strong growth at bacterial group in the control milk, active LP system inhibited significantly development of psychrotrophic bacteria in the stabilized milk. The "a" coefficient was 0.74 and "c" coefficient amounted to 2.85.

Thermoduric count in the control milk, during 48 h observations, was insignificantly lowered. Under these circumstances, the observed drop in the count of that bacterial group in the stabilized milk ("b" = 0.21), through very high, cannot be explained by the activity of LP system only. It seems that thermoduric bacteria in mixed population at the examined temperature, do not find favourable conditions for growth.

Lipolytic count in the control milk increased 8.29 times during 48 h. At the same time in the stabilized milk the count of this bacterial group decreased. The "b" coefficient related to this drop was 0.93. These values show evidently a strong and long-lasting activity of the LP system in relation to lipolytic bacteria.

Proteolytic count in the control milk did not show a significant growth. In the experimental lot, active LP system inhibited growth of this bacterial group only for 24 h ("a" = 0.65). After 48 h, "b" coefficient reached as high as 4.65.

The results obtained are an evidence of strong but short-lasting activity of the LP system in relation to proteolytic bacteria.

The statistical analysis revealed high significance of influence of the active LP system on the growth of all the examined bacterial counts during 24 h. For the 48 h period, high significance of differences was demonstrated only for coliforms, psychrotrophs, thermoduric and lipolytic counts.

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TABLE 1. Bacteriological quality of untreated and LP-stabilized milk during 48 h of storage at 10°C /mean value/.

Type of count	LP-system	Viable count /CFU/ml/ after various times /h/ 0	24	48
Standard Plate Count	no	3.65×10^6	1.15×10^7	2.63×10^7
	yes	2.36×10^6	1.35×10^6	1.23×10^7
Coliform	no	1.20×10^4	6.65×10^4	2.31×10^5
	yes	2.68×10^4	3.70×10^5	1.49×10^4
Psychrotroph	no	4.84×10^5	2.51×10^6	7.15×10^6
	yes	3.52×10^5	2.46×10^5	1.62×10^6
Thermoduric	no	1.01×10^5	7.63×10^4	7.77×10^4
	yes	2.11×10^5	8.23×10^4	2.16×10^4
Lipolytic	no	1.00×10^5	4.51×10^5	8.76×10^5
	yes	4.64×10^5	2.38×10^4	8.12×10^4
Proteolytic	no	1.62×10^5	3.50×10^5	5.78×10^5
	yes	1.40×10^5	8.98×10^4	5.31×10^5

The LP—System as a Mean for Milk Storage

by J. Schmekel[1] and G. Härnulv[2]

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The lactoperoxidase (LP) system has been studied in field trials in different tropical countries.

To stabilize the milk, 12 ppm sodium thiocyanate in liquid form and 10 ppm sodium percarbonate as powder, the H_2O_2 generator, were added to every litre milk just after milking. That means that the natural levels of SCN^- (3-5 ppm) and of H_2O_2 (2 ppm) were increased to about equimolarity, i.e. 0.25 mM.

In these trials, milk quality was measured as percentage of milk samples rejected certain hours after LP-stabilization. A ten-minute resazurin test was used as indicator.

	Ambient temp.		Percentage rejected milk after (hours) 2	5	7	10
Srl Lanka	30	Control	20	95	100	100
		LP	0	0	0	0
Pakistan	30	Control	0	15	70	100
		LP	0	0	0	0
	45	Control	0	15	100	100
		LP	0	0	0	0

The results clearly show the possibility of using LP-stabilization as a mean to preserve milk under tropical conditions without cooling.

The LP-system is temperature-dependent. The total lag-phase is approximately:

Temp (°C)	Lag-phase (hours)
5	~120
15	24-26
20	15-16
25	11-12
30	7-8

The effect of the LP-system is dependent upon the original milk quality. A tenfold increase of a bacterial number of 800,000 in raw milk decreases storage time from 12 hours and more to 8 hours at an ambient temperature of about 30°C. Control samples were spoiled at a time period of about 5 hours shorter (Ganegoda).

From different results available it is possible to conclude that the activation of the LP-system enables:

- Storage and transportation of milk up to 8 hours at 30°C.
- Overnight storage at max. 15°C.
- Storage of milk up to 5 days at 4°C.

Problems with milk collection such as:

- high ambient temperature,
- long transportation time,
- lack of electricity for cooling,
- uncontrolled use of various chemical preservatives, and
- adulterations

seem possible to solve.

POSTER 14

The Lactoperoxidase System: a Defence System of Nature with Importance for Animal Health

by Ms B. Gudmunsson
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The Lactoperoxidase System (LPS) is one of the naturally non-specific antibacterial systems occurring in milk and also in other important biological fluids.

Increasing knowledge of the LPS has enabled the system to be activated on an adequate biological level by a special method, developed by EWOS AB, Sweden. This activation of LPS has led to a number of feeding experiments with young calves, since much of the present research deals with an activated LPS in milk replacers.

The results from 7 different experiments conducted so far in the Scandinavian countries, comprises 275 calves.

LPS stimulates the appetite !

The calves were individually fed in all trials except one (Denmark) and calf health was carefully observed. There were some differences in the calf environment and the feeding regime, but in almost all experiments a definite trend has been observed for calves fed a milk replacer containing an activated LPS.

When the calves had free access to concentrate, the trend was for the "LPS-calves" to have a higher concentrate consumption and a better live-weight gain.

The reason for this appetite stimulation probably is that the LPS stabilizes the ecological environment in the gastro-intestinal tract.

Calf health

In several experimental cases it was judged that the LPS was of importance for the calf health. The conclusion is that the LPS fills the "immunological gap" during the first weeks of life, while the calf is developing its own defence system.

LPS in the future

The promising results from experiments with LPS in calf feeding, hopefully give reason for more research on LPS, also as concerns other domestic animals.

Furthermore, it is important to identify how today's feeding methods interfere with natural defence mechanisms.

Another research aim: LPS against Salmonella infections.

PROBIOTIC instead of antibiotic.

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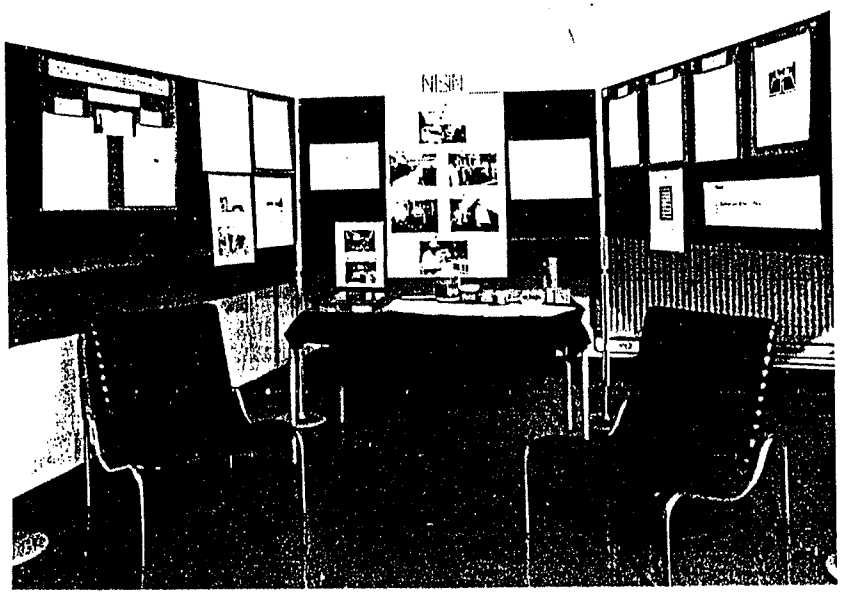

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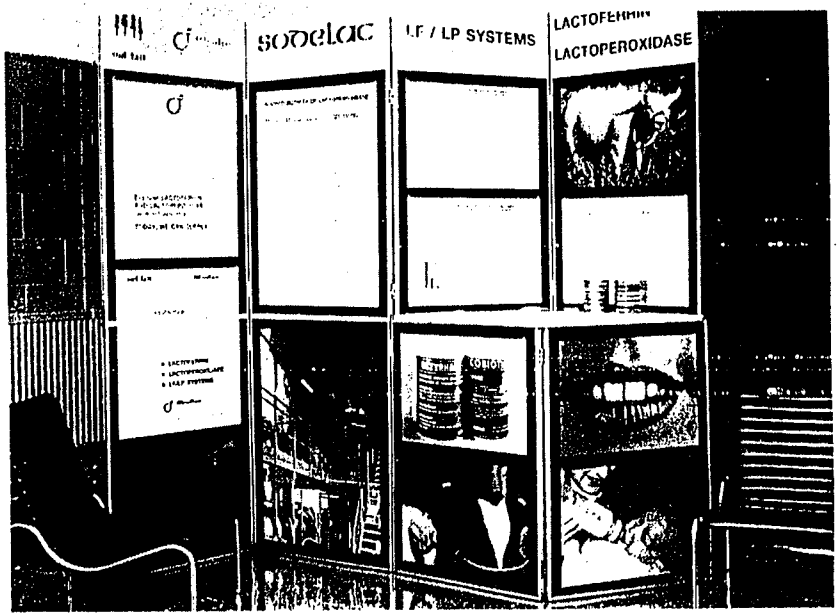

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APPENDIX C

Protective Proteins in Milk

Biological Significance

And exploitation

International Dairy Federation, Brussels, Belgium 1985

LLETIN OF THE INTERNATIONAL DAIRY FEDERATION N° 191/1985

BULLETIN

PROTECTIVE PROTEINS IN MILK-
BIOLOGICAL SIGNIFICANCE AND EXPLOITATION

LYSOZYME, LACTOFERRIN, LACTOPEROXIDASE, XANTHINEOXIDASE

INTERNATIONAL DAIRY FEDERATION
BULLETIN No 191 / 1985

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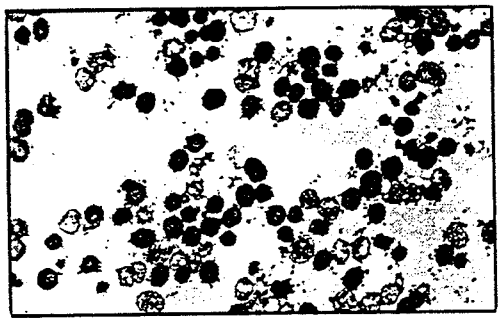

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CONTENTS

The IDF Bulletin for 1984 includes the following publications:

168 – Somatic cell counting in milk

169 – Use of unmarketable milk on the farm

170 – World market for butter

171 – Addresses in Oslo on cheesemaking (MM Lenoir, Oterholm, Schipper) in English and French

172 – World market for ice-cream

173 – Consumption statistics for 1982

174 – Balance tanks for dairy effluents

175 – Interim cheese market report

176 – Computerized bulk milk collection (Toronto Workshop of September 1983)

177 – Quality assurance (means of promoting efficiency in dairying) in English and Spanish (proceedings of a Seminar in Valdivia, Chile, November 1983)

178 – General Code of Hygienic practice for the dairy industry

179 – Fermented milks (Avignon Seminar, May 1984) in English and French

180 – Inventory of IDF/ISO/AOAC methods of analysis

181 – Methods for detecting mixtures of cow's, ewe's and goat's milk

182 – Thermization of milk; on farm use of membrane systems

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THE BIOLOGICAL SIGNIFICANCE AND EXPLOITATION OF THE NON–IMMUNOGLOBULIN PROTECTIVE PROTEINS IN MILK : LYSOZYME, LACTOFERRIN, LACTOPEROXIDASE, XANTHINEOXIDASE

Bruno Reiter, National Institute for Research in Dairying,, University of Reading, U.K. (retired)
Hon. Research Fellow Department of Pediatrics, University of Oxford, U.K.

DEDICATION

I would like to dedicate the work reported in this paper to the late Professor J.W.G. Porter, Director of the National Institute for Research in Dairying, who gave me so much support and encouragement.

INTRODUCTION AND PRECIS

Increasingly the medical profession is recommending for health reasons changes in people's diet. High on the list of changes is the restriction of the intake of foods containing saturated fats which includes, of course, dairy products. It is, therefore, to be expected that the dairy industry is facing a decline in sales; no doubt, the dairy industry will take the appropriate measures to deal with this problem.

Milk, however, does not only contain nutrients but like other biological secretions – saliva, tears, bronchial and nasal secretions, pancreatic fluid, etc. – a complete immune system. This consists of leucocytes, antibodies, complements, lysozyme, lactoferrin, lactoperoxidase, xanthine oxidase, etc. Incidentally, the same or similar enzymes are involved in the intracellular killing by leucocytes. While the antibodies are specific, depending on the antigenic experience (infection) of the dam, the non-antibody protective proteins are present *a priori,* augmenting and complementing the immune system.

Similar to the nutritive composition, the concentrations of those proteins vary according to species. While it is recognized that the concentration of the nutrients varies according to the needs of the offspring – maturity at birth, rate of growth, digestive system and environment it is born into – it is not known what determined the variation of the non-antibody protective systems; they may be related, like the class of immunoglobulins, to the evolutionary status of the animal species.

Bovine milk is specifically "designed" to the needs of the calf only and has to be adjusted nutritionally when fed to human infants or the newborn of other species. Similarly, it begins to be recognized that bovine milk ought to be also adjusted immunologically particularly as the protective proteins are heat sensitive and usually destroyed during manufacture. Preservation of these proteins or their replacement through isolation from raw milk are a feasible aim and is the concern of this review.

In the rest of the introduction a precis will be presented: for detailed information the remainder of this contribution is based on a draft for a chapter in Vol. 3: Developments in Dairy Chemistry, editor P.F. Fox, Applied Science Publishers Ltd, London. The editor and the publishers have kindly given permission for this use of the text.

Human milk is rich in lactoferrin and lysozyme in contrast to bovine milk in which lactoperoxidase (and xanthine oxidase) are the main protective proteins. Lactoferrin and lysozyme inhibit a wide range of bacteria and are more effective in synergy with a certain class of immunoglobulins (secretory IgA) which also occurs in other secretions and in the intestines. While the *in vitro* effect is well established, the role of lysozyme, lactoferrin and the lactoperoxidase system in man and animal begin to emerge. So far, investigations had to be restricted to small laboratory animals because the enzymes could not be isolated from milk on a sufficiently large scale to permit experiments on domestic animals and man. Recently a method for large scale production of milk enzymes has been established which allows extensive trials. Preliminary results feeding calves with lactoferrin and lactoperoxidase are promising.

The lactoperoxidase system consists of the enzyme lactoperoxidase, hydrogen peroxide and thiocyanate. It is capable of suppressing growth of sensitive lactid acid bacteria (e.g. in starters) and kill bacteria such as coliforms, salmonellae, etc. The system can be activated in raw milk by adding extremely low concentrations of thiocyanate (~ 10 ppm – milk contains normally only up to ~ 4 ppm) and hydrogen peroxide (~ 8 ppm). Through these

(*) *The present paper was submitted as a special address to the meeting of Commission F during the IDF Sessions in Prague, on 17 September 1984.*

additions refrigerated milk can be preserved for several days because the psychrotrophic spoilage bacteria can be suppressed. Uncooled milk can also be temporarily preserved by inhibiting its lactic acid bacterial flora. This method has been found to be useful in developing countries where adequate cooling facilities are lacking. Depending on the ambient temperatures, milk can be prevented from going sour from 8 - 18 hours. Sucessful trials were conducted in Kenya, Mexico, Sri Lanka, Pakistan and India. This method is now under consideration by the World Health Organization and the Food and Agriculture Organization.

The lactoperoxidase system occurs naturally in saliva and in the stomach. For calves it was shown that raw milk or specially prepared calf starters based on milk can provide the enzyme; thiocyanate is secreted in the stomach and hydrogen peroxide generated by lactic acid bacteria colonizing the intestinal tract. Under favourable conditions, the lactoperoxidase system is naturally activated. It was shown to kill coliforms and permit a desirable lactid acid flora to become dominant. Complementing, however, milk with thiocyanate and hydrogen peroxide insured the reduction of ingested coliforms by 99.99%. Large scale feeding trials proved that feeding the active system to calves, increases appreciably live weight gains and reduces scouring.

Since human milk contains only appreciable amounts of peroxidase two weeks post partum, the main source of lactoperoxidase in the human infant is saliva. Investigations are now in hand to survey the level of salivary peroxidase and thiocyanate in premature and full term babies and assess the proportion of hydrogen peroxide producing lactic acid bacteria in the intestinal tract.

The antibacterial systems in milk have been studied at different times from 1963 onwards at the National Institute for Research in Dairying, U.K. but their exploitation began only recently because the dairy industry was reluctant to consider their application and FAO rejected the suggestion to employ the lactoperoxidase system to preserve milk in developing countries. The suggestions to fortify baby milk preparations with some of the protective proteins were also rejected at the time.

