Muhammad Abubakar · Ali Saeed
Oguz Kul *Editors*

The Role of Biotechnology in Improvement of Livestock

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Preface

Biotechnology is undoubtledly one of the most important scientific areas in the future of mankind. In the second millennium, the cooperations between the scientific branches that are much more open to novelty such as molecular genetics, genetic engineering, proteomics, and targeted production technologies, have generated great opportunities for human and animal welfare, nutrition and health issues. Novel biotechnological products and techniques developed for the improvement of existing classical agricultural and animal husbandry will also be environment and nature friendly.

As biotechnology is a complexity of all the areas of cell and tissue culture, molecular biology, microbiology, genetic sciences, and almost all engineering technologies, it is targeted at development of new technologies and/or products that are closely related to productivity of animals, animal yields, or health. For example, recombinant DNA technologies have been commonly used to produce specific enzymes and proteins that are not sufficiently synthesized by the organisms themselves or that are not present in nature. Whereas the use of biotechnology in veterinary practice finds a very wide range from animal health to animal husbandry, generally it takes enormous and important duties for improvement of quality and quantity of animal products, development of industrially integrated biological products, reduction of waste products of animals, and environmental sensitive solutions. In this book, it is also emphasized the positive contributions of veterinary biotechnology on the following subjects:

- Embryo cloning, cryopreservation, embryo sexing, and transfer techniques
- Animal health, DNA, and recombinant vaccine technologies
- Recombinant drug, enzyme, and protein production using transgenic techniques
- Biotechnological approaches to animal nutrition and feed efficiency
- Impacts of biotechnology on the environment

Biotechnology has taken the first place among all the sciences that hit the mark in the twenty-first century, having close collaborations with nearly all other biological, engineering areas; it is an interdisciplinary branch. Thus, I and all contributing

authors believe that this book will be of use to students and experts who are studying veterinary, animal husbandry, biology, chemistry, medicine, pharmacy, agriculture, and other disciplines in engineering.

Contents

Chapter 1
Biotechnology and Animal Reproduction

Ahmad Yar Qamar, Aman Ullah Khan, Aatka Jamil and Muhammad Abubakar

Biotechnology has great impact on breed improvement, reproductive rate, and animal production.

The most common reproductive applications that are integrated with biotechnology are artificial insemination (AI), semen preservation, fertilization capacity of sperms, sperm sexing, synchronization and fixed-time insemination, superovulation, embryo transfer (ET), and in vitro embryo production (IVEP).

1.1 Artificial Insemination

Artificial insemination has been practiced on many domestic animals for hundreds of years. It is one of the earliest reproductive biotechnologies and permits the use of superior males for breeding purposes. This technique involves semen collection from superior males, its dilution, freezing, and deposition in the female reproductive tract. The first successful artificial insemination (AI) was reported in a water spaniel bitch in 1780 by the Italian scientist, Spallanzani and got three puppies. Spallanzani's work was confirmed 2 years later by another scientist Rossi (Roberts 1971). After initial work on bitches, AI was done in mares by Pearson. The AI technique in different farm animals is based on AI techniques of horses developed by Ivanow (1907).

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1.1.1 Semen Collection Methods

There are many methods of semen collection in domestic animals. Primitive methods involved semen collection directly from the vagina of an estrum female with the help of a spoon or syringe with a long nozzle. But the semen was contaminated, contained mucus, and the quantity recovered was minute. It had also a limitation of estrus female. Rowson in 1947 devised a method of making fistula in the urethral opening leading toward the vaginal fornix. Rowson's method involved semen aspiration directly from vaginal fornix by using catheter after natural breeding but it resulted in fibrosis, urinary tract infection, and also had the same limitation as the primitive method. After that, massage method and electro-ejaculator methods were developed.

1.1.1.1 Massage Method

This method was devised in the bull for the first time by Case in 1925 and modified by Miller, Evans, and Goodwin in 1934 (Roberts 1971). This method involves the massage of ampulla and seminal vesicle per rectum.

Indications

- Used in the case of aged bulls unable to mount.
- Used for crippled bulls.
- Used for bulls with decreased libido or with the problem of impotency.
- Used for bulls that are unwilling or unable to copulate.

Procedure

- Restrain the bull properly, handle it quietly, and keep it relaxed.
- Wash, rinse, and dry with a brush and cotton pledgets or with a clean towel the prepuce and the preputial hairs and the region around the preputial opening with warm physiological saline.
- During washing, stroking of sheath should be done to induce urination, which will not cause any contamination during collection.
- Operator, wearing a glove gently inserts a lubricated hand and forearm into the bull's rectum emptying it of feces.
- Massage the vesicular gland a few times in backward and downward fashion toward urethra. This will result in release of cloudy fluid containing few spermatozoa.
- The ampulla is then massaged in the same fashion. The semen is stripped with pressure against floor of pelvis.
- Sometimes pelvic urethra is also massaged.
- Another person holds a rubber cone attached to a collection vial placed in a plastic bag attached to a metallic ring about 7.5 cm in diameter with a long handle.

Limitations of Massage Method

- Skill and experience is needed for massage.
- Semen samples collected are not usually clean and contain many bacteria, as semen dribbles through the prepuce and drips from preputial hairs.
- More secretions of accessory sex glands and low sperm concentration.
- Sometimes sample may also be contaminated with urine.