It is perhaps significant that only scientists from outside the dairy industry recognized the potential use of the protective proteins, both in preserving milk and feeding calves, infants and adults. Pediatricians and gastroenterologists are now seriously interested in "antibacterial milk" and milk products. The adverse image for milk may yet be reversed.

Discovery of protective proteins in milk

"------ apart from phagocytosis and the bactericidal power of blood fluids, the tissues and secretions have also primary antiseptic properties" ---- "its (lysozyme) importance in connection with natural immunity does not seem to be generally appreciated" (1, 2).

The above quotations refer to the first defined non-immunoglobulin antibacterial agent lysozyme and express the disappointment with the poor response of the medical profession. Today, so many years later, the general attitude is beginning to change.

The bacteriocidal and bacteriostatic properties of milk were observed at about the same time as those of blood (e.g. 3), but, naturally, have not attracted the same degree of attention. However, medical workers were interested in the spread of diseases such as cholera, salmonellosis, "scarlet fever" *(Streptococcus pyogenes)* through consumption of contaminated milk (e.g. 4). Veterinarians investigated the anti-streptococcal activity of milk in relation to inflammation of the bovine udder (e.g. 5); dairy bacteriologists were concerned with the sporadic cessation of lactic acid production by starter streptococci in cheese making (other than those caused by bacteriophage) and the keeping quality of milk (6, 7).

Those inhibitors were referred to by the trivial name "lactenins" until they were partly identified as agglutinins (specific antibodies to streptococci) or associated with lactoperoxidase (8, 9). Eventually, the complete "lactoperoxidase system" was demonstrated to include H_2O_2 (10) and thiocyanate (11) *.

Lysozyme – "the extraordinary bacteriolytic agent" – was demonstrated to occur in tissues, secretions and egg white (1, 2) and in human but not in bovine milk (12). Fleming (2) and Bordet (12) using a more sensitive assay organisms *(Micrococcus lysodeicticus)* detected low concentrations of lysozyme also in bovine milk, particularly milk from Jersey cows. In the same paper, Fleming (2) cites the findings of Rosenthal & Lieberman (13) that lysozyme could be detected in the faeces of breastfed but not of "artificially fed" infants. He concurred with the suggestion that the lysozyme of human milk has "a marked influence on the bacterial flora of the intestine of infants". This appears to be only the second instance of maternal milk being considered beneficial to the newborn, other than as a nutrient. Ehrlich (14) was first to suggest that mouse milk could transfer passive immunity (against ricin) to the off-spring and that antibodies may be derived from mammalian tissue and pass unaltered through the digestive tract.

The possible *in vivo* role of lysozyme remained largely ignored and the enzyme became a tool in biophysics, chemistry, physiology and clinical medicine. It is now time to reassess its biological role. While the lytic activity of lysozyme was described first and the enzyme purified much later (15), lactoferrin was first isolated as an iron-containing red protein from human milk by several workers simultaneously (16 - 18) and later from other

---

* *Footnote: The demonstration of the "complete" lactoperoxidase system was erroneously attributed to references 9 and 235 in the F–Doc 96, 1983.*

secretions and leucocytes (see Ref. 19). The antibacterial activity of lactoferrin was demonstrated simultaneously in bronchial mucus against *"Staphylococcus albus"* (20) and in bovine milk and the secretion of the non-lactating bovine udder against *Bacillus stearothermophilus* (21, 22).

Lactoperoxidase was purified from bovine milk (23) long before the lactoperoxidase/H_2O_2/thiocyanate system was recognised. The peroxidative activity of human milk is much lower than that of cow's milk and is now known to be derived from polymorphonuclear leucocytes (myeloperoxidase) and peroxidase (24). It is very likely that the low peroxidative activity of human milk is supplemented by salivary peroxidase which is more akin to lactoperoxidase (25). Since bovine milk lacks high concentrations of lactoferrin and lysozyme but is rich in lactoperoxidase, it is not surprising that the role of the lactoperoxidase system can be best investigated in calves.

Bovine, but not human milk, is also rich in xanthine oxidase, mainly associated with the fat globule membrane. This molybdenum- and iron-containing enzyme was first purified and characterized by Ball (26). In vivo, its primary function is the catabolism of purines by converting them to uric acid; in this reaction, both superoxide (O_2^-) and H_2O_2 are generated. Normally, milk does not contain purines but milk cultured with lactic acid bacteria contains variable but appreciable amounts of acetaldehyde. Xanthine oxidase reacts faster with aldehydes than with purines and the H_2O_2 generated becomes bacteriocidal and/or a source for the activation of the lactoperoxidase system (unpublished, see later).

Summing up, there is little doubt that the total antibacterial effect of the non-immunoglobulin proteins and immunoglobins is greater than the sum of each acting individually because of their interaction (see section on synergism). These proteins are not restricted to milk but occur in all secretions bathing mucous membranes.

The functions of the protective milk proteins can be two-fold; protection against infection of the mammary gland and/or protection of the newborn. The latter appears to be more important or at least we have more evidence for their *in vivo* activity. For historic accounts and reviews see (27-38).

LYSOZYME (MURAMIDASE) (N–ACETYLMURAMYL HYDROLASE, E.C. 3.2.1.17)

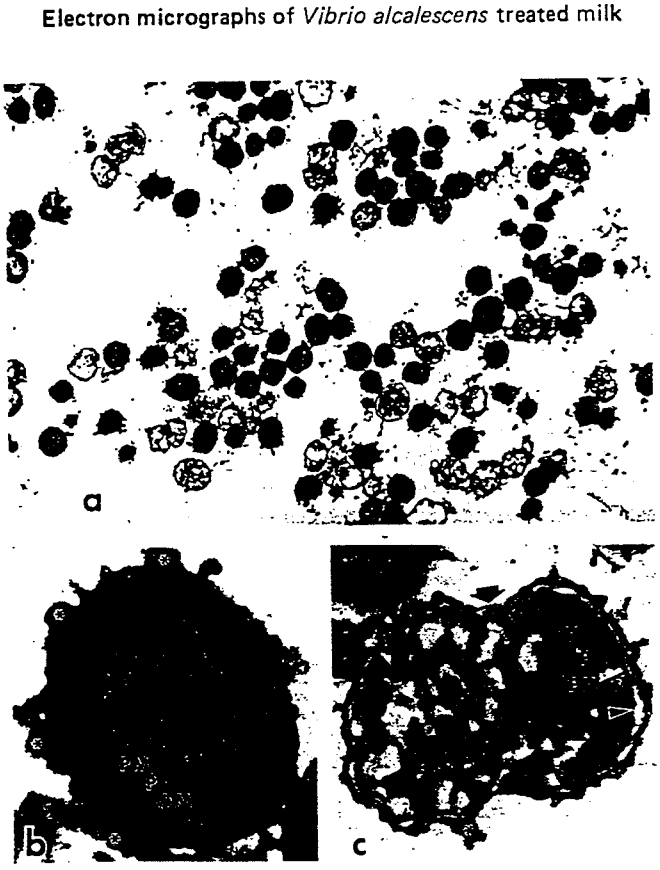
Lysozyme (for review see Ref. 39) has recently attracted renewed interest as a component of the antibacterial systems of milk which influence the intestinal bacterial flora of the neonate, possibly also affecting the general immune system. Lysozyme also inhibits the outgrowth of spores and their vegetative cells (see later).

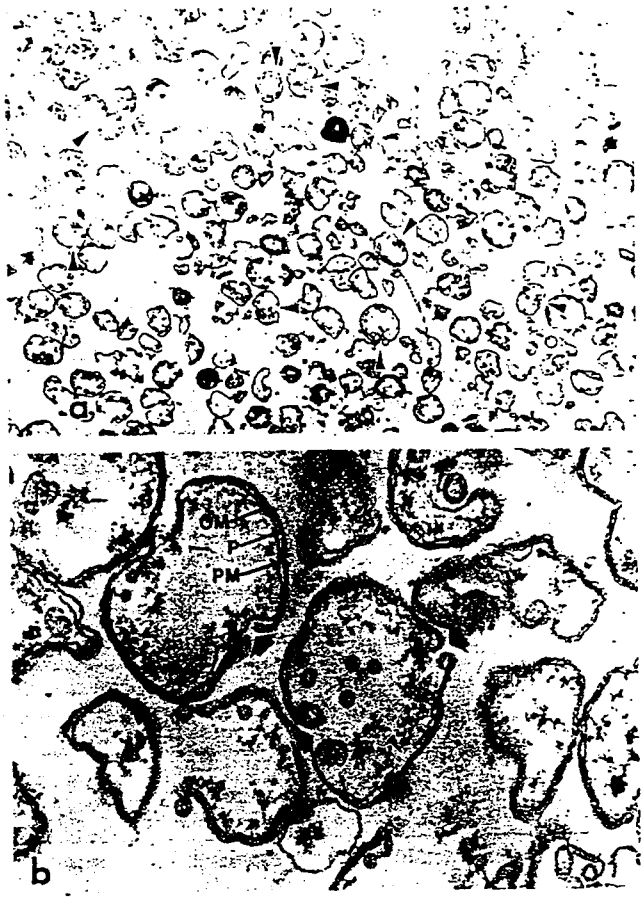
Mode of Action

Lysozyme is a small basic protein (MW ~ 15,000) which attaches itself to the basic bacterial surface in the presence of electrolytes and has a low energy of activation (rate of activity at low temperatures) (40). Its bacteriocidal and lytic action depends not only on the concentration of the enzyme and the ionic strength but also on the nature of the anions: SCN^- and HCO_3^- are now known to promote lysis (Plate 1) both being more effective than Cl^- or F^- (41 - 44). The enzyme is stable at low pH and lysis is also promoted when it is transferred from a low pH to a high pH (45, 46, Fig. 1). Considering the low pH in the stomach (particularly during fasting when the stomach is empty), the active secretion of SCN^- (see later under lactoperoxidase) and the high pH in the intestinal fluid buffered by HCO_3^-, it is surprising that the *in vitro* results have so far not been related to and investigated *in vivo*.

Plate 1

Electron micrographs of *Vibrio alcalescens* treated milk





Human lysozyme only

a) x 13.200, few lysed cells

b) x 90.000, unlysed cell but with dense material attached

to → outer membrane; note
also → peptidoglycan (P)
and → plasma (inner) membrane (PM)

c) x 61.000, lysed cell:
➧ separation of septum
➧ damaged area with portions of OM, P and PM missing

Human lysozyme followed by $NaHCO_3$

a) x 13.200, nearly all cells lysed

b) x 132.000, OM, P and PM intact or completely absent in damaged area

(symbols as above)

(Ref.: 45)

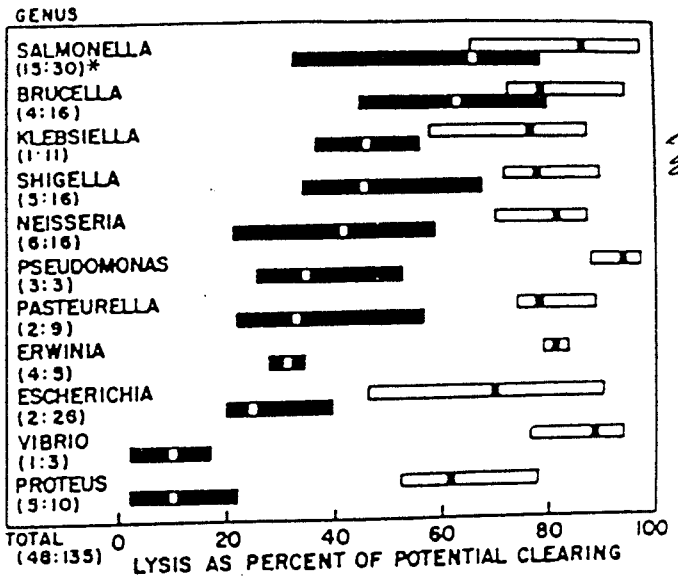


Figure 1. Lysis of Gram-negative genera by eggwhite lysozyme at low pH (3.5); after transfer to high pH (98).

Averages: □, ■

Note: these organisms are not lysed at neutral pH. (Ref. 60)

Sources

Bovine milk contains an average of only 13 ug lysozyme/100 ml but human milk contains 10 mg/100 ml (30, 47). The enzyme is generally considered to be synthesized in the mammary gland but so far *in vitro* synthesis by mammary tissue has not yet been attempted. Tissues of the gastrointestinal and respiratory tract, normal and inflamed, monocytes and macrophages incorporate C^{14} labelled amino acids thus proving *in vitro* synthesis (48). Gastric mucosa appears to synthesize both secretory component (for the transport of circulating blood dimeric IgA) and lysozyme. The Paneth cells appear to be the main source of lysozyme in the crypt lumen (49).

It has now been established (50a, 50b) that in the calf, adult cattle, goat and sheep, the abomasum but not the duodenal tissue secretes lysozyme. It was found to be absent from bovine saliva and nasal secretions, as well as from bovine leucocytes (56). The question arises now whether the lysozyme concentration in the abomasum is high enough to compensate for the low concentration in the bovine milk.

The so-called β- lysin (e.g. 51) of blood serum, which has been known to be bacteriocidal for some Gram-positive organisms, is now recognised to be identical with lysozyme (52). We must therefore also consider whether the enzyme diffuses into the mammary gland or intestinal tract when inflamed. The faeces of infants with diarrhoea, for instance, contain more lysozyme than that of healthy infants (53, 54). It is not clear whether this increase can be ascribed to greater synthesis of lysozyme, to infiltration of leucocytes or to direct diffusion from the blood. No comparable data ara available for animals. However, infected udders secrete milk containing increased numbers of leucocytes and, according to Korhonen (55), increased levels of lysozyme. Since bovine leucocytes are not known to be lack lysozyme (56), they cannot be the source. The inflamed udder, therefore, either increases the synthesis of lysozyme by analogy with lactoferrin (see later) or lysozyme (β - lysin) diffuses from the blood like other blood proteins.

Table 1 — The lytic effect of bovine milk, human milk and egg white lysozyme on live *Micrococcus lysodeikticus*

Source of lysozyme	Rate of lysis Δ% T min^{-1} * (buffer)
Bovine milk	1-82
Human milk	1-70
Egg white	0-63

* Determined in spectrophotometer at 540 mμ
Buffer: phosphate buffer pH 6-2.
Compiled from ref. 47.

Possible Biological Significance

In vivo, we have, so far, no direct evidence that either lysozyme of ingested milk or intestinally secreted lysozyme affects the intestinal bacteria, particularly Gram-negative, potential pathogens. Claims have been made that in rats the addition of egg white lysozyme to formula feeds reduces the pH of their faeces and increases the number of bifidobacteria, thereby bestowing a "clinical" benefit (57). Numerous attempts to detect any benefit from feeding lysozyme to infants are even more difficult to interpret.

Theoretically it is possible that hydrolysates of cell walls — N — acetyl — glucosamine and N — acetylmuramic acid-stimulate the growth of bifidobacteria, e.g. *Lactobacillus bifidus* var. pennsylvania, which is unable to

synthesize peptidoglycan in the absence of N-sugars (which occur only in human milk at adequate levels). The organisms, for instance, grew adequately in a synthetic medium in which *M. lysodeicticus* had previously been lysed by lysozyme (unpublished). Recently Spik et al. (58) demonstrated that some ammonium sulphate-precipitated fractions of human milk yield growth promoting factor(s) for bifidobacteria *in vitro;* their nature has not yet been established.

An entirely new hypothesis for the function of lysozyme was proposed by Jollès (59); he suggested that the hydrolytic products of peptidoglycan may act as an adjuvant or immunomodulator, (Table 2). Indeed, without apparent knowledge of this hypothesis, Lodinova & Jouja (60) reported that feeding lysozyme to infants increased the immunoglobulin (secretory IgA) level in the faeces compared with the faeces of infants given formula feed only; the immunoglobulin level in the blood was not affected. Other workers (61), acknowledging the Jolles hypothesis, showed that injection of hepatitis B antigen into the foot pads of guinea pigs increased the circulating hepatitis antibodies in the blood and stimulated cellular immunity (skin and corneal tests for hypersensitivity). Digest of bacterial cell walls by lysozyme and pronase or N-acetalymuramyl dipeptide also increased the serum and blood antibody levels but not as efficiently as Freund's complete adjuvant.

Table 2 – IgA levels in stool filtrates

Groups of infants		No of infants		No of stool samples		
		Total	sIgA* positive	sIgA positive	sIgA negative	Total
A	Breast fed	20	19	75	112	187
B	Artifically fed	13	1	3	127	130
C	Artificially fed plus lysozyme	15	10	52	113	165

* If sIgA was detected in stool at least once – such infants considered sIgA positive

Significance: A : B x^2 = 21.63 $p < 0.01$

A : C = not significant

(compiled from ref. 60)

A novel application for lysozyme is now being established in the cheese industry. Preparations of lactoferrin isolated from secretions of the non-lactating udder were shown to inhibit vegetative cells and apparently the germination of *B. stearothermophilus* spores which could be reversed by iron (22). Subsequently, it was discovered that the lactoferrin preparation had been contaminated with lysozyme which was responsible for the inhibition. Egg white lysozyme was found to be also effective; furthermore iron did reverse the inhibition when the spores were suspended in phosphate but not citrate buffer, hence the previous erroneous conclusion of the involvement of lactoferrin. Lactoperoxidase and lactoferrin (61a, 61b) form a strong complex with an electrophoretic mobility distinct from that of lactoferrin and lysozyme. It is therefore not surprising the the "purest" preparation of bovine lactoferrin contains traces of lysozyme (and lactoperoxidase) (182).

Egg white lysozyme is now being used to prevent "butyric acid blowing" of cheese (62 - 65) and appears to be a feasible alternative to the addition of the toxicologically undesirable nitrates. It appears that lysozyme inhibits the overall outgrowth of spores of *Clostridium tyrobutyricum* into vegetative cells but without affecting the early stages of germination such as optical refractibility and heat sensitivity. Considering the greater activity of milk lysozyme it would be of interest to determine its effect on various species of spores.

In this context, it is interesting to recall that peasants traditionally fed raw eggs to piglets and calves to relieve scouring. Recently, it was reported that the count of clostridia was markedly reduced in the faeces of breast-fed compared with artificially fed infants (66). This could be ascribed to the high lysozyme content of human milk (some clostridia are, of course, known to cause diarrhoea in man and animals).

Finally, it can be speculated that lysozyme promotes protection through its effect on leucocytes. Human lysozyme at concentrations of 10-100 mg/ml significantly ($P < 0.001$) stimulated phagocytosis of yeast cells by polymorphonuclear leucocytes in the absence of any serum factors (opsonins, complements); egg white lysozyme was ineffective (67).

LACTOFERRIN

It is now well established that iron-chelating proteins such as transferrin, lactoferrin and ovotransferrin have antibacterial activity *in vitro*. While transferrin is mainly a blood and extravascular protein, although it occurs also in milk, lactoferrin is exclusively a secretory protein. It occurs in milk, saliva, tears, nasal and intestinal secretions, pancreatic juice and seminal fluid as well as in the secondary granules of neutrophils. Ovotransferrin, also referred to as conalbumin, is exclusively an avian egg white (albumin) protein.

Mode of Action

Lactoferrin is a single glycoprotein, MW ~ 76,500, to which two carbohydrate groups are attached. The molecular functional and evolutionary comparisons with transferrin and ovotransferrin have been reviewed (19, 37, 58, 68-72). The protein possesses 2 metal-binding sites, each of which can bind a ferric ion (Fe^3) together with a bicarbonate ion. In the native state, lactoferrin is only partly saturated with iron (8-30%) which is physiologically important because iron can be chelated and thus inhibit bacteria by depriving them of iron essential for growth. Iron-saturated proteins are devoid of antibacterial activity (Fig. 2, Table 3) (20-22). The capacity of lactoferrin to bind iron depends on the presence of bicarbonate, mole per mole (73), but is inversely related to citrate concentration (Table 3) (74, 75, 75a). Citrate can exchange iron chelated by lactoferrin (and conalbumin) and iron citrate can be actively taken up by microorganisms through outer membrane receptor proteins (76a). Bovine and human milks contain 4-8 mM and 2.3 mM citrate, respectively; thus, citrate can counteract the bacteriostatic activity of lactoferrin unless the bicarbonate concentration is high, as in the intestinal fluid where bicarbonate is the main buffer. Also, it was reported (in calves) that citrate is rapidly absorbed in the upper duodenum (33). Lactoferrin (and ovotransferrin) differs from transferrin in its ability to retain iron at very low pH (up to 2.2) and therefore should pass through the acid gastric fluid unharmed.

Bacteriostasis in only temporary because some gram-negative pathogens adapt to iron restrictive conditions *in vitro* and *in vivo* by synthesizing low molecular weight iron-chelators (siderophores) that can remove iron from lactoferring (or transferrin). The iron-laden siderophores are taken up by newly synthesized outer membrane proteins. *E. coli* produces two types of siderophores: enterocholin (gene controlled) and aerobactin (plasmid controlled) (77, 77a, 77b, 77c, 77d). (It is of interest that the outer membrane proteins act also as receptors (for bacteriophages and colicins).

It was generally assumed that lactoferrin inhibits only bacteria with high iron requirements (e.g. coliforms) while lactic bacteria, with low iron requirements, remain unaffected (78). This explanation may be insufficient because it has been reported (79-82) that apolactoferrin (iron-free) has a bacteriocidal effect against a wide range of microorganisms — Gram-positive, Gram-negative, aerobic and anaerobic bacteria, as well as yeasts. This effect

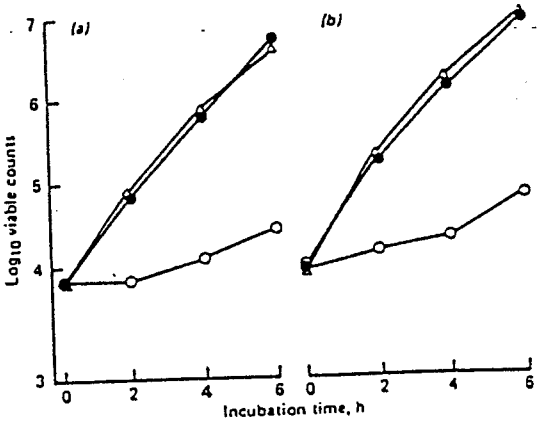


Figure 2. Inhibition of *E. coli* in carbon-ammonium salts medium by lactoferrin isolated from bovine whey.

●—●, control (no added lactoferrin or Fe^{1+}); ○—○ medium containing 2.5 mg/ml lactoferrin; △—△, medium containing 2.5 mg/ml lactoferrin saturated with Fe^{1+}. (a) *E. coli* serotype 0101 (serum-resistant); (b) *E. coli*, NCTC 9703, serotype 0111 (serum-susceptible).

(Ref. 182)

Table 3 — Effect of citrate and bicarbonate on the bacteriostatic activity of dialysed colostrum (74)

HCO_3 ($\mu M/ml^{-1}$)	Citrate ($\mu M/ml^{-1}$)			
	0	0.1	1	10
	Increase in $\log_{10}$ viable count after incubation (6 h)			
0	0.7	1.9	2.6	2.4
0.7	0.7	0.9	2.5	2.5
7.0	1.1	1.1	1.1	2.4
70.0	1.0	0.7	0.7	0.8

(Ref. 74)

appears to be strain-specific because the strains of species previously shown to be inhibited only, are not killed. It will be necessary to demonstrate that the bacteriocidal effect is valid for 'native' lactoferrin, which is partly saturated, and tested under physiological conditions, since the bacteriocidal effect was observed only with suspensions of bacteria in distilled water or saline; under conditions of growth, viability was maintained but the uptake of glucose, production of lactic acid and the subsequent synthesis of DNA, RNA and protein was impaired. The enhancement of the antibacterial activity of lactoferrin by antibodies is discussed in the section on synergism.

Sources

Bovine milk contains between 0.02 and 0.35 mg/ml of lactoferrin, depending on the period of lactation, while human milk contains 2-4 mg/ml. The relatively high levels of lactoferrin in human colostra (6-8 mg/ml) and bovine colostra (~1 mg/ml) decline rapidly within the first few weeks of lactation (30, 84, 85).

Lactoferrin is synthesized *in situ* in the mammary gland, intestinal tract, salivary and haderian glands and in neutrophils. Blood contains only traces of lactoferrin so that it is unlikely to contribute to the levels in the mammary gland. Artificially infected bovine udders produce milk with increased lactoferrin concentration which appears to be a direct response to infection because it was calculated that the increased number of leucocytes does not contain sufficient lactoferrin (83). So far, a possible increase in the level of transferrin by diffusion from the blood has not been investigated. This could be of particular importance in the case of coliform infections of the mammary gland, which are known to cause the rapid appearance of blood proteins in the milk.

Biological significance

It is unlikely that lactoferrin plays a significant role in the defence of the bovine mammary gland against infections during lactation: its concentration is too low and the citrate level too high. In the non-lactating udder, however, the conditions for antibacterial activity are very favourable: the lactoferrin concentration increases up to 70 mg/ml, the citrate is rapidly reabsorbed after lactation ceases; and at the same time, bicarbonate diffuses from the blood. Indeed, the secretion of the non-lactating udder is very inhibitory to *E. coli* and can be completely reversed by the addition of iron. *In vivo* experiments showed that the non-lactating bovine udder is exceptionally resistant to *E. coli* (86) (see later, Tables 3 and 4).

There is some indication that lactoferrin may have a role in the protection of the newborn:the breast-fed human infant ingests ~3,000 mg lactoferrin per day during the first weeks of life, declining to ~1,000 mg during the following 3 weeks (30). Unfortunately, we have no data for the calf but, assuming that the calf drinks 1-2 litres colostrum and 4 litres of milk per day, it can be calculated that a calf ingests 1,000 - 2,000 mg lactoferrin per day while consuming colostrum, declining to 80 - 1,400 mg per day when consuming "normal" milk (related to relative body weights, the calf ingests only 10% as much lactoferrin as the human infant).

Whatever the intake of lactoferrin, it is important to know how well the protein resists digestion and survives passage through the intestinal tract. *In vitro* experiments show that purified lactoferrin and the lactoferrin in milk resist digestion by trypsin and chymotrypsin but not by pepsin (87, 88). Lactoferrin can be recovered from the faeces of breast-fed infants, still capable of binding iron (89, 90). The faeces of articially fed infants contain no lactoferrin unless a feed based on cow's milk is supplement with human or bovine lactoferrin. Lactoferrin, besides its bacteriostatic activity, may be also involved in the absorption of iron by mucosal cells. There is some evidence for specific lactoferrin receptors in the human intestine and *in vitro* experiments have shown that iron is transported across the duodenal brush border (91). However, based on *in vivo* results, some authors disputed that lactoferrin is involved in iron transport (92, 93).

In vivo Experiments

While the antibacterial activity of lactoferrin *in vitro* is well documented, only a few animal experiments have been reported.

The non-lactating bovine udder was chosen for a limited investigation (86) to test the effect of lactoferrin *in vivo*. A strain of *E. coli* was selected which was known to cause severe mastitis in lactating udders or even death of the cow if not treated in time. 13 out of 17 quarters became infected when small numbers of microorganisms (250 - 300 cfu/quarter) were infused during lactation; in contrast, none of the 14 dry quarters became infected. However, when 2 quarters were infused immediately before parturition, both became infected (Table 4). Although the latter experiment ought to be repeated with a larger number of quarters, the observation agrees with the well-known fact that coli-infections do frequently occur after calving. Two dry quarters infused with both iron and *E. coli* became infected, indicating that lactoferrin (besides antibodies, complements leucocytes and lactoperoxidase) plays a part in preventing the multiplication of infused microorganisms. The failure of lactoferrin to prevent infection in the quarters prior to parturition can be explained by the changed composition of the secretion:

Table 4 — Response of lactating and non-lactating udders to the infusion of 250-300 cfu/quarter *E. coli*

State of quarter	No of quarters		
	Infused	Infected	% infection
Lactating	17	13	76
Non-lactating	14	0	0
2 days pre-calving	2	2	100

(Ref. 86)

according to Peaker & Linzell (94), citrate, which they regarded as the "harbinger of parturition", reappears during the formation of colostrum. We may also assume that the level of bicarbonate decreases at that time. The favourable conditions for the antibacterial activity of lactoferrin in the dry udder are therefore reversed and any "dormant" bacteria can multiply, causing mastitis.

Bullen et al. (95) investigated the effect of lactoferrin on the intestinal bacterial flora of sucking guinea piglets which were chosen because guinea pig milk is as rich as human milk in lactoferrin. Shortly after birth, the piglets were dosed with *E. coli* 0111 but developed a normal intestinal flora within 6 days: lactobacilli became the dominant flora in the small and large intestines and *E. coli* counts declined to $\sim 10^2$/g intestine within 2 days. When guinea piglets were fed an artificial diet, coliforms remained the dominant microorganisms; lactobacilli appeared only after 3 days, remaining well below the coliform numbers. When sucking guinea piglets were also given haematin, as a source of iron, the coliform count increased 10,000 fold in the small intestine compared with piglets without a source of iron; the increase was smaller in the large intestine, only 100 fold, but still significant. Stephens et al. (96) showed that guinea pig milk is not only rich in lactoferrin but also in lactoperoxidase — $\sim$ 20 fold higher than in bovine milk. They found that guinea pig milk 3 days *post-partum* inhibited *E. coli* 0111 and that the inhibition could be reversed by iron. However, milk obtained 7 days *post-partum* killed *E. coli* and this bacteriocidal effect could not be reversed by iron but only by reducing agents such as cysteine or penicillamine which indicates that the lactoperoxidase thiocyanate was involved but there was no indication of the source of H_2O_2 required to activate the lactoperoxidase system. However, there may be a possible explanation: it is well known that guinea pigs have a massive requirement for ascorbic acid when fed artificially, and it can therefore be assumed that guinea pig milk contains naturally high levels of ascorbic acid which could be oxidized to dehydroscorbic acid in the presence of copper, thus producing H_2O_2. Ascorbic acid was considered (97) as an alternative source of H_2O_2 in bovine milk and it has since been proven that physiological levels of copper and ascorbic acid effectively activate the lactoperoxidase system in milk *in vitro* (unpublished).

The problem of inhibitors other than lactoferrin which may interfere with the interpretation of *in vivo* experiments was overcome by using a preparation of conalbumin (Ricordati, Milano*) as an alternative source of an iron-chelating protein. Conalbumin can replace lactoferrin in the presence of bicarbonate *in vitro* and *in vivo* (98-101). When newborn guinea piglets were orally infected with *E. coli* 0111 and fed milk powder plus conalbumin, a similar protection was provided as when suckling the dam. These favourable results encouraged the planning of large-scale trials in which infants with acute enteritis were treated in about 30 hospitals. The preliminary results appear to be favourable (102, and to be published); it was found that infants given conalbumin in a cow's milk formula feed improved their general status and normalized the albus sooner than the 'controls' ($P < 0.01$) (Table 5).

Although we have no direct evidence as yet that lactoferrin contributes to the suppression of *E. coli* in the human infant, it seems plausible (see also Ref. 58).

* *Brit. patent 1463327 — Method of isolating transferrins from biological materials*

Table 5 — Normalization of bowels of infants with acute enteritis (6 months to 1 year)

Patients treated	3 days	3-6 days	6-9 days
With conalbumin * (m. 3.70 ± 0.36) n : 20	11 (55%)	7 (35%)	1 (10%
Without conalbumin (m. 3.70 ± 0.36) n : 20	1 (5%)	11 (55%)	8 (40%)

* = $P < 0.01$ in favour of the group treated with conalbumin "t" test.

(Ref. 120).

THE LACTOPEROXIDASE SYSTEM

Wright & Tramer (9) associated the inhibition of some starter strains with lactoperoxidase. They observed a high correlation between the inactivation of the enzyme by heat, low pH or sodium azide and the inhibitory activity of raw milk against some strains of lactic acid streptococci. Since reducing agents such as cysteine or sodium sulphite reversed the inhibition they suggested the formation by lactoperoxidase and H_2O_2 of an inhibitory oxidation product having a quinonoid structure. The role of lactoperoxidase was rapidly confirmed when it was shown that purified lactoperoxidase edded to milk, heated to destroy the native enzyme, restored the original inhibition of susceptible strains (103). Next, the necessity of H_2O_2 was established (10) and the oxidizable substrate identified as thiocyanate (11). Inhibition was also confirmed in a synthetic medium, provided all three components – lactoperoxidase, H_2O_2 and thiocyanate – were present (104, 105).

The inhibition or killing of many bacterial species *in vitro* by the lactoperoxidase system is now well established (for reviews see Refs 106-108 a, b).