1.1.1.2 Electro Ejaculator Method

This technique was first described and used in rams by Gunn in 1936. The probes used for this purpose are of different sizes and shapes depending on the species. This technique is painful for bulls and so was criticized by the Animal welfare personnel (Roberts 1971). That is why, it is only used in animals as in the below indications.

Indications

- Used in the case of aged bulls unable to mount.
- Used for crippled bulls.
- Used for bulls with decreased libido or with the problem of impotency.
- Used for bulls that are unwilling or unable to copulate.

Procedure

- Restrain the bull properly, handle it quietly, and keep it relaxed.
- Wash, rinse, and dry with a brush and cotton pledgets or with a clean towel the prepuce and the preputial hairs and the region around the preputial opening with warm physiological saline.
- The preputial hairs should be clipped.
- Operator, wearing a glove, gently inserts a lubricated hand and forearm into the bull's rectum emptying it of feces.
- Now the probe is inserted into the rectum placing it in the midline against the floor of rectum. Probe should also be lubricated with a noninsulating material like "K.Y" jelly.
- After the proper placement of probe, 3–5 V of current is applied for 3–5 s. It will result in erection and dripping of seminal fluids.
- After 3–5 s of current application, animal is given rest for 3–5 s.
- After resting, again the same amount of current is applied for the same time, then again rest is given. This process is repeated at least 5 times.
- Now current is increased up to 10–15 V. This current is applied for 3–5 s and after that the animal is given rest for 3–5 s as done with the low voltage. This will result in semen ejaculation.
- High voltage of 10–15 V is applied for 5 times with intervals as applied in low voltage.

- Another person holds a rubber cone attached to a collection vial placed in a plastic bag attached to a metallic ring about 7.5 cm in diameter with a long handle.

Limitations of Electro Ejaculator Method

- Skill and experience is needed for massage.
- Semen samples collected are not usually clean and contain many bacteria, as semen dribbles through the prepuce and drips from preputial hairs.
- More secretions of accessory sex glands and low sperm concentration.
- All bulls stiffen and show arching of back due to pain. Sometimes, bull may lean to one side or raise and extend one or both the rear limbs.
- This may lead to ataxia.
- Sometimes, choking may lead to death of animal due to pressure exerted by rear limb extension.

1.1.1.3 Artificial Vagina Method

This is the most widely used method. The early models made by Russians consisted of a bag like artificial vagina placed inside the vagina of a cow or the dummy. The AV used these days were developed in England, Perry, and Maule (Stephen). It is preferred over the other methods because the semen collected by this method is clean; complete ejaculate is obtained which is closer to natural ejaculation. There are two types of artificial vagina for bulls: a triple layer type or winter-type AV and a double layer type.

The artificial vagina of a *triple layer type* consists of three major layers, an outer casing made of plastic, a rectangular inner sleeve A made of rubber and a triangular inner sleeve B made of rubber. The inner sleeve A is fitted to the outer casing, and the inner sleeve B is fitted to the inner sleeve A. At one end of the inner sleeve B, there is a collection vial. Semen flows into the collection vial. This arrangement protects semen from temperature shock due to changing of temperature. But the main disadvantage of the triple layer AV is that it is longer and heavier compared to the double layer type.

The artificial vagina of a double layer type is shorter and lighter than the triple layer AV. It is used in tropical and warm regions. Due to shorter size it is easier to use. It consists of an outer casing, inner rubber liner, a cone, a collection vial, and an insulating jacket. About half to two-third of the chamber formed between inner rubber liner and the hard casing is filled with warm water. The water should be 125–180 °F, 50–70 °C. At the time of collection, the temperature of AV should be between 40 and 50 °C (MacMillan et al. 1966). Only small amounts of lubricant should be used for lubricating the inner liner. More quantity of lubricant will contaminate the semen. For this purpose, white sterilized Vaseline, K.Y. jelly, or pure white mineral oil are used (Fig. 1.1).