Distribution of the Components

Lactoperoxidase: Bovine milk contains ~30 ug/ml of this enzyme (23). The values of unit activity/ml reported in the literature are difficult to compare because different substrates, e.g. phenylenediamine, 0-dianisidine or pyrogallol, have been used to measure colour formation catalysed by lactoperoxidase/H_2O_2. In a large-scale survey, Kiermeier & Kayser (109) reported that mid-lactation milk contains 1.7 - 17.6 'phenylendiamine' U/ml. Colostrum contains little or no lactoperoxidase and reaches a peak at 5 days *post-partum* and declines thereafter. The enzyme level also depends on the sexual cycle of the cow and varies according to summer or winter season, feeding regime and breed (110). Korhonen (55), also using phenylenediamine but under different conditions, reported average values of 0.46 (range 0.3 - 0.6) U/ml in samples of milk containing less than 5×10^6 leucocytes/ml and hence was assumed to be from healthy udders; the values were slightly higher in milks with higher cell counts. Since we now know that leucocytes suspended in milk phagocyte (ingest) casein micelles and fat globules (111 - 113), it is likely that the increased peroxidative values were caused by leakage of myeoloperoxidase, the peroxidase of polymorphonucleated leucocytes; phagocytosis induces a metabolic burst, generating H_2O_2 and diffusing H_2O_2 as well as myeoloperoxidase (Korhonen & Reiter, cited in Ref. 107).

Recently, a more sensitive assay has come into general use employing 2, 2 azino-di-(3-ethyl benathiazoline-6-sulphonic acid) (ABTS) as substrate (114). According to Stephens et al. (96), bovine milk contains, on av. 1422 (range 738 - 3889) ABTS mU/ml. It is important that all the peroxidative values for bovine milk quoted are far above the minimum requirements for the lactoperoxidase/H_2O_2/SCN^- system. For instance, it was found that as little as 20 ABTS mU/ml in human milk are sufficient to kill *E. coli* in the presence of SCN^- and H_2O_2 (36) (Fig. 3). However, all the peroxidative values in human milk have now been shown to be derived from leucocytes and hence are due to myeloperoxidase and not lactoperoxidase (24). This is the probable explanation why the milk of Gambian mothers were found to contain persistently, for several months, high levels of lactoperoxidase in contrast to the milk of women obtained from local hospitals; the breasts may have been infected, hence the milk would contain high cell numbers which were probably lysed after freezing for transport and subsequent thawing (36). The general distribution of lactoperoxidase in various secretions is similar to all the other protective proteins (including immunoglobulins) but it is not clear whether its occurrence in intestinal mucosal scrapings is due to *in situ* synthesis or is derived from eosinophilis (115). The contribution of human salivary lactoperoxidase system

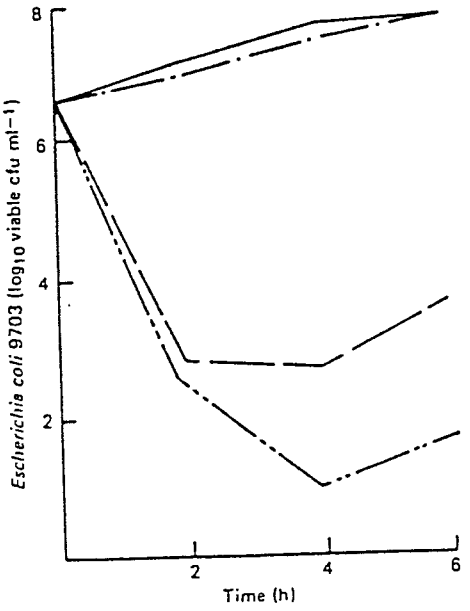


Figure 3. Bactericidal effect of human milk (10 days after parturition) containing 20 mU/ml^{-1} lactoperoxidase

Control: ———
Additions: 1) SCN^-, 0,225 mM: — —
2) as 1) plus 200 mU/l^{-1} lactoperoxidase:— - - —
3) as 2) plus lmM $Na_2S_2O_4$: — - —
(reversing bactericidal activity)

(Ref. 36)

first suggested in 1963 (11) to the suppression of lactic acid bacteria, and hence of caries-producing oral streptococci, is now well documents (116, 117).

Human salivary peroxidase may have yet another function, in particular for the new born infant: it may compensate for the lack or paucity of lactoperoxidase in human milk. The concentration of salivary peroxidase varies greatly, from nil to 2353 mU/ml amongst infants and varies during the day. The enzyme level is very low in calf's saliva up to 1-2 weeks after birth, 265 mU/ml up to 3 days, but rises to >5000 mU/ml (118). The gastric juice of fasting calf contains no lactoperoxidase because saliva normally enters the rumen and the abomasum only during feeding (119). This is, of course, in contrast to the human infant whose saliva enters the stomach all the time. Since the enzyme is resistant to low pH and proteolysis, gastric juice should always contain lactoperoxidase (to be investigated).

Thiocyanate (SCN^-): this anion, which permeates freely, occurs ubiquitously in animal tissues and secretions. It is found in the mammary, salivary and thyroid glands, in the stomach and kidney, and in fluids such as synovial, cerebral, spinal, lymph and plasma. SCN^- is derived endogenously during the detoxification reaction between thiosulphates and metabolic products of sulphur amino acids, cyanide and exogenously from foods containing glucosides which yield SCN^- after hydrolysis. Cows on natural pastures with clover (CN^--containing) and 'non-grasses' (e.g. cruciferae containing glucosides) give milk with higher concentrations of SCN^- (0.02 - 0.25 mM) than cows on winter feed or lay pastures (120, 121). The health of the udder also seems to influnece the SCN^- level: milk containing less than 5×10^5/ml leucocytes contains less SCN^- than milk with higher cell numbers (55); obviously, the increase is derived from the blood plasma by diffusion. The saliva of man and animals also contain high levels of SCN^- but is not a major source in the gastric juice. Gastric juice in the abomasum of the calf contains very high concentrations of SCN^- independently of the level in the ingested milk (up to 0.45 mM before feeding) Table 6.

Table 6 — Concentration of thiocyanate (SCN^-) and lactoperoxidase (LP) in calf abomasal fluid before and after feeding raw milk

	Time of sampling			
	Before feeding	After feeding		
		30 min	60 min	120 min
SCN^- concentration (mM)	0.45 ± 0.14 (17)	0.15 ± 0.08 (30)	1.15 ± 0.08 (37)	0.20 ± 0.09 (38)
LP concentration (u/ml)	0.009± 0.004 (75)	1.4 ± 9.7 (20)	1.14 ± 0.88 (18)	1.09 ± 0.68 (19)

Mean values with standard deviation are shown. Figure in brackets is number of samples. Four calves were sampled over a period of 50 days. The milk contained 1.9 - 0.85 u/ml LP (n = 11). At 5 h, no LP was detected.

(Ref. 119)

It is now assumed that the same parietal cells in the gastric mucosa which secrete HCl also secrete SCN^-. While HCl secretion is delayed in the calf (to prevent digestion of immunoglobulins) the gastric juice of the human baby at birth as a pH of 1.5 - 2, so that it may secrete SCN^- as well; however, this needs to be established. The gastric juices of mice, rats, guinea pigs and hamsters have been known for a long time to contain SCN^- (122).

H_2O_2: Some catalase-negative microorganisms, e.g. lactic acid bacteria, generate metabolically H_2O_2 under aerobic conditions. In the presence of lactoperoxidase and SCN^-, as in raw milk, sensitive streptococci, therefore, become self-inhibitory; some streptococci are 'resistant' to the lactoperoxidase system (see later). Catalase-positive organisms, e.g. coliforms, dismutate any H_2O_2 that is formed and can therefore only be inhibited or killed by an exogenous supply of H_2O_2 or generated enzymatically (e.g. by glucose oxidase, xanthine oxidase, etc.) (123, 124, 125) or provided by catalase-negative organisms (e.g. lactic acid bacteria) in ecological systems as in the intestinal tract (119) or oral cavity (126, 127, 129, 130, 144), could activate the native lactoperoxidase system instead of inactivating the enzyme by high concentration of H_2O_2 advocated for the preservation of milk.

The oxidation products of Thiocyanate

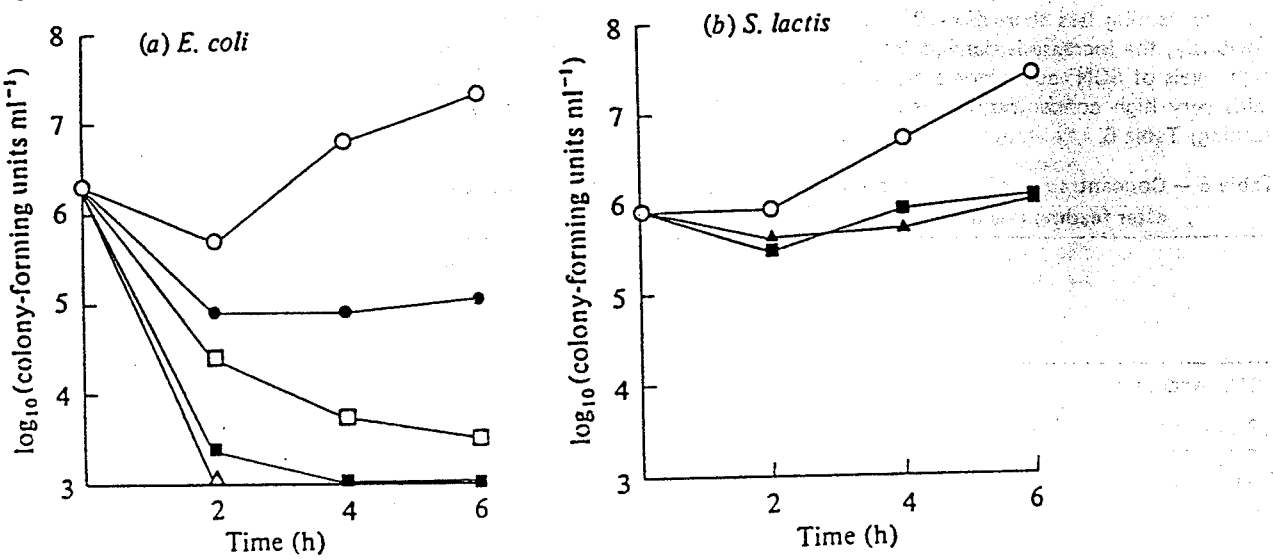
Although H_2O_2 *per se* is a strong oxidizing agent at high concentrations, it reacts only very slowly with biological material; hence 10-20 mM H_2O_2 are required to kill *E. coli.* However, 0.01 - 0.02 mM H_2O_2, catalysing the oxidation of SCN^-, Br^- and I^- are inhibitory. The end products of the oxidation of thiocyanate (CO_2, NH_4^+, SO_4^{2-}) are inert but the intermediate oxidation product(s) are inhibitory (97, 128). One of the intermediate products has now been definitely identified and chemically synthesized: hypothiocyanite ($OSCN^-$), the anion of hypothioxyanous acid — HOSCN, which are in acid-base equilibrium; a pK_a value of 5.3 was calculated for HOSCN (126). Further oxidation by excess H_2O_2 yields higher anions at cyanosulphurous acid (O_2SCN^-) and cyanosulphuric acid (O_3SCN^-) (116, 130-132).

Mode of Action

$OSCN^-$ appears to have a choatropic effect on the inner membrane of bacteria because within minutes of exposure to it (or to the complete lactoperoxidase system), K^+ and amino acids are leaked in to the medium (133); the uptake of carbohydrates, amino acids, etc. is inhibited and subsequently the synthesis of proteins, DNA and RNA (33, 134-136, 139, 140, 142). This inhibition of nutrient uptake is due to the effects of the lactoperoxidase system on the generation of a proton gradient in membrane vesicles and whole cells of *E. coli* (145). Thus, the

early observations of inhibition of oxygen uptake, enzyme inhibition, acid formation and growth of lactic streptococci are explained (11, 104, 97, 139, 140). While lactic streptococci are only temporarily inhibited (Fig. 4), Gram-negative organism such as coliforms, pseudomonads, salmonellae, shigellae and multiple antibiotic-resistant strains are killed (Fig. 5), and eventually lysed (125) (Plate 2). The difference in response between Gram-positive and Gram-negative organisms is not yet fully understood but appears to be related to the structure and composition of the gram-positive cell wall respectively the outer membrane of the Gram-negative organisms. (141, 137, 138). The cellular components most commonly oxidized are sulphydryl (SH) groups (139, 141, 141a) and nicotinamide nucleotides NADH, NADPH (104, 97, 131). The SH groups include the cysteine residues of specific proteins (e.g. albumin), free cysteine or reduced glutathione. The SH groups may be oxidized to disulphides (–S–S) sulphenyl thiocyanates (–S–SCN$^-$) or sulphinic acid (–S–OH). The oxidation products can be converted back into SH groups by reducing agents. SH depending enzymes of the glycolytic pathway such as hexokinase can therefore be inhibited (97, 142), although a non-SH depending enzyme such as D-lactate dehydrogenase was also found to be irreversibly inhibited, imparing the uptake function for nutrients (145). Amino acid and K^+ leakage studies indicate that the inner membrane of *S. lactis* is affected less than that of *E. coli* because less leakage occurs (Fig. 6). It appears therefore that the streptococcal cell wall is a greater barrier to the penetration of $OSCN^-$ than the outer membrane of *E. coli* (133).

Figure 4. Effect of various concentrations of $OSCN^-$ on the viability of *E. coli* and *S. lactis*

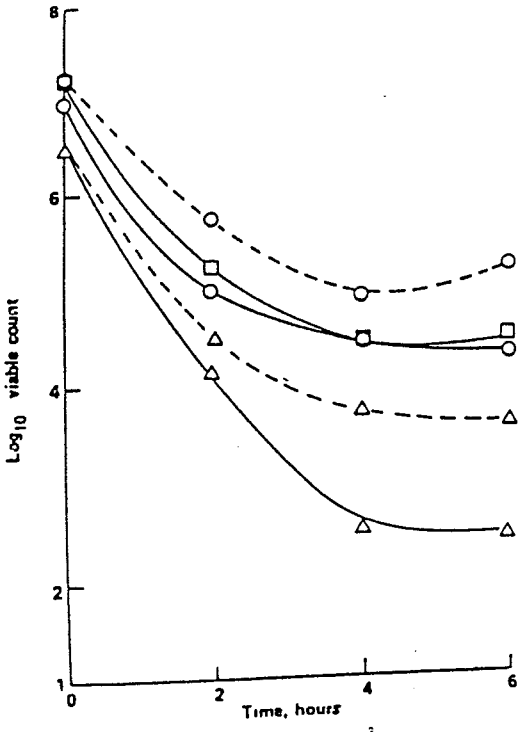


Synthetic medium, pH 5.5.

$OSCN^-$ concentrations; ○, O; ●, 5 μM; □, 10 μM; ■, 20 μM; △, 25 μM; ▲, 35 μM

(ref. 133)

Figure 5. Bactericidal effect of the lactoperoxidase system against some Gram-negative pathogens



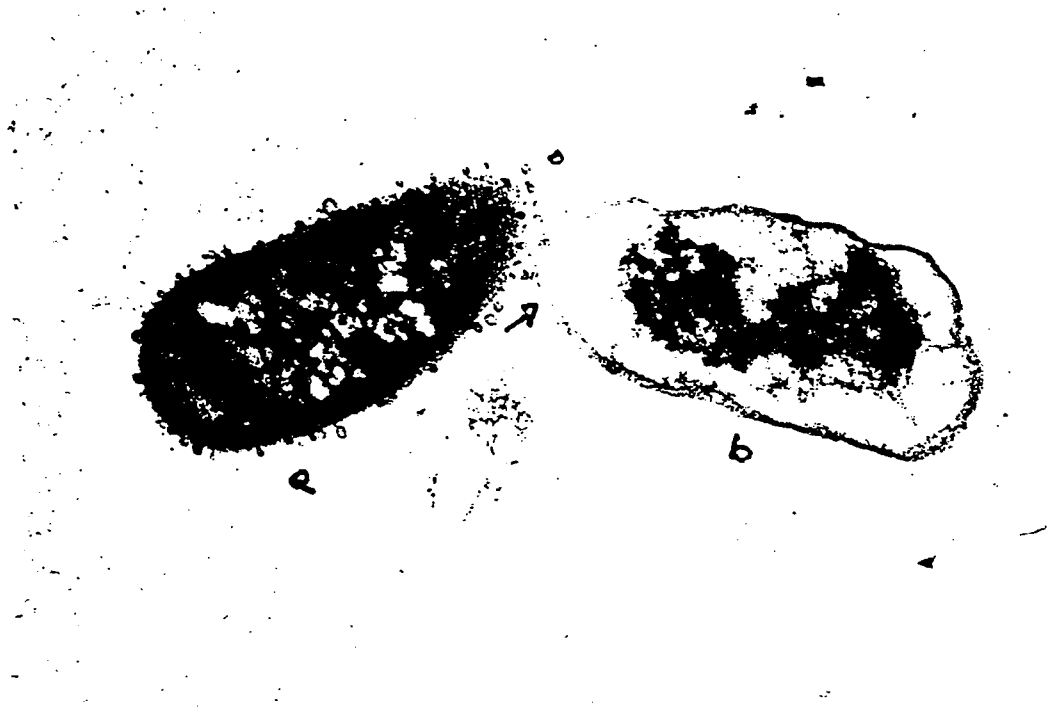
E. coli 0101 (serum resistant) at a concentration of 0.15 mM SCN^- (△---△) and 0.30 mM SCN^- (△——△) against *P. aeruginosa* at 0.15 mM SCN^- (○---○) and against *S. typhimurium* at 0.15 mM SCN^- (□).

(LP – 1.50 μ/ml; Glucose oxidase 0.1 μg/ml)

(Ref. 125)

Plate 2

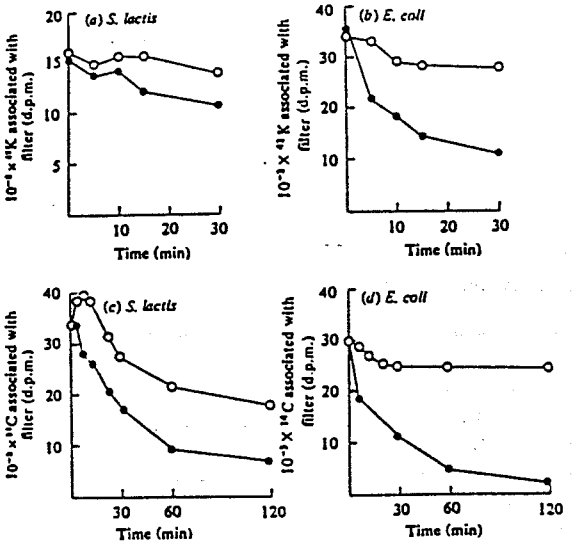
Electron micrograph of *E. coli* 9703, showing damage and lysis after exposure to lactoperoxidase system



note "blibbing" of cell a) and ghost with damage to outer membrane of cell b) (compare with Plate 1)

(By courtesy of Dr B.E. Brooker, NIRD and ref.: 31)

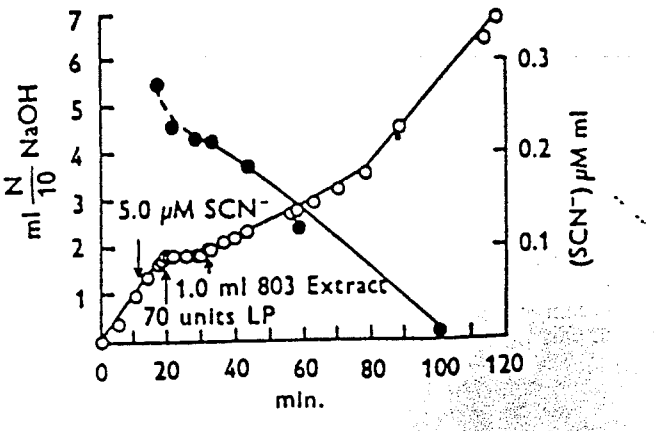
Figure 6. Effect of lactoperoxidase system on leakage of K^+ and amino acids



Leakage of $^{42}K^+$ from *S. lactis* (a) and *E. coli* (b) and on leakage of ^{14}C-labelled amino acids from *S. lactis* (c) and *E. coli* (d): ○, no $OSCN^-$; ●, 25 M $OSCN^-$ $OSCN^-$ produced by lactoperoxidase system.

(Ref. 133)

Figure 7. Inhibition of *Str. cremoris* (972) and its reversal by a cell free exctract of a "resistant" strain *Str. cremoris* (803)



Approximately 5.10^{10} washed cells were added to 20 ml of M 100 K phosphate pH 6.8 containing 1% glucose. Acid production was measured as the amount of N/10 NaOH (○—○) required to maintain the pH at 6.8. Values for SCN^- (●—●) are all corrected for volume changes.

(Ref. 104)

Besides the resistance, depending on the cell envelope and sulphydryl level of the bacteria, there exist also a different kind of resistance. Jones & Simms (5) found that certain streptococci were killed *(S. pyogenes)* or inhibited in raw milk *(S. agalactiae)* while others multiplied *(S. diagalactiae)* as well as in heated milk. Wilson & Rosenblum (4) extended this observation to the then known Lancefield's serogroups of streptococci and found that different strains of the same serogroup could be either susceptible or resistant (9), which led to initially to the identification of SCN^- as the oxidizable substrate because other oxidizable substrates occurring in milk, such as iodide and indican, inhibited both susceptible and resistant organisms (104, 105). The early observations of resistance amongst streptococci (Fig. 7) (Group N - *S. lactis* and *S. cremoris)* proved eventually to be particularly important in ecological systems such as the intestinal and oral flora. As far as starters are concerned, the obvious solution to the problem was to test the starter strains for inhibition (in raw milk free of bacteriophage) and avoid their use. Resistant strains were shown to possess a 'reversal' factor (enzyme) which reverses the inhibition of glycolysis: it catalyses the oxidation of $NADH_2$ in the presence of an intermediate oxidation product (now known to be $OSCN^-$). This factor was partly purified from resistant strains and was absent in susceptible strains (128, 97). The resistance factor has now been confirmed to be present in some oral streptococci *(S. mutans)* and renamed NADH:$OSCN^-$-oxidoreductase, which converts $OSCN^-$ into the inert thiocyanate (SCN^-) (143). There is some circumstantial evidence that $OSCN^-$ only inhibits *E. coli* while the higher oxyacide are bacteriocidal (146). The existence of very short-lived higher oxyacids was first proposed as antistreptococcal agents by Hogg & Jago (131). Pruitt et al. (147), in their kinetic and pologràphic studies on the oxidation of SCN^-, came to the conclusion that the higher oxyacids are produced at increased H_2O_2 concentrations. These findings explain why SCN^- appeared to be abruptly further oxidized at SCN^-: H_2O_2 ratios above 1:1 (97, 104). Carlsson et al. (148) confirmed that $OSCN^-$ at increased H_2O_2concentration becomes bacteriocidal and cytotoxic because of the higher oxyacids produced.

Biological Signicance and *in vivo* Activity

Research on the intestinal flora of the calf established at a high proportion of the lactobacilli colonising the oesophagus, abomasum and upper duodenum produce H_2O_2 (119, 149). That this natural source of H_2O_2 could activate the lactoperoxidase/thiocyanate system was proven as follows: a culture of *E. coli* was fed to cannulated calves which were either given raw milk or milk heated to destroy the lactoperoxidase. Samples withdrawn at intervals from the abomasum showed that *E. coli* were killed when raw milk was fed (reduction by ~95%) but no reduction occurred with heated milk. Addition of a solid source of H_2O_2 (magnesium peroxide, MgO_2) reduced the inoculum by ~99.99% (150) (Fig. 8). So far, it has not been possible to detect any oxidation products of SCN^- in the abomasal fluid because it was not possible to clarify it. Samples from the duodenum should be more suitable because raw milk fed to calves is rapidly clotted in the abomasum and clear straw-coloured whey flows through the pylorus within minutes of feeding.

The natural occurrence and activity of the lactoperoxidase system has now been established in a different way. Research on dental caries showed that saliva contains not only SCN^- but also the oxidation product, hypothiocyanate ($OSCN^-$), catalysed by salivary peroxidase and H_2O_2 generated by resistant oral streptococci. When freshly collected saliva was incubated, the $OSCN^-$ level increased appreciably, H_2O_2 being the limiting component. This was the first time that $OSCN^-$ was detected in a secretion under physiological conditions, thus proving that the lactoperoxidase system operates naturally *in vivo.* Concurrently, acid production in dental plaques is inhibited suggesting that the system reduces caries (e.g. 126, 129, 144).

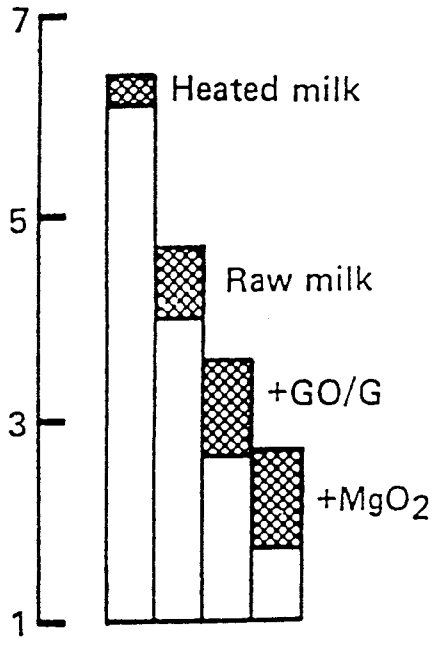


Figure 8. Failure to kill *E. coli* in abomasum of a cannulated calf when feeding heated milk (absence of lactoperoxidase); killing of *E. coli* when feeding of raw milk (presence of lactoperoxidase) without and with addition of an exogenous source of H_2O_2.

1. Heated milk (no lactoperoxidase)
2. Raw milk
3. Raw milk plus glucose/glucose oxidase
4. Raw milk plus MgO_2.

(Ref. 150)

Considering that the lactoperoxidase system is known to inhibit many of the bacterial species which cause bovine mastitis, it is surprising how few *in vivo* experiments have been performed to date. The non-lactating udder has long been recognised to be a latent source of bacteria which affect the udder after calving (e.g. 151). Intensive research led subsequently to the development of the so-called NIRD method of mastitis control which consists of hygiene measures, routine antibiotic treatment of the udder after drying-off (see Ref. 152) and milking machine control (153). The "NIRD method" of mastitis control produced a marked decrease in intramammary infections caused by staphylococci and streptococci with the exception of *S. uberis* (and *E. coli*) which are now considered the major mastitis-causing organisms (e.g. 154). However, although treatment with antibiotics proved to be successful against many pathogens, and is being widely used, there is always the doubt about the indiscriminate use of antibiotics and the emergence and spread of multiple antibiotic-resistant strains. It was and remains, therefore, of theoretical and practical interest to study the natural defence mechanisms, both to understand hopefully to increase their effectiveness.

Early attempts (32, 155) to evaluate the *in vivo* role of phagocytosis, complements and antibodies in mastitic infections proved to be inconclusive but yielded unexpected results (Table 7). A limited number of glands were infused with 2000 c.u.f. of either *S. uberis* or *Staph. aureus* on the day of drying-off and 14 days afterwards. *S. uberis* failed to infect any glands (0/6) when infused at day 0 (immediately after drying-off) but infected 4 of 6 glands 14 days after drying-off (2 delayed infections). *S. aureus* infected every gland at day 0 and 4 or 6 glands at day 14 (2 delayed). Although the small number of glands investigated did not allow firm conclusions, it was suggested that at the beginning of the dry period, the lactoperoxidase system may inhibit *S. uberis* but not *Staph. aureus*. This interpretation, however, depends on the presence of oxygen which would allow the formation of H_2O_2. Previous attempts to measure the O_2 pressure of milk in the bovine teat sinus indicated a 15% saturation; these results were obtained by cannulating milk through a layer of paraffin and need to be confirmed by direct measurements by introducing an O_2 electrode through the teat canal. 15% O_2 saturation was found to be sufficient to inhibit *S. uberis* in raw milk (unpublished). There is an analogy in phagocytosis: intracellular killing is reduced under anaerobic conditions and the leucocytes of children with chronic granolomatous disease can kill catalase-negative bacteria *in vitro* (e.g. H_2O_2-producing streptococci), but catalase-positive bacteria (e.g. staphylococci) survive because the leucocytes are metabolically unable to generate sufficient H_2O_2. The infections such children suffer, support the *in vitro* data (e.g. 156). Determination of the O_2 pressure *in vivo* could also have an important bearing on the efficiency of the leucocytes in preventing infections both in the lactating and non-lactating mammary gland. At the same time it would be of interest to investigate whether the intracellular killing by mycloperoxidase depends, at least partially, on the level of SCN^- as previously suggested (21, 128), instead of Cl^- (204).

Recently, the experiments involving the infection of non-lactating udders with *S. uberis* were repeated (157): 30-600 c.f.u. of *S. uberis* were infused into glands at drying-off (day 0) and after 7, 14 and 21 days. The rate of infection was: day 0: 0/9; day 7: 1/8; day 12: 2/6; day 21: 8/10. After 21 days and up to 1 day pre partum, 100% of the glands became infected (total 18). Those results basically confirm the suggestion of Reiter et al. (155) and results by Roguinsky (158) who concluded that the lactoperoxidase system should be most active against *S. uberis* at the beginning of the dry period. Brown & Mickelson (159) attributed the declining lactoperoxidase activity against *Streptococcus agalactiae* to the increasing concentrations of cystine and cysteine. The authors regarded the former as a growth stimulant, counteracting the system, while the latter would reduce the oxidized $OSCN^-$ to SCN^- (160-163).

The most convincing way to determine the role of the lactoperoxidase system in the bovine udder would be to attempt to reverse the lactoperoxidase system by a strong reducing agent such as sodium sulphite or mercaptoethanol, or better yet, to assay whether $OSCN^-$ is formed in the lactating and/or non-lactating bovine udder.

Table 7 – Rate of infection after infusion of staphylococci or streptococci into quarters immediately after last milking, or into quarters dry for 14 days

	Number of quarters infected after infusion with			
	Staphylococci		Streptococci	
No of days quarters dry	20 CFU/qr	2000 CFU/qr	20 CFU/qr	2000 CFU/qr
0	1/6	6/6*	0/6	0/6
14	1/6	4/6x	1/6	4/6x

* Infection delayed in 1 quarter
x Infection delayed in 2 quarters

Six cows infused with staphylococci, 6 cows infused with streptococci. In each cow, 2 drying-off quarters, 2 dry quarters infused with low (20 CFU/qr) and high (2000 CFU/qr) inocula, respectively.

(Ref. 155).

Practical Applications

From 1975 (164a) onwards, animal trials were undertaken to feed new-born calves with whole raw milk containing the complete activated lactoperoxidase system, a source of H_2O_2 (glucose oxidase/glucose) and increased SCN^- concentrations. Trials performed under various conditions showed that scouring (diarrhoea) was reduced and the live weight gains significantly increased especially when the incidence of scouring in the controls (no

artificially-activated lactoperoxidase system) was high, Fig. 9. When scouring was low (as in the herd of the National Institute for Research in Dairying, Shinfield, U.K.), live-weight gains were still higher, but less so, for the calves fed milk with the lactoperoxidase system compared with the control animals (164b) (Table 8).

All these calf trials were performed by feeding milk and not calf starters; this means that even the natural, host-specific milk can be improved by artificially activating the lactoperoxidase system. Most calf starters are based on skim milk powders and it was shown that lactoperoxidase can be preserved and activated by a suitable source of H_2O_2. Successful trials have been made with such milk powders in the rearing of calves (165, 166).

Another use of the lactoperoxidase system was first suggested to the Food & Agricultural Organization, Rome, in 1967 (Reiter cited in Ref. 167). It was argued that the supply of low concentrations of H_2O_2 (0.2 mM) or the continuous production of H_2O_2 by enzymes such as xanthine oxidase, native to milk and hypoxanthine, xanthine or meat extract could activate the natural lactoperoxidase system instead of inactivating lactoperoxidase by high concentration of H_2O_2 advocated for the preservation of milk. Such a method might be used to preserve milk collected under difficult conditions and subjected to long journeys at high ambient temperatures. At that time, this approach was considered to be impractical. However, Reiter et al. (123), in cooperation with Alfa-Laval, Sweden, showed that psychrotrophs were killed in cooled milk by the activated lactoperoxidase system (see also 124, 125 168) (Fig. 10). To investigate the effect on cheese making, milk was experimentally inoculated with *Pseudomonas fluorescens,* incubated for 3 days at 4°C, and pasteurised before being made into cheese; the cheese became rancid and unacceptable (169). However, when milk of the same delivery inoculated to the same level, was treated by the lactoperoxidase system, the cheese developed normal flavour, without rancidity. Although pasteurization killed the psychrotrophs, their enzymes, which are extremely heat resistant, survived in the control cheeses and spoiled the flavour (Table 9).

The early suggestion to preserve milk by activating the natural lactoperoxidase system has now been proven in large scale trials (Kenya, Mexico, Sri Lanka, Pakistan). The lactoperoxidase system was activated by the addition of SCN^- and a source of H_2O_2 to preserve raw, uncooled milk collected under difficult conditions at ambient temperatures of up to 38°C. The keeping quality of the milk was increased appreciably measured by the 10 min Resazurin test (170). The preservative effect was not due to the reduction of the Gram-negative flora but to the suppression of acid development by contaminating lactic acid bacteria (150, 171, 172) (Fig. 11 and Table 10)*.