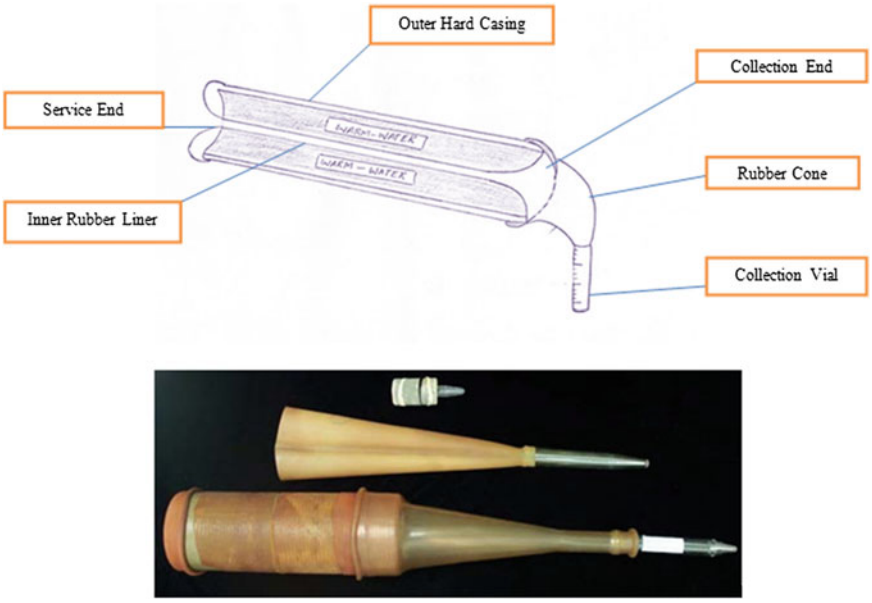

Fig. 1.1 Double layered artificial vagina

1.1.2 Advantages of Artificial Insemination

1. Artificial insemination not only increases the use of superior male animals but also makes their use more efficient. More people can be benefited from superior male. Use of the proven sires in dairy herds markedly increases milk production up to 30 % compared to natural breeding, Van Vleck.
2. Artificial insemination helps in great genetic improvement of farm animals. The selection and efficient use of superior bulls improves production.
3. Artificial insemination helps in controlling different venereal and other diseases like trichomoniasis, Vibriosis, brucellosis, etc.
4. The danger and expenses of keeping and handling bulls that prove to be inferior males can be eliminated.
5. It is easier to transport semen doses over long distances than to transport male animals.
6. Artificial insemination makes it possible to use the semen even after the death of a male.
7. Widespread use of artificial insemination in the dairy industry helps in proper breeding records.
8. Artificial insemination makes possible breeding of animals with size differences without injuries.

9. Artificial insemination made the use of those sires that are not capable of copulating, like aged or crippled sires.
10. It is a pre-requisite for embryo transfer.

1.1.3 Disadvantages of Artificial Insemination

1. Artificial insemination is an advanced and sophisticated technique, so well-trained personnel are required to supervise semen collection, examination, extension, freezing, shipping, and insemination of females.
2. Widespread use of artificial insemination increases the possibility of transmission of genetic abnormalities, for example COD, spastic syndrome, poor conformation especially of feet and limbs, and lack of libido.
3. Artificial insemination uses a limited number of elite bulls. This limited gene pool may improve milk production but it has a reverse effect due to increased inbreeding, which results in genetic abnormalities because of expression of recessive genes.

1.2 Sex Sorted Semen

Sex of the fetus is determined by the sperm because the sperm may carry either X or Y sex chromosome. Sperm having X sex chromosome when fertilizes an oocyte will result in a female and a sperm having Y sex chromosome when fertilizes an oocytes will result in a male offspring. The desire to separate X and Y bearing sperms is driven by the fact that one sex has more economic importance than the other for certain species. As in the dairy industry, the female calves are more important than the males because of maximum utilization of AI. As the major income of a dairy farm comes from milk, so it is advantageous to have more female calves that will become future producers (Senger 1999).

1.2.1 Procedure

The technique used for separation of X-bearing sperms from Y-bearing sperm is known as "Flow cytometry or cell sorting". Experiments have resulted in 80–90 % of successful separations of sperms in rabbit, cattle, and swine. It is well known that the X and Y chromosomes have different quantities of DNA. It is said that the X-bearing sperm has 2.8–4.2 % more DNA compared to Y-bearing chromosome depending on the species (Senger 1999). On the basis of difference of DNA, we can separate the X-bearing sperms from Y-bearing sperms. For this purpose, a DNA

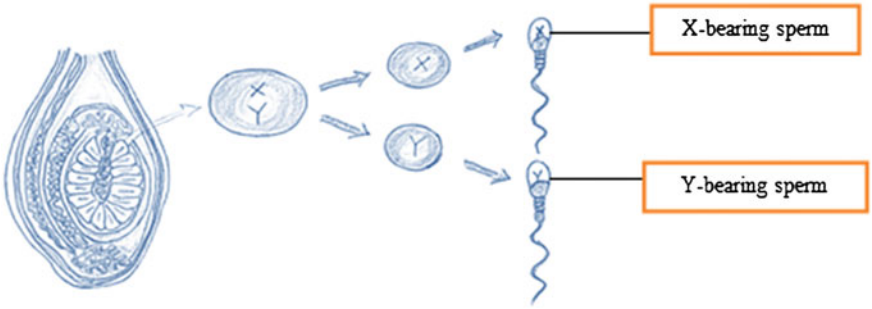

Fig. 1.2 X and Y bearing sperms produced by testes

stain or dye is used called Fluorochrome. The X-bearing sperm will take more DNA dye compared to the Y-bearing sperm. Vital stains used for sperm staining have a property of emission of light of specific wavelength when excited or activated by a light of specific wavelength.

Step 1: Collection of the semen from the male by using different methods as explained earlier (Fig. 1.2).

Step 2: Treatment of semen with Fluorochrome (Sperm staining dye). X-bearing sperms will take on more stain compared to Y-bearing sperms (Fig. 1.3).

Step 3: Once the spermatozoa enter the flow cytometer chamber, they pass single file through a small nozzle. After staining, the stained spermatozoa are excited by a laser beam. As a result of excitation, the X-bearing sperms emit more light compared to Y-bearing sperms. Sperms will emit light of different wavelength depending on their liveability and DNA contents. Dead sperms emit a very low beam of light when excited by laser beam, so they are easily differentiated (Fig. 1.4).

Step 4: After being excited, sperms pass through a light sensing device that is coupled with a computer. This device will determine the amount of light emitted by sperms and also order the passage of each sperm through a column below the nozzle.