* *Footnote: The use of the activated lactoperoxidase system is protected by the British patent 1 468 405 (Method of improving the keeping qualities of milk and other liquids) and 1 546 747 (Antibacterial pharmaceutical and feedstuff compositions).*

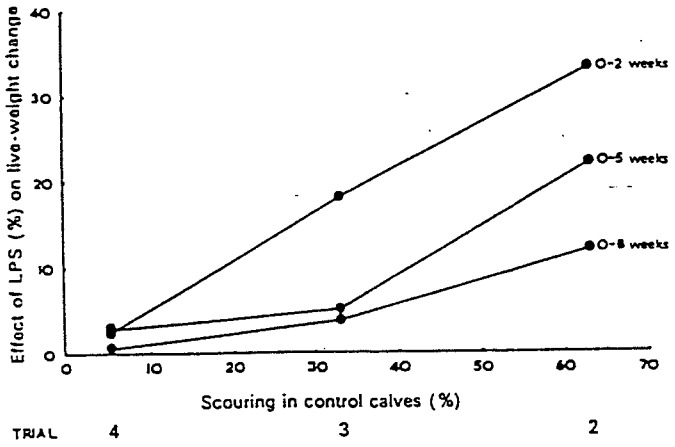


Figure 9. Relationships between the effect of the LPS (%) on live-weight change and the level of scouring (%) in calves given whole milk only.
(Ref. 164)

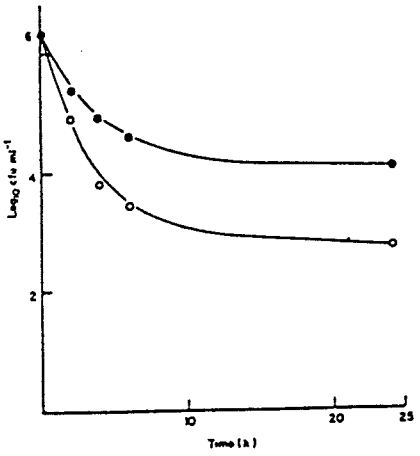


Figure 10. Effect of LP system on *Ps. fluorescens* in milk at 4°C

Glucose (0-3%) and glucose oxidase (0.1 ml^{-1}) were added to aseptically drawn milk. *Ps. fluorescens* in the presence of 0-17 mM SCN - (●); *Ps. fluorescens* in the presence of 0-26 mM SCN- (○)
(Ref. 169)

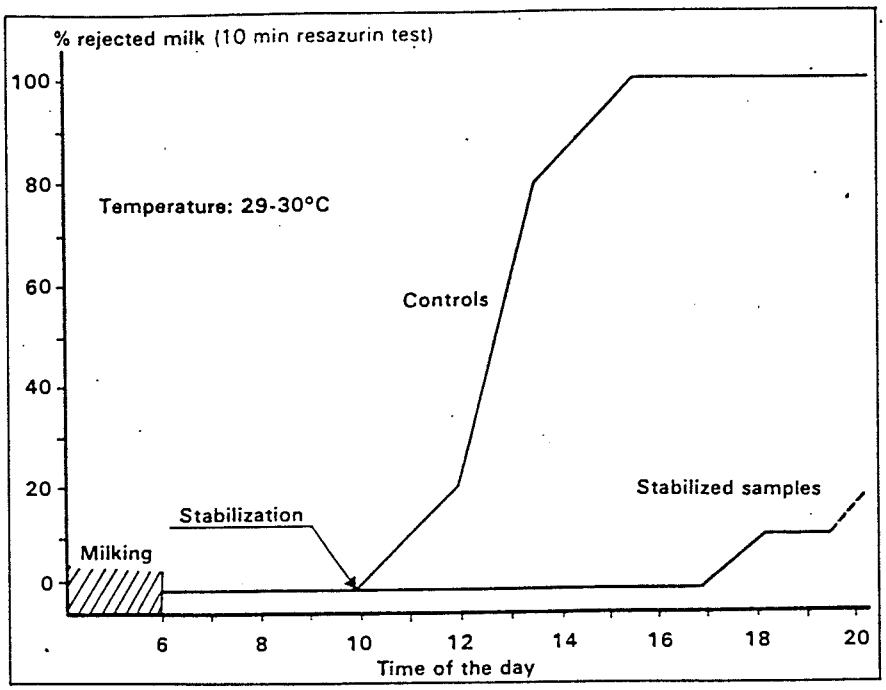


Figure 11. Effect of stabilisation of milk by activation of the LP-system on the keeping quality of raw milk delivered to the Narahenpita collection centre. Sri Lanka.

(Ref. 150)

Table 8 – Overall mean live-weight changes for calves fed whole milk with or without the lactoperoxidase system (LPS) (164)

	Treatment A Whole milk + LPS	Treatment B Whole milk	Difference ± S.E.[x]
No of calves	92	93	
Birth weight (kg)	36	37	– 1
Live-weight change (g/d)			
0–1 week	306	269	37 ± 35.0
0–2 weeks	340	294	46* ± 21.7
0–3 weeks	368	322	45** ± 16.8
0–5 weeks	371	341	31* ± 12.4
0–8 weeks	477	454	23 ± 13.2

Levels of significance:

* $p < 0.05$

** $0.001 < p < 0.01$

x S.E. based on the variation between calves within treatments and trials

(Ref. 164).

Table 9 – Effect of lactoperoxidase system on multiplication of *Pseudomonas fluorescens* and cheese quality

Treatment of milk	No of *Ps. fluorescens* (cfu $ml^{-1} \times 10^1$) in milk stored at 5°C for (days) 0	1	2	3	Cheese quality at 4 months FFA* (μmol $10\ g^{-1}$)	Flavour assessment
None	15	29	150	1400	248	Rancid
LP system	21	1.1	0.2	0.1	50	Normal

* Free fatty acids

(Ref. 169).

Table 10 — Development of acidity in samples of milk taken at the Giriulla Collection Centre, Sri Lanka. Cows were milked at 4-6 a.m. and samples were stabilized by activation of the lactoperoxidase system at 7.30 a.m. Ambient temperature exceeded 30°C at around 9 a.m. and reached 32°C in the afternoon (150).

	Acidity (% lactic acid) at (time)				
	10 a.m.	12 noon	2 p.m.	4 p.m.	6 p.m.
Stabilized milk (LP)	0.15	0.15	0.16	0.16	0.17
Untreated controls	0.16	0.17	0.19	0.22	0.26

(Ref. 171).

SYNERGISM

The best known example is the complement-mediated bacteriocidal effect of specific antibodies, mainly of the IgM class. The multiple lesions produced in the outer membrane of Gram-negative organisms facilitate the access of lysozyme to the peptidoglycan layer, thus rapidly lysing the organism. Secretory IgA (sIgA) by itself is not complement-binding but may bind it in the presence of lysozyme (173a, 173b) although this has been disputed (174). Secretory IgA has also been shown to enhance the antimicrobial effect of the lactoperoxidase system against *S. mutans* (175). The main function of sIgA is recognised to be the prevention of the attachment of pathogens (e.g. *E. coli, Vibrio cholera)* to the epitheliumof the intestine *in vivo.* The sIgA must be specific for the adhesion antigen (plasmid-controlled) which determines the capability of an organism to attach in a particular host (Plate 3). Exposure to the lactoperoxidase system has a similar effect as specific sIgA *in vitro;* it prevents the non-specific attachment to brush-borders isolated from either pig or calf (33, 36) (Table 11). This effect needs to be confirmed *in vivo* because it may be an important defence mechanism, replacing or augmenting the action of sIgA. Moreover, if motility contributes to the process of attachment, the system also immobilizes bacteria because it inhibits the energy metabolism.

Under physiological conditions, the most likely limiting factors in the lactoperoxidase system are SCN^- and H_2O_2 the former is derived from the diet or feed and the latter from various sources. Catalase-negative organisms can produce enough H_2O_2 to become self-inhibitory in the presence of lactoperoxidase and SCN^- or, if resistant to the system, provide H_2O_2 to inhibit or kill other bacteria as in ecological systems such as in the intestinal tract or oral cavity. Eukaryotic cells (e.g. in the salivary gland) are also capable of producing sufficient H_2O_2 to oxidise SCN^- because $OSCN^-$ was detected in sterile samples of stimulated human parotid saliva collected directly from the Stenson's duct (176).

In preliminary experiments (unpublished) it was also found that streptococci can provide an indirect source of H_2O_2 by producing acetaldehyde which can serve as a substrate for the milk xanthine oxidase. Xanthine oxidase itself can produce sufficient H_2O_2 from as little as 1.5 mM xanthine to kill a variety of pathogenic Gram-positive and Gram-negative organisms (177) (this phenomenon incidentally can interfere with bacterial counts when undiluted or low dilutions of milk are plated on agar containing meat extract, *ergo* xanthine, hypoxanthine). Hence the original suggestion to preserve milk by adding xanthine or hypoxanthine (or meat extract). Recently it was shown (178) that as little as 0.05 mM hypoxanthine (and 0.05 mM SCN^-) reduced *E. coli* in raw milk by 99.9% in 2 h at 37°C (90% without added SCN^-). Unfortunately, however, the keeping quality of milk on the farm and during collection is largely determined by the rate of acid development (Table 9) to which coliforms contribute very little. It would be therefore of greater interest to test the role of the xanthine oxidase/lactoperoxidase system on the development of acidity under field conditions.

It is well known that xanthine oxidase oxidizes aldehydes more rapidly than purines and that acetaldehyde is also a metabolic product of many lactic acid bacteria. It is therefore not surprising that preliminary experiments showed (unpublished), that yogurt made from low heat treated milk contains sufficient acetaldehyde to activate the xanthine oxidase/lactoperoxidase system and kill *E. coli.* The consumption of such yogurt could provide a potential antibacterial activity in the intestinal tract.

Several workers reported that specific antibodies promote the bacteriostatic activity of lactoferrin (89, 95, 179, 180) while others disputed it (181, 182). Interestingly, it appears now that the antibodies are not directed towards the somatic antigens, as thought previously, but to the low molecular iron chelators (siderophores) synthesized by the organisms to overcome iron deficiency (180, 183) and outer membrane protein receptors (e.g. 77d). Human sera have now been shown to certain enterocholin (183) and outer membrane antibodies (77d). Preliminary results indicate that human milk contains also antibodies against enterocholin (J.H. Brock, pers. com.) and outer membrane receptors (E. Griffin, pers. com.). The contradictions may be resolved by ascertaining in future whether the organisms used in experiments synthesizes siderophores or not because they are probably plasmid-linked, and hence labile.

A direct interaction between lysozyme and lactoferrin was observed with *Micrococcus luteus* (61b): the protoplasts produced by the lytic activity of lysozyme were strongly agglutinated by lactoferrin; it is suggested that the lysozyme-lactoferrin complex may increase this effect. The capacity of lactoferrin to form complexes may even affect the attachment of pathogens to intestinal epithelia. It was suggested (58) that the fucose residues of lactoferrin-glycans may complex with fucose-rich glycopeptides of intestinal mucus and thus inhibit fucose-sensitive adhesion of some pathogens.

Lactoferrin was reported (186) to increase haemolysis of red blood cells by activating, or at least stimulating, complement. Riviers et al. (187) reported that transferrin increases the susceptibility of *E. coli* to the complement-mediated bactericidal activity of specific antibodies possibly because iron-deprived organisms become energy-deficient and are therefore less able to repair damage to their cell envelope.

Table 11 — Attachment of *E. coli* 0149 : K_{91}, K_{88} to brush border cells

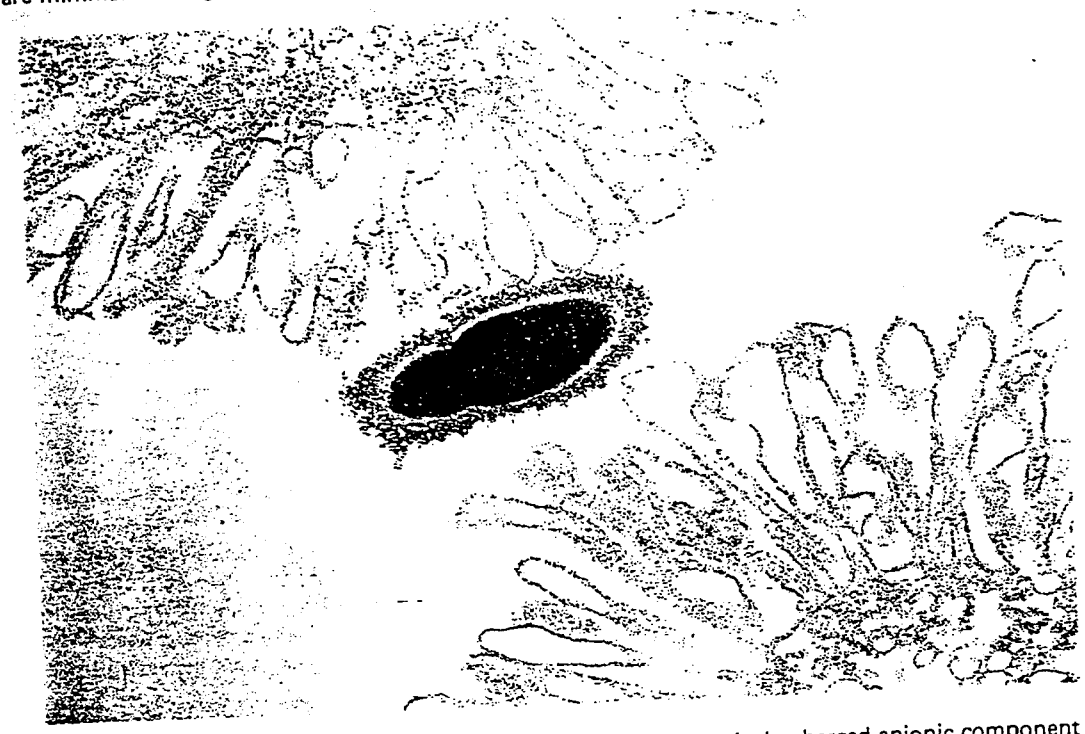
Control	Treatment with lactoperoxidase system
14, 17, 18, 16	0, 0, 4, 3
17, 20, 12, 8	3, 7, 6, 3
16, 17, 14, 16	2, 4, 0, 5
15, 18, 20, 12	1, 0, 3, 3
11, 14, 14, 13	4, 2, 6, 3
x = 14.6	x = 2.95
S = ±2.9	S = ± 2.00

(unpublished)

Plate 3

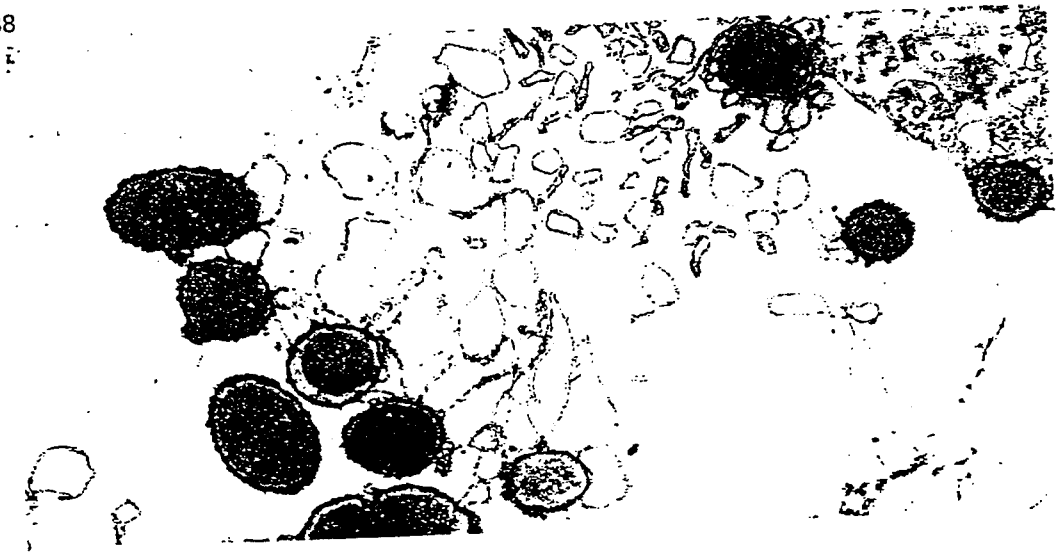
Attachment of *E. coli* 0149: K_{91} K_{88} ac to porcine bruh border cells revealing surface antigen (s) (adhesin)

a) Stained with cationic ferritin
compare minimal staining of brush border cells and large bacterial capsula (k)



x 25.000

b) Stained with colloidal iron hydroxide at pH 1.8 — revealing highly negatively charged anionic component of K_{88}



x 18.000

(By courtesy of B.E. Brooker, NIRD)
(See also ref. 33)

COMMENTS AND PROSPECTS

Bovine milk is an important source of human food but its antimicrobial activity has so far not been exploited. Our knowledge about the occurrence, distribution and mode of action of the protective proteins offers new opportunities for its utilization.