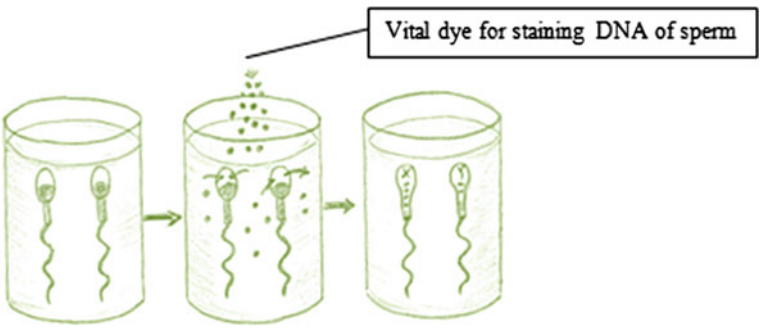

Fig. 1.3 Ejaculated sperm treatment by DNA dye Flurochrome

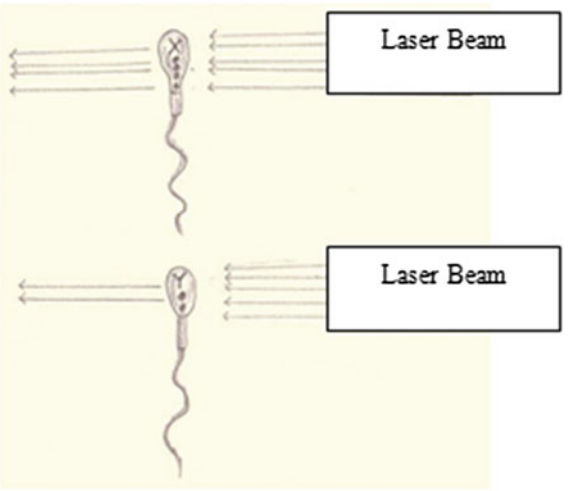

Fig. 1.4 X and Y bearing spermatozoa emitting different intensity of light when excited by a laser beam

Step 5: After that, sperms are charged on the basis of their DNA contents as they pass through the charged plates. Sperms are assigned either positive or negative charges depending on their DNA content (Fig. 1.5).

Step 6: Then the micro droplet containing a single sperm is passed through an electromagnetic field; the computer applies an appropriate charge and directs the droplet to one side or the other. Dead sperms are directed into the central tube. There are three vessels for sperm collection, one having higher proportion of X-bearing sperms, one having higher proportion of Y-bearing sperms, and one vessel having low proportion of dead sperms.

1.2.2 Limitations

Regardless of the problems associated with the separation of X-bearing sperm from Y- bearing sperm, the main limitation of this procedure is the cost of equipment. Also, the separation rate of this flow cytometer is slow.

1.3 Controlled Breeding and Synchronization

Early detection of estrus is becoming a major concern with the extension of the number of cows reared, the improvement of milking cows with high milk production, and the changes in the circumstances of feeding and management of cows.

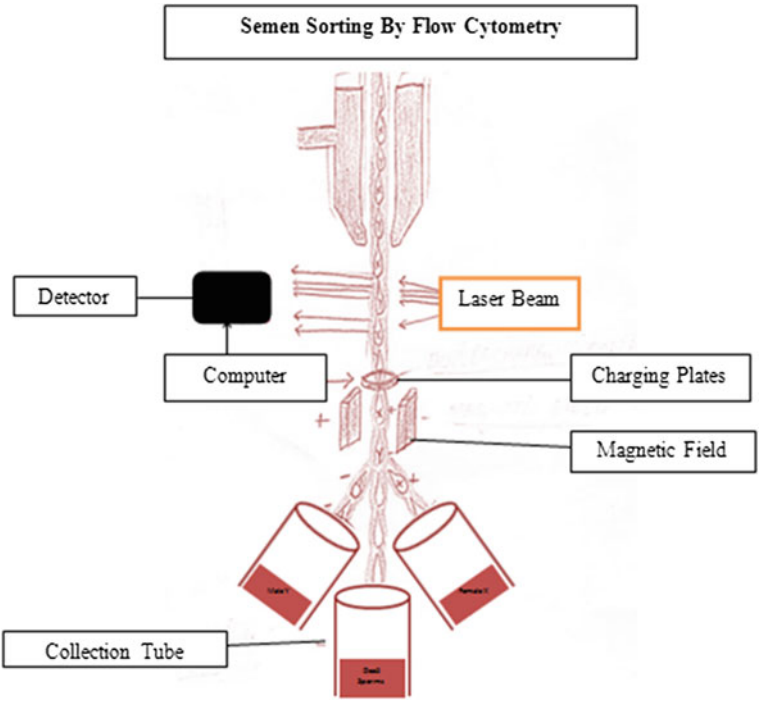

Fig. 1.5 Separation of X- and Y-bearing spermatozoa by flow cytometry

Estrus synchronization or controlled breeding is grouping of females for parturition at the same time. It is used at commercial dairy farms for uniform milk production throughout the year. It is closely linked with AI and is also a pre-requisite for embryo transfer, or is the first step of embryo transfer. Estrus detection is a major problem but by use of synchronization, we can reduce the time required for estrus detection with timed insemination (Sa'Filho et al. 2009).

1.3.1 Principle

- **Prolonging the luteal phase**
  - with P_4 for 9–14–21 days, on withdrawal follicular growth ensue, estrus, and ovulation occur within 2–8 days.
- **Shortening the luteal phase**

  $PGF_{2\alpha,}$ also E_2 regress CL within 1–3 days and estrus and ovulation occur.

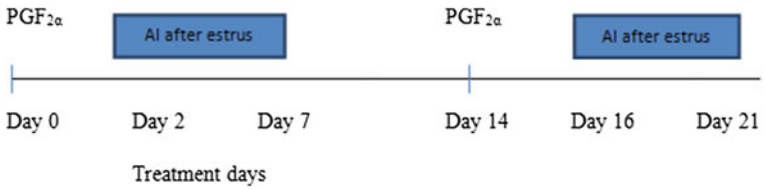

Fig. 1.6 Double PG protocol

1.3.2 Estrus Synchronization Protocols

1.3.2.1 Double PG Protocol

The main objective of double PG protocol is to have a high percentage of animals in Diestrus at the time of second injection. Double PG protocol involves an injection of prostaglandin (PGF2α) at random stage of the cycle. Animals with a mature corpus luteum (CL) will undergo regression after PGF2α injection and will come in heat within 2–5 days (Morrow 1986). A second shot of PGF2α is given after an interval of 11 or 14 days. The second shot will bring the remaining animals in heat. Artificial insemination is done after 3 days of the second shot (Fig. 1.6).

1.3.2.2 Ov-synch Protocol

Ovsynch protocol involves the use of $PGF_{2\alpha}$ and GnRH to synchronize ovulation in dairy animals. This protocol involvs the use of time AI (TAI), resulting in conception rates similar to that of AI after a detected estrus.

Procedure
Day 0: Inject GnRH to ovulate follicle and start a new follicular wave
Day 7: Inject $PGF_{2\alpha}$ to regress CL
Day 9: Inject GnRH to ovulate follicle
Day 10: Timed AI 16–20 h after second GnRH (Fig. 1.7)

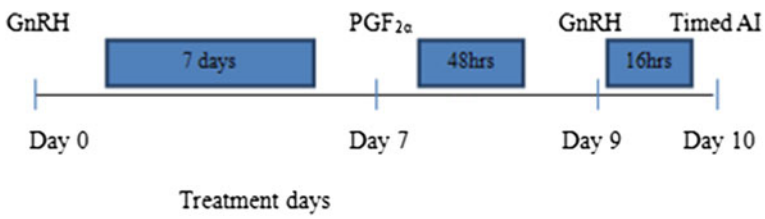

Fig. 1.7 Ovsynch protocol

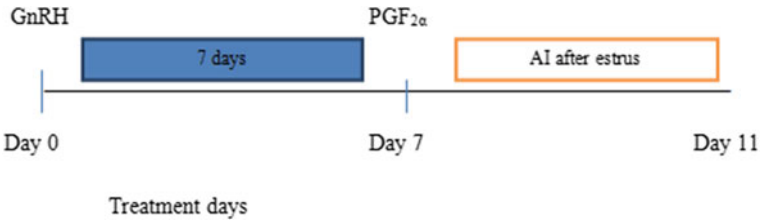

Fig. 1.8 Select synch protocol

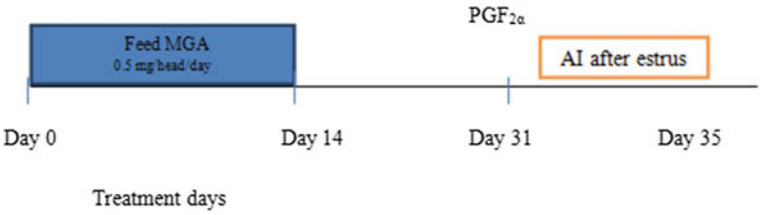

Fig. 1.9 MGA protocol

1.3.2.3 Co-synch Protocol

It is a modified method of Ovsynch. In this protocol fixed timed insemination is conducted at the same time of the second GnRH injection. Conception rate is low compared to Ovsynch, but labor is reduced in this method.

1.3.2.4 Select Synch

In the select synch program, the second GnRH is not administered. $PGF_{2\alpha}$ is injected 7 days after the first GnRH injection. After $PGF_{2\alpha}$ injection, the animal is observed for heat and is inseminated at the proper time. For the cow in which estrus is not seen, the fixed timed insemination should be conducted 3 days later. Select synch can be used in conjunction with TAI in a program called hybrid synch, by inseminating cows detected in heat after $PGF_{2\alpha}$ and conducting TAI+GnRH injection for the animals not detected in estrus by 84 h after $PGF_{2\alpha}$ (Fig. 1.8).

1.3.2.5 MGA Protocol

Malengesterol acetate is used only in the case of heifers, and not recommended in the case of heifers. It is generally fed in a grain carrier and either top dressed onto

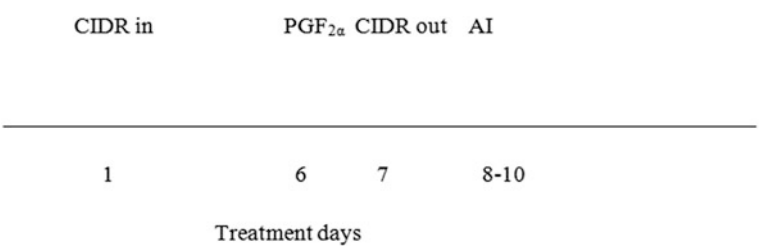

Fig. 1.10 CIDR protocol (designed by authors)

feed. MGA is given in feed (0.5 mg/head/day) for 14 days and $PGF_{2\alpha}$ is administered on either the 17th day or after the conclusion of MGA supplementation. Insemination is done 12 h after estrus (Larson et al. 1995) (Fig. 1.9).

1.3.2.6 CIDR Protocol

In this protocol, a progesterone-based intra-vaginal device CIDR is placed in the vagina of the dairy animals for 7 days. $PGF_{2\alpha}$ is injected on the 6th day of CIDR placement. CIDR is removed on the 7th day. Insemination is done after 12–24 h of estrus detection (Naseer et al. 2011) (Fig. 1.10).

1.4 Embryo Transfer

Embryo transfer is a technique in which embryo before implantation stage is collected from a donor female and is transferred to a recipient female that will serve as a surrogate mother for the remaining pregnancy. This technique has been applied to a large number of domestic and wild animals.

1.4.1 History of ET

The first successful transfer of rabbit embryos was done in 1890 and in bovines, embryos were collected successfully in 1930. The technique was developed in the 1970s and 1980s on a commercial basis. Initially, this technique utilized surgical procedures and is the main reason for early rejection in early days. ET grew in

popularity with the development of nonsurgical method. In buffalo, birth of calf by embryo transfer was done by Drost et al. in 1983. Birth of a buffalo calf from a frozen embryo was done by Nemat Ullah in 1987.

1.4.2 Basic Steps for Embryo Transfer

- Synchronization of donor and recipients
- Superovulation (FSH, luteolysis with PGF)
- Insemination at estrus (Day 0)
- Collection of embryos using flushing media (PBS) (Day 5)
  - Surgical (laparotomy) or nonsurgical (trans cervical)
- Grading of embryos-Cryopreserved
- Transfer (Fresh or Frozen) in recipients (Day 5)
  - Surgical or nonsurgical
- Pregnancy test.

1.4.3 Selection of Donor Cow

The selection criteria for donor depend on the species and also on the demands of breeder that will be more on the basis of economic issue than genetic issues. In bovine, the donor is selected on the basis of the following criteria.

- Most important is the performance records of the animal, show ring appeal, or both.
- The reproductive tract of the donor should be normal. For that purpose, properly palpate it through rectal palpation.
- Embryo transfer should be done after 60 days of previous parturition.
- Animal should have a regular estrous cycle beginning at a younger age and normal estrus interval.
- It should have a history of no more than 2 breeding pre-conception.
- First three calves born within two calendar years.
- No conformational or detectable genetic defects.
- No calving difficulties or reproductive irregularities.
- From 3 to 10 years of age. In older cows, muscles in the perineal region have less tone and tend to suck air into the uterus and rectum after the administration of epidural anesthesia. This is reduced by completely full rumen or by lifting the fore limbs resulting in positive pressure.

Table 1.1 Super ovulation treatments in cow

Day	Time	Treatment 1	Treatment 2	Treatment 3
10	AM PM	2500 IU PMSG	5 mg FSH 5 mg FSH	5 mg FSH 5 mg FSH
11	AM PM	Recipient receives $PGF_{2\alpha}$	4 mg FSH 4 mg FSH	5 mg FSH 5 mg FSH
12	AM PM	Donor receives $PGF_{2\alpha}$	3 mg FSH 3 mg FSH	5 mg FSH 5 mg FSH
13	AM PM		2 mg FSH 2 mg FSH	5 mg FSH 5 mg FSH
14	AM PM	AI	AI	AI
15	AM PM	AI AI	AI AI	AI AI

1.4.4 Superovulation of Donor

The most favorable and optimal time for superovulation treatments is between the 8th and 14th day of the cycle (Morrow 1986). Superovulation treatment will result in release of multiple eggs at a single estrus. Cows or heifers that are properly treated can release 10 or more viable eggs. Nearly 85 % of the donors respond to superovulation treatment protocols.

Treatments. Superovulation treatment protocol involves the use of single injection of 1500–3000 IU of equine chorionic gonadotropin (eCG) or previously known as pregnant mare serum gonadotropin (PMSG) in cow (Eldsen et al. 1978). However, response was better in terms of ovulations, embryos recovered, and pregnancies reported after superovulation treatment protocol using follicle stimulating hormone (FSH) twice daily in decreasing doses for 5 days or 5 mg FSH twice daily for 5 days (Eldsen et al. 1978) (Table 1.1).

1.4.5 Insemination of Animals

Most of the superovulated females are inseminated multiple times. One example: Inseminate the superovulated cow at 12, 24, and 36 h after onset of standing heat. Site for semen deposition: body of uterus or into the entrance of each uterine horn.

1.4.6 Selection and Preparation of Recipient

It is a critical step for embryo transfer success. Recipient must have the following characteristics.

- Recipient must have proper nutrition (BCS 6).
- Recipient should have gone through herd health program.
- Synchronization of estrus (within 1 day) between the donor and recipient cow.
- Recipient must not have any problem in reproductive tract.
- Recipient should not have weak CL.

1.4.7 Embryo Collection

In the early days till 1975, surgical techniques were used for ET. Surgical methods resulted in adhesions of the reproductive tract which in turn reduces the fertility of a very expensive elite animal. In addition, requirement of different facilities and recovery of embryos at farm level was impossible by surgical method. As non-surgical method was developed which was preferred over the surgical method as it was not damaging for the reproductive tract, repeatable, and could be performed at farm level. Nonsurgical method involves the recovery of embryos from uterus by penetrating the cervix during early diestrus.