As detailed in this bulletin, the colostrum and milk of all species contain a multi-factorial antibacterial system, albeit at different concentrations and in different proportions. [The antiviral properties have so far been concerned with specific antibodies although non-antibody factors are beginning to be investigated – e.g. (188)]. These proteins protect the newborn and thus help to bridge the immunological gap until the newborn synthesizes its own defence systems. The same protective systems appear to contribute to the defence of the mammary gland against infections but the evidence for it is scant as yet.

There is little doubt that natural feeding provides the best protection for the newborn. Artificial feeding depends for its success on the nutritive composition and degree of hygiene obtainable but remains unsatisfactory as long as the protective proteins are not either preserved during manufacture, taking account of their different heat sensitivity (189, 190), or added as purified proteins afterwards. Feeding dried bovine milk causes difficulties even in the rearing of calves, partly because the heat treatment destroys or reduces the clottability of the reconstituted milk and partly because the lactoperoxidase is destroyed (see experiments with heated milk). Dried bovine milk is even more unsuitable for other animals such as piglets, rabbits, guinea piglets which cannot be successfully reared. The human infant seems to be remarkably resilient; its digestive system deals with cow's milk rather well. However, the standard of hygiene needs to be adequate to avoid infections, as events in developing countries have highlighted.

At least it is now recognised that the milk of each species is nutritionally best suited to its own offspring whose requirements depend on the maturity at birth, digestive system, rate of growth and the environment, it is born into (the newborn whale or porpoise does need ~45% fat in the milk). It took a very long time of paediatricians and nutritionists to appreciate the differences in the composition of different milks but nowadays babies are given nearly exclusively "humanized" cows's milk. It is to be hoped that the significance of the heat-sensitive protective proteins will eventually be appreciated as well.

Immunoglobulins

Although these proteins are not the subject of this chapter, they interrelate with the other protective proteins and at least need to be summarized.

Long before secretory IgA was discovered, Peterson and his collaborators (191, 192); see also (33, 34, 36) fought for the concept of the mammary gland as an "exocrine endothelial gland" capable of producing antibodies against bacteria and viruses. [(The latter was confirmed by intramammary infusion of bacteriophage as a model for viruses, resulting in high antibody titres against the specific phages (193)]. It was also demonstrated (191, 192) that the process of suckling by calves infected with salmonellae stimulated the production of specific antibodies in the udder. This "on demand" antibody producion was termed "diathelic" (i.e. incoming through the duct). In 1975, Salajka et al. (194) confirmed the phenomenon with *E. coli*-infected piglets suckling their dam.

Petersen aimed to produce "immune" milk by vaccinating cows with human pathogens; this concept has now been taken up and led to the manufacture of "Milk Immunoglobulin Concentrate" (e.g. 195-198) which contains antibodies against the main serotypes of strains of *E. coli* (and rotavirus) affecting infants. Good results have been reported in the treatment of pre-term, low-weight and hospitalized infants in two trials (196,199). It is suggested that the bovine class of immunoglobulins, IgG, representing 73-81% of the preparation, withstands proteolytic digestion similarly to secretory IgA (200, 201). In this context it is of some interest that bovine colostrum contains naturally specific antibodies, bactericidal in the presence of complements, against human enteropathogenic serotypes of *E. coli*, indicating a considerable cross-reaction between human and bovine strains (33, 34). Bovine and human rotavirus strains are known to have common antigens and hence it is not surprising that human rotavirus antibodies occur in bovine colostra (197).

Lactoperoxidase

The major anti-body protective protein in bovine milk is lactoperoxidase which was first exploited to preserve milk and then to improve the growth of newborn calves. The importance of using, whenever possible, "host-specific" milk or at least milk adjusted in its composition was experienced during a series of experiments with newborn piglets. Cow's milk frequently causes a bolus in the stomach which could become extended to 3 times its normal size. Failure to empty the stomach regularly, influenced the results greatly and the benefit of the lactoperoxidase system remained equivocal. In a later exploratory experiment, diluted colostrum proved to be nutritionally more acceptable and appeared to give clear cut, albeit limited, results. Eight piglets were infected with *E. coli* 0149, K_{88}, at birth (no colostrum) and hand-fed with diluted bovine colostrum with and without activated lactoperoxidase system. All the piglets (4) receiving the lactoperoxidase system remained well and unaffected while 3/4 of the controls developed severe diarrhoea within 48-60 h and 1 piglet died. Out of 10 infected piglets which remained with the dam, 5 died and 4 remained unaffected (B. Reiter & M. Knutsson, Ewos, A B, unpublished).

Before the treatment of milk with the lactoperoxidase system instead of H_2O_2 (203) (or indeed the direct treatment of infants and adults by the activated system) can become accepted by the FAO and WHO (202), it must be proven that the system occurs naturally in man and has no undesirable side effects. A detailed review has now been published. Besides proving that the lactoperoxidase system occurs naturally, a number of investigations have indicated that the system has no toxic or damaging effects. Erythrocytes, which are considered to be useful models for investigating the damaging effects of agents on mammalian membranes, were not lysed following exposure to the lactoperoxidase system which is in complete contrast to the effect of the myeloperoxidase-H_2O_2-Cl^- system which not only lysed erythrocytes, but also toxic for spermatozoa (reviewed by Clark, 204). Bovine spermatozoa exposed to the lactoperoxidase system were not killed but showed decreased motility and penetration into the cervical mucus (205), caused by the interference with the energy metabolism.

The concentration of SCN^- required for the preservation of milk is so low (i.e. 12 ppm) that it falls within the limits observed in the milk of cows on natural pastures. Alternatively, kale or rape seed which contain glucosides that yield SCN^- or clover which contains CN^-, could "naturally" increase the SCN^- level. Activation of the system requires little H_2O_2 (~ 8 ppm) which is in contrast to the 300-800 ppm recommended by the FAO (203) for the preservation of milk produced under difficult conditions such as lack of cooling facilities, high ambient temperatures and long transport. The addition of H_2O_2 or SCN^- could not be regarded as "adulteration" of milk.

If it can be shown that $OSCN^-$ occurs in milk as in saliva, this would indicate that H_2O_2 occurs naturally in milk oxidizing SCN^-; the addition of SCN^- would only correct a shortage because of current feeding regimes. Furthermore, H_2O_2 is rapidly used up in the oxidation of SCN^- and $OSCN^-$ is both short-lived and heat-sensitive to pasteurization. This is in contrast to the high concentrations of H_2O_2 recommended by the FAO for the preservation of milk the surplus of which has to be removed by the addition of catalase. (It is also possible that the intestinal tissue provides a source of H_2O_2 because the O_2 requirement is enormous, about 30 times higher than in muscle).

More stringent criteria will have to be applied before the lactoperoxidase system can be approved for the treatment of diarrhoea in infants and adults. It will be necessary to continue to assay salivary peroxidase and SCN^- in the gastric juice and ascertain whether the lactic acid flora in the stomach and duodenum is resistant to the lactoperoxidase system, and capable of producing H_2O_2 and possibly also acetaldehyde.

As detailed above, the lactoperoxidase system uniquely affects the inner or cytoplasmic membrane and membrane-bound enzymes. Respiration is immediately inhibited in bacteria but does not affect for instance whole liver cells; only the respiration of isolated mitochondria is inhibited. The lack of haemolysis and inhibition of respiration of these two cell types indicates that $OSCN^-$ does not penetrate the mammalian cell membranes (S. Finch, D. Baum & B. Reiter, to be published). The effect on the isolated mitochondria is not surprising because they are commonly considered to be "internalized" bacterial membranes.

It appears therefore that the lactoperoxidase system is not toxic; indeed it may even be protective. During clinical investigations on a toothpaste containing H_2O_2-producing enzymes for the activation of salivary peroxidase, it was observed that patients who had suffered from recurrent aphthous lesions (oral ulcers) were cured in the majority of cases and generally remained free from lesions (117, 206). This apparent healing and protective effect provoked research with various cultured cells. Hanström et al. (207) and Tenuovo & Larjava (208) showed, independently, that human gingival fibroblasts and HeLa and Chinese hamster ovary cells could be protected against the toxic effect of H_2O_2 retaining their capacity to proliferate. Their rate of lactic acid production in the presence of glucose was, however, reduced like in bacteria but at much higher concentrations of $OSCN^-$, although mammalian glyceraldehyde-3-phosphate dehydrogenase was inhibited like the bacterial enzyme. NADH : $OSCN^-$ oxido-reductase, which occurs in resistant bacteria (97, 148), was not detected in mammalian cells.

H_2O_2 by itself does not only damage the mammalian cell membrane (209) but causes also single-strand breaks in DNA being mutagenic (210). In contrast to the H_2O_2 effect, White et al. (211) demonstrated that the lactoperoxidase system does not oxidize calf thymus DNA (no change in UV spectra) and has no mutagenic effect on mutagen-sensitive strains of *Salmonella typhimurium* and *Saccharomyces cerevisiae*.

All these observations bear out the axiom that oxygen metabolism leads to toxic end-products such as oxygen free radicals and H_2O_2 but that enzymes such as superoxide dismutase [which occurs also in milk [12]], catalase and peroxidase protect procaryotic and eucaryotic cells.

It is obvious that this system ought to be exploited with infants who cannot be breast-fed. Only when the contribution of salivary peroxidase is known can it be decided whether lactoperoxidase itself will have to be added to artificial feeds or whether it suffices to add a source of H_2O_2 or whether to increase the SCN^- level.

Reiter (35) and Korhonen (107) discussed the question whether the antibacterial factors in milk, and particularly the lactoperoxidase system, protect the mammary gland or the newborn against infection. Both authors deplored the lack of *in vivo* evidence for the role of the lactoperoxidase system in mastitis. However, recent confirmation (156) of early work, 32, 154) that the lactoperoxidase system may protect the drying-off bovine udder against infection by *S. uberis* (but not *Staph aureus*) should stimulate further work. It is clear that positive information on the O_2 pressure in the lactating and non-lactating udder (H_2O_2 production by *S. uberis*) and identification of the intramammary oxidation product of SCN^- — $OSCN^-$ — are required. The influx of leucocytes during drying off may generate an additional source of H_2O_2 during the ingestion of fat and casein. Also an attempt ought to be made to demonstrate whether the high numbers of leucocytes actively phagocyte and kill bacteria in the dry udder which may also, at least partially, depend on the O_2 pressure.

Early work (97, 32) suggested that the myeloperoxidase of polymorphonuclear leucocytes may oxidize SCN^- to antibacterial oxidation products of SCN^-. This concept was completely over-shadowed in the medical literature by the concept that myeloperoxidase utilizes I^- and later Cl^- (reviewed by Clark, 204). I^- was discarded (104) from the beginning because its concentration is far too low in secretions such as milk and Cl^- was of no interest for milk because lactoperoxidase, unlike myeloperoxidase, does not oxidize Cl^-. Recent findings may revive the concept that leucocytes utilize SCN^-. In the past it was accepted that human milk contains lactoperoxidase (118) and it has been shown that *E. coli* are killed only after addition of SCN^- (and a source of H_2O_2) (36). Indeed, Hewitt, Public Health Laboratory Dolwich Hospital, London (pers. com.) successfully preserved raw human milk at 4°C in 1976. Considering that milk contains Cl^-, it appears that the enzymes oxidized SCN^- in preference to Cl^-; furthermore, the myeloperoxidase-SCN^- system proved to be dramatically more bacteriocidal than the bovine lactoperoxidase-SCN^- system because this strain was relatively resistant to the latter (see under mode of action.

These unexpected results appear to warrant a reassessment of the contribution of the myeloperoxidase-Cl^- system to the intracellular killing of bacteria by polymorphonucleated leucocytes (204). Cl^- is oxidized to OCl^- which is highly toxic; amongst other effects it lyses erythrocytes and would be expected to damage the membranes of the vacuola and lysosomes of PMN during the intracellular killing. It is therefore of interest to investigate whether the highly permeable anion SCN^- diffuses from the blood into the phagocytic cells and thus becomes oxidized instead of Cl^- (32, 36, 97).

Lactoferrin

So far it was not feasible to consider the fortification of artificial feeds based on bovine milk with milk lysozyme and lactoferrin, which are the major non-immunoglobulin protective proteins in human milk. It now appears that a technically feasible isolation and purification method for lactoperoxidase and lactoferrin has been developed (J-P. Perraudin & J-P. Prieels, s.a. Oleofino N.V. Bruxelles, personal communication) and it is to be hoped that this process can be extended to the purification of lysozyme. This advance in dairy technology increases the significance of the results obtained on feeding a formula feed fortified with conalbumin to infants with diarrhoea (120). Considering that adults with chronic diarrhoea (achlorhydric), who did not respond to antibiotic treatment, appear to benefit from conalbumin (Cornelli, Ricardoti, S.P.A. Milano, personal communication) it is expected that the effect of lactoferrin (and/or some of the other protective proteins) could become useful both for infants and adults.

Lysozyme

There is enough evidence available to show that lysozyme is resistant to digestion but there is no direct evidence that intestinal bacteria are lysed. Ideally, germ-free animals, mono-contaminated with susceptible bacteria, ought to be fed with lysozyme (preferably milk lysozyme) to assess its activity in the intestinal tract instead of trying to deduce its activity by analyzing the faecal flora which reflects the intestinal flora very poorly. Other interesting aspects are its synergistic activity with the other system and its possible adjuvant (immunomodulator) effect.

Recent research appears to show that endotoxins (lipopolysaccharides) of Gram-negative organism regulate some immune responses, at least in mice and piglets. Endotoxins seem to have an important role in inducing precursor surpressor cells in the Gut Associated Lymphoid Tissue (GALT) and in mediating one form of oral tolerance to small dietary changes in the new born (e.g. foreign proteins, ovalbumins, fed orally (214-218). It appears, therefore, that the natural encounter of antigens with GALT in the presence of endotoxin (as B-cell mitogen) may have profound effects on the host and mediate one form of oral tolerance in the newborn.

It follows that the Gram-negative intestinal flora has a role to play. The natural defence systems appear to be superior because they do not eradicate the Gram-negative flora unlike broad spectrum antibiotics but establish a balanced intestinal flora.

Xanthine oxidase

So far it has only been established that lactic acid bacteria colonizing the intestinal tract are capable ot producing H_2O_2. However, it is well known that acetaldehyde is a metabolic by-produce of lactic acid streptococci. It is therefore, possible to consider whether the enzyme-substrate reaction can produce sufficient H_2O_2 *in vivo*. Preliminary work was shown that the lactoperoxidase system can be "self-activated" in yogurt if both enzymes, i.e. xanthine oxidase and lactoperoxidase, are preserved (unpublished). Present practices of preheating milk at 80°C in the manufacture of yogurt destroy the enzymes. Since Metchnikoff's book "The Prolongation of Life — Optimistic Studies" (1907), wherein he retrieved the folklores on the "health.giving properties" of yogurt, a library could be filled with contradictory literature on the subject. However, it seems to have been entirely forgotten that the original yogurts were undoubtedly made from raw and not heated milk and an attempt ought to be made to investigate the effect on the intestinal flora by yogurt made from milk wherein the indigenous milk enzymes are preserved.

Milk Fat Globule Membranes

Although this subject has not been treated in the main text, it warrants future attention because earlier work has now been confirmed which ought to increase the discussion on non-immunoglobulin glycopeptides and glycoproteins in colostrum and milk (219). The milk fat globule (FG) is surrounded by a true cell membrane (unit membrane).

This can be shown by electronmicroscopy and is supported by the serological cross-reaction (agglutination) between bovine fat globule membranes (FGM), bovine red blood cells (RBC), fat globules are also agglutinated by the cold agglutinins present in blood and milk (220). The agglutination of RBC by *E. coli* possessing K_{88} or K_{99} antigens is inhibited by FG or FGM. The enzyme produced by *Vibrio cholerae* which destroys the RBC receptors, also destroys the receptor on the FG and FGM for coliform (221). The agglutination of RBC's however is non-specific because RBC of several species can be used for agglutination. The ability of *E. coli* to directly attach to FG or FGM was therefore investigated on the supposition that the FGM, as a true cell membrane, may possess the same specific receptors as intestinal brush borders. Indeed, it was found that entero-pathogenic bovine strains of *E. coli* possessing K_{99} antigen attach only to bovine FGM, porcine enteropathogenic strains possessing K_{88} antigen attach only to porcine FGM, while human enteropathogenic strains possessing CF_1 and CF_2, colonization factors, attach to human FGM but there was also a low attachment rate to porcine FGM (Plate 4) (for review on attachment, see 222). The bovine and porcine strains failed to attach to FGM after exposure to the lactoperoxidase system while the human strains remained unaffected. The latter strains which are mannose-sensitive (i.e. fail to attach to RBC's in the presence of mannose) were also inhibited by mannose from attaching to FGM. Clearly, the mode of attachment between these strains differs. These experiments raise the question whether this attachment is so strong that it hinders attachment to the intestine *in vivo* (36).