1.4.8 Preparation of Donor

- Restrain the donor by placing in a squeeze chute. Rectum is evacuated of feces and air.
- Check the number of CLs on both the ovaries.
- Now administer the epidural anesthesia.
- After epidural anesthesia, wash the perineal area, and tie the tail.
- After epidural anesthesia, ballooning of rectum may occur. So remove the excess of air by using stomach tube or pump.

1.4.9 Catheters

There are basically three types of catheters used in the nonsurgical method of embryo collection. Most commonly, two-way and three-way Foley catheters are used. Foley catheter is mostly used because it is low cost and easily available. However, it is soft and difficult to thread into the uterine horns.

Sometimes we may also use “Modell Neustadt” two-way catheter. This is stiff and long compared to the Foley catheter. It is more useful in those animals with long uterine horns.

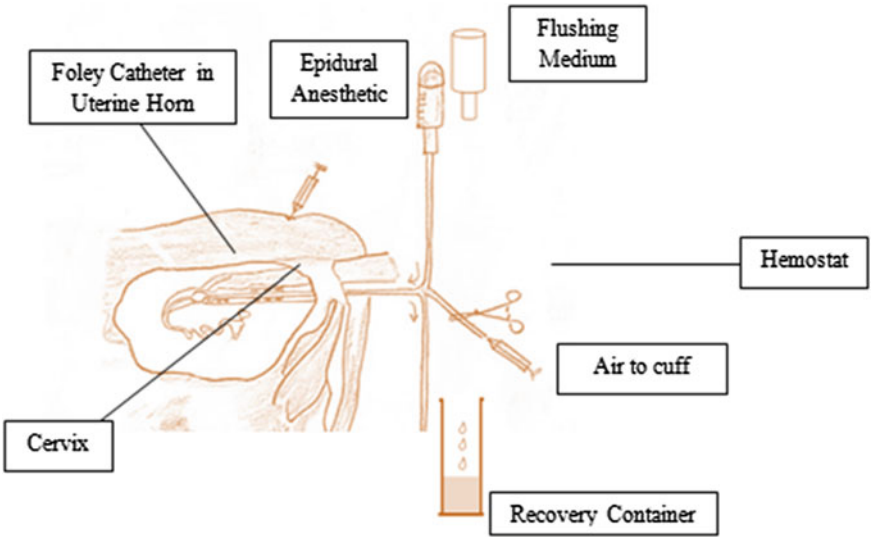

Fig. 1.11 Embryo collection by three-way Foley Catheter

1.4.10 Techniques

There are two methods of collection: the continuous way, closed-circuit system and the interrupted-syringe system. Closed system helps in maintaining sterility. It also prevents the loss of media and embryos. But extra tubing provides extra potential for contamination from either bacteria or chemicals. Interrupted method allows for the use of completely disposable equipment with the exception of catheter.

1.4.10.1 Interrupted Syringe Technique

The lubricated catheter with the stilette is passed through the vagina and the cervix. Sometimes cervical dilators are used before passing the catheter. The catheter is directed into the right uterine horn and stilette is gradually removed as the catheter is threaded down the horn. The catheter is so placed that the inflated cuff is present approximately half way between the uterine body and the tip of the horn. Inflate the cuff with 5 ml of sterile saline, then palpate with additional saline until the cuff completely fills the uterine lumen.

Collection is done with a disposable syringe using 25–35 ml of media per flush. The embryo is usually found in one to the first four flushes. Each horn is flushed at least eight flushes placed in 500 ml graduated cylinder. Normally, 85 % embryos are found in first four flushes. Do not force the media with pressure into the horn as it may lead to injury to uterus. In this method, first, inflate the uterine with media,

agitate the horn, and then remove this fluid. After repeating this procedure with both horns, each horn is infused with 30–40 ml of an antibiotic solution. $PGF_{2\alpha}$ may be given at this time or 1 week later to prevent pregnancy (Fig. 1.11).

1.4.10.2 Continuous-Flow Technique

Embryo collection is usually done using the three-way Foley catheter. The lubricated catheter with the stilette is passed through the vagina and the cervix. Sometimes cervical dilators are used before passing the catheter. Catheter is manipulated into the selected horn so that the inflated cuff is situated at the bifurcation of the horns. Inflate the cuff with 15 ml of the air or fluid, more can be added according to need. Approximately 500–800 ml of the media having temperature of 37 °C is placed in an Erlenmeyer flask fitted for infusion with tygon tubing. The tubing from the flask is attached with the inflow tube of Foley catheter. Another tube will be connected with the outflow of catheter carrying the flushed media into a 1000 ml graduated cylinder.

First, 20–30 ml of media is allowed to freely move in the system to check for blockages or to clear mucous or blood clots. Outflow tubing is then clamped and horn is filled with the media from inflow tubing. Massage the uterus gently and agitate the horn to dislodge the embryo from endometrial folds. Now outflow clamp is removed and inflow is clamped. Fluid is drained out of uterus into horn. After flushing wash the endometrium three times with media. $PGF_{2\alpha}$ may be given at this time or 1 week later to prevent pregnancy.

1.4.11 Searching of Embryo

Media after flushing is allowed to settle down for 35 min in a straight-sided cylinder. A siphon of sterile silastic tubing may then be set up to remove all but bottom 50 ml of medium. Remaining media is agitated, swirled, and aspirated into a syringe with a uterine infusion pipette. Place the media in a sterile, disposable petri dish. Observe the aspirated media under a stereo microscope (Fig. 1.12 and Table 1.2).

1.4.12 Transfer of Bovine Embryo

There are two methods of embryo transfer in bovines.

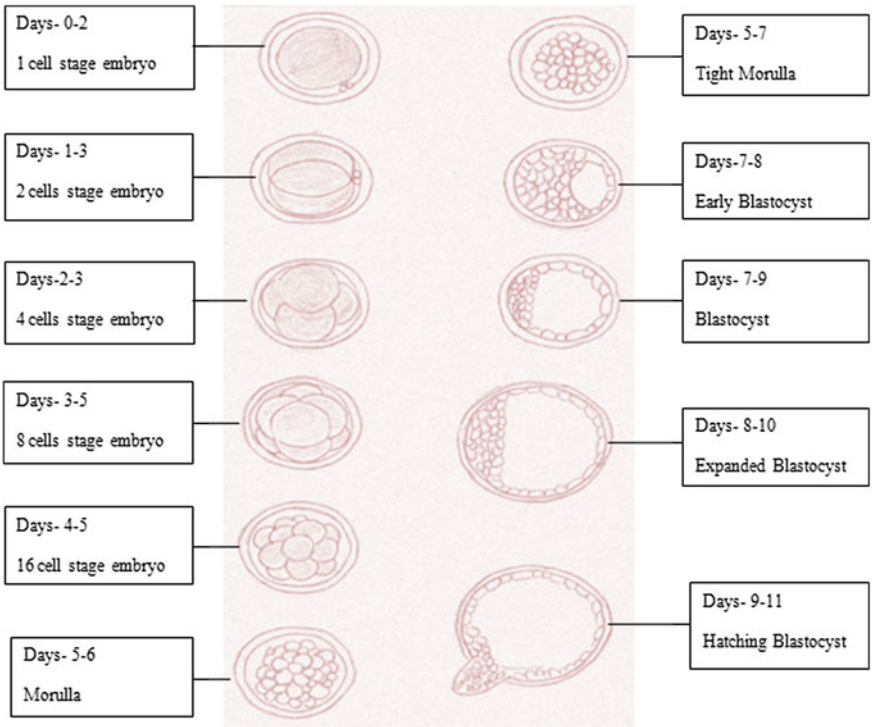

Fig. 1.12 Stages of development in pre-attached embryos

Table 1.2 Grading of embryo

Grade	Quality	Typical characteristics of embryo
1	Excellent	Embryo perfectly symmetrical, showing even granulation, and with a well-defined, distinct outline; no blastomere extrusion. The embryo should be at the expected stage of development for its age
2	Good	Embryo showing even granulation with a well-defined distinct outline; some blastomere extrusion and some minor blastomere degeneration; occasionally somewhat asymmetric in shape
3	Fair	Embryo intact but with a hazy outline in parts; obvious defects apparent such as extruded cells, vesiculation, and some degenerate blastomeres
4	Poor	Embryo showing uneven granulation with a hazy outline; much blastomere extrusion and degeneration evident; sometimes shaped abnormally
5	Degenerate	Degeneration so pronounced that it may not be possible to determine the exact developmental stage; sometimes shaped abnormally

1.4.12.1 Surgical Transfer Technique

Embryos are transferred by surgical method either by flank approach or by mid-ventral technique. This generally involves use of general anesthesia of the recipient, placing her in dorsal recumbency, surgical preparation of the mid-line just anterior to the mammary gland, and surgical invasion of the abdominal cavity. Once the uterus has been located and a suitable CL has been confirmed in one of the ovaries, a small puncture is made into the lumen of the uterine horn ipsilateral to the CL. The pipette loaded with the embryo is introduced through the puncture and the embryo is deposited. Routine closure, extubation, and post-operative recovery follow. To facilitate the procedure, most firms withhold feed (24–48 h) and water (12–24 h) from the recipient prior to midventral surgery.

1.4.12.2 Nonsurgical Transfer Technique

Many methods had been used in the past for transfer of embryo through nonsurgical technique. These include the use of telescoping stainless steel rods with flexible polyethylene tubing to deposit embryo in the cranial portion of uterine horn, transvaginal technique using stainless steel needles to circumvent the cervix and polyethylene tubes for embryo deposition. However, the most commonly used equipment for nonsurgical embryo transfers in the bovine is the Cassou AI gun and either 0.5 or 0.25 ml French straws. The recipient is examined for the presence of an appropriate CL and a normal uterus, and an epidural anesthesia is given to eliminate rectal contractions. The straw is loaded into the AI gun and the sheath is placed over it. The vulvar area is cleaned and wiped dry. The Cassou gun is inserted to the cervix and crossed through the cervix into the horn that is ipsilateral to CL.

1.5 In Vitro-Fertilization (IVF)

In vitro-fertilization is the collection of oocytes from a donor female that are matured in the lab and fertilization of that matured oocyte in a laboratory dish. The embryo resulting from that fertilization is cultured in a specific media for a few days and ultimately transferred into a female recipient. The eggs after collection are placed in CO_2 incubators in the IVF laboratory. Most viable spermatozoa are recovered after processing for inseminating the eggs. Because of the thick layer of zona pellucida and thousands of follicular cells around the ova, embryologists usually add approximately 100,000 spermatozoa for an egg. The addition of large number of viable spermatozoa to each ova will disperse the follicular cells and also

ensure fertilization of egg by one spermatozoa. IVF has been used to treat many infertility issues, i.e., when both fallopian tubes are blocked, fertilization