An attempt was also made to develop a simple test for the determination of attachment of strains of *E. coli.* Wright & Tramer (8) showed that some strains of *Str. cremoris* are agglutinated in milk and on standing are carried with the cream to the upper layer. This phenomenon explained why some starters are inhibited when grown in raw milk. For a long time a similar test has been used to detect brucellosis using the "Ring test" for *Brucella abortus* agglutinins whereby the organisms also rise to the cream layer if the milk contains specific antibodies (agglutinins). A photomicrograph published by Kenyon et al. (223) clearly shows the clustering of fat globules by brucella organisms and IgA and IgG; these immunoglobulins were actually isolated from fat globule surfaces. IgA described by the authors was probably secretory IgA. sIgA and the attachment of the major part of sIgA to fat globules, as well as a similar test based on agglutination of fat globule membranes have recently been described (224, 225).

E. coli specifically cluster on fat globules, which can be observed under the microscope (Plate 5). Alternatively, $10^7 - 10^8$ cfu/ml of the organisms can be inoculated into whole milk, which after incubation for 30 min at 37°C, is centrifuged to separate the fat. The bacterial could in the skim milk indicates the degree of attachment to the fat; reduction to $10^4 - 10^5$ cfu/ml was common (unpublished) (Table 12). The brush border membranes of susceptible and resistant phenotypes of pigs show different glycolipid patterns; this galactosyl residues are important (226, 227). Carbohydrate compositions of erythrocyte receptors from *E. coli* possessing K_{99} (calf enteropathogen) and CFA/II adhesins (human) were suggested by Faris et al. (228).

Considering the well-known similarities between the structural and chemical nature of RBC and FGM, it is reasonable to speculate that some of their constituents (glycolipids and glycoproteins) with various inhibitory activities, could be isolated from various milk fractions. Indeed, various milk fractions, non-immunoglobulin in nature, were shown to have interesting biological activities: inhibition of heat-labile enterotoxins of *E. coli* and *Vibrio cholerae* adherence (haemagglutination) (230, 231, 232) and a rotavirus inhibitor (188).

It appears, therefore, that the FGM can be used as a model for the intestinal brush border and the comparison between the composition of the membranes of RBC, FG and brush border, ought to be fruitful. If it emerges that the FGM play an active part in the prevention of attachment to the intestinal brush border *in vivo,* the dairy industry could produce an unlimited supply of membranes isolated from butter-milk.

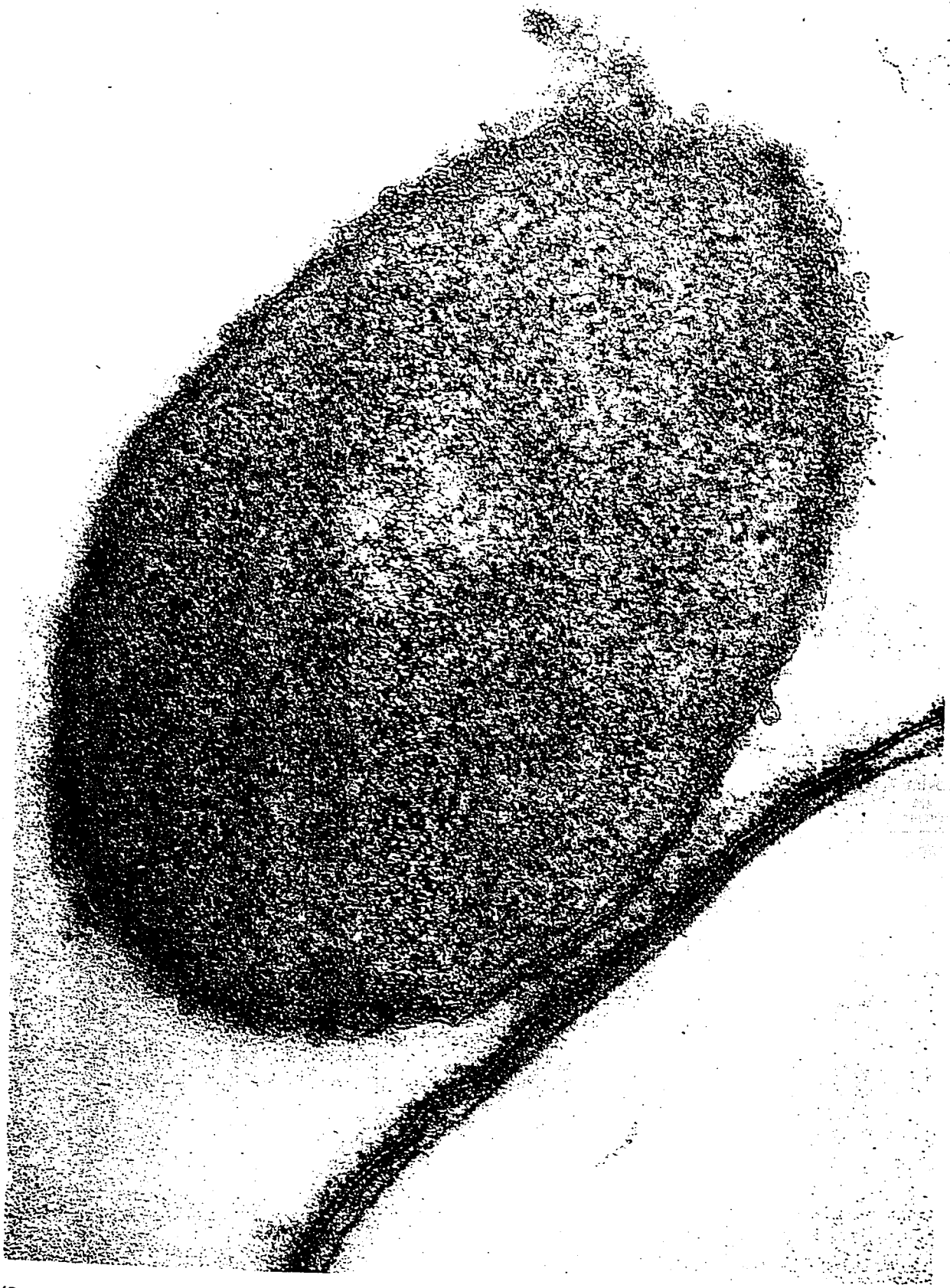
In this context it may be of interest to cite a paragraph written by Freter (233) in his contribution to the symposium "Association of enterotoxigenic bacteria with the mucosa of the small intestine": "Many foodstuffs, especially milk, contain toxins as well as oligosaccharides and proteins, some of which resemble similar substances in the intestine and therefore may also resemble receptor for bacterial adhesion (attachment). It should also be possible to enrich foodstuffs by the addition of known toxins and receptor materials. Many foodstuffs may therefore block bacterial association with mucosa by competing with mucosal receptor for adhesins ot with bacterial receptors for toxins ..... such mechanisms may explain some of the still enigmatic relations between diet and susceptibility to diarrhoeal diseases and may be responsible for at least part of the well known protective effect of mother's milk discussed elsewhere in this symposium". In 1969 Weiser et al. (234) were first to acknowledge the various non-antibody protective proteins: "Indeed, the attention of immunologists, which continues to be focussed largely on antibodies, has led to the virtual neglect of a second broad area in immunology concerned with non-antibody factors of host resistance to harmful parasites and other foreign agents, which play an important role by augmenting antibody action, but also by affording protection before the specific immuno response is effective".

This statement can now be rephrased and extended. There is such a strong analogy between phagocytosis and intracellular killing by leucocytes and milk – complements, immunoglobulines, lactoferrin, lysozyme, peroxidases so that ..... " ..... if we accept that leucocytes are the primary defence against invading organisms, we could regard colostrum and milk as liquid leucocytes because so many of the antimicrobial factors are common to both (33).

Hopefully, the dairy industry will eventually realise some of the aims discussed in this contribution.

Plate 4

Attachment of *E. coli* 09, K_{99}, K_{30} to bovine milk fat globule membrane.
Note the unit membrane structure x 100 000



(By courtesy of B.E. Brooker, NIRD, see also ref. 36)

Plate 5

Attachment to bovine fat globule

x 10.000

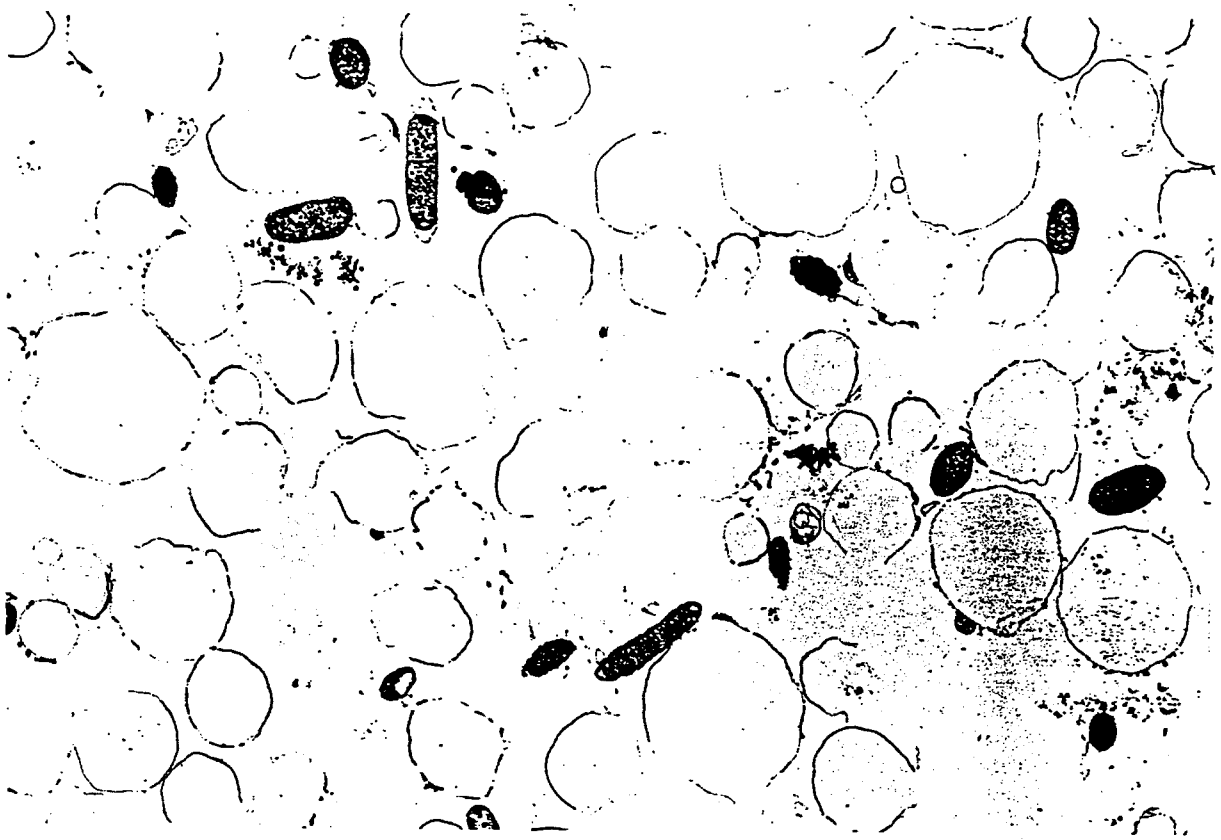


Table 12 — Specific attachment of *E. coli* to Fat Globule Membrane (FGM)

	FGM		
E. coli antigen	Porcine	Bovine	Human
Porcine K_{88}	2.5 - 3.5*	0	< 1.0
Bovine K_{99}	0	2.5 - 3*	0
Human CFA/II	0	0	2 - 3*

* *E. coli:* $\log_{10}$ difference between inoculum and number of bacteria attached to cream layer after centrifugation.

(unpublished)

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APPENDIX D

Some Differences between

Raw and Pasteurised Milk

Jan Aland

SOME DIFFERENCES BETWEEN RAW AND PASTEURISED MILK

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During the past 12 months or so, the proprietors of the last six raw goats' milk dairies in Queensland have battled to retain their licences. Two research booklets were purchased from the International Dairy Federation in Brussels, Belgium: "Protective Proteins in Milk – Biological significance and Exploitation" and "Natural Antimicrobial Systems in Milk", both published in 1985. In the process many other research articles were unearthed.

Along with nutritional components of protein, fat, carbohydrate, minerals and vitamins, raw milk contains antimicrobial protective proteins which can protect the infant from infections essentially within the gastro-intestinal tract (immunoglobulins, lactoferrin, lysozyme, complement components, active leucocytes) and enzymes (lactoperoxidase, lipase, xanthine oxidase, etc.) They play a role in digestion as well as protection against infection.

Raw goats' milk most closely resembles human milk in these components and scientific research has shown raw milk consumption essentially boosts the immune system, especially in children and invalids. It is easily digested, higher in minerals, higher in beneficial short-chain fatty acids and glycerol ethers (important for infants), higher in riboflavin, niacin and vitamin A.

"Actual factual proof that goats' milk is the best alternative to breast milk is being gained by biopsies of the lining of the gut of babies: at last doctors are producing indisputable proof of something which goat-keepers have known instinctively for years". (United Kingdom, 1977)

A strong synergism (combined total greater than components) exists between lactoferrin, lactoperoxidase, (hydrogen peroxide formed as a by-product) and thiocyanate (produced from feeds, especially tropical, and found to be high in goats), lysozyme, immunoglobulins and other protective proteins. So strong is this effect that inoculation of raw milk with Salmonella typhimurium, E.coli and Campylobacter jejuni and others results in the pathogens being destroyed and only lactobacilli and streptococci surviving.

Depending on the temperature, this can take up to 24 hours. Even clostridial spores are lysed (destroyed) in raw milk. This natural system is destroyed by pasteurisation, which alters milk and negates its role in the immune/digestive system.

Xanthine oxidase is one enzyme which survives pasteurisation. But, without its natural system, homogenisation allows some into the circulation where it creates havoc by attacking plasmalogen tissue within the artery walls. Further damage is done to the heart muscle. These lesions eventually harden into calcified plaques covered with cholesterol and obstruct blood flow, leading to high blood pressure and angina.

The heart disease death rate skyrocketed after the homogenisation of milk became commonplace in the USA. Circulatory disease is rare in countries in which raw milk is consumed. Further evidence in the case against heated milk protein was documented in 1972 and again in 1980, when antibodies to heated milk protein were linked to coronary heart disease.

A-lactoglobulin from raw cows' milk has a lower allergy-causing reaction than pasteurised.

A recent study (since verified) has linked pasteurised cows' milk consumption with incidence of insulin-dependent diabetes mellitus (IDDM) in 13 OECD countries. In 1996, researchers in Sydney found that exposure to formula in the first three months of life doubled the risk of diabetes, confirming a similar study in Finland.

The loss of enzymes make digestion very difficult for young babies whose own enzyme factory, the pancreas, does not become fully operational until three months of age. Those who cannot breastfeed will attest to a scenario of unsettled, crying babies with colic, excess mucus and even eczema (so-called easily-digested, heat-denatured milk proteins blocking the capillaries in the skin). Some babies even become sensitised to these proteins in utero.

Many experiments have been done on these enzymes and it is now known that though they are easily destroyed by heat, they can survive the wide range of pH change from the acid stomach to alkaline small intestine, to continue their role in the complexities of digestion. Losses in pasteurisation are critical for infants who derive most or all of their nutrition from milk.

Goats' and Cows' milks are presumed to be lower in folate than human milk. Three research papers found that provided oats received sufficient green feed, goats' milk contained folic acid, vitamins B12 and D at approximately the same level as human milk.

Research has shown raw milk consumption gives a higher haemoglobin as the transferring/lactoferrin systemassures the correct assimilation of iron and folate.

Raw milk is known to give better brain development in babies.

Anemia is a well-known side-effect of pasteurised milk if it is not fortified with ion. Thiis available iron, without the essential enzyme complex, allows growth of pathogens (and not beneficial lactobacilli and streptococci), causing diarrhoeal disease from; such as Salmonella, E.coli, Campylobacter, Vibrio cholerae etc. Nectrotising enterocolitis and other infections (especially aural) were problems with pasteurised formulas.

Recent research, not yet completed, has linked pasteurised milk to Attention Deficit Disorder.

Crohn's disease is thought to stem from atypical bacteria which survive pasteurisation, but not raw milk.

The risk of Sudden Infant Death Syndrome is related to allergy responses and anaphylaxis, specially with pasteurised milk and formulas.

Lactose intolerance is not such a problem in raw goats' milk because the enzyme lactase is still present.

Whey proteins with a higher nutritional value are affected by heat.

Our main thrust in meetings with the decision makers was that <u>there is a place for all milks.</u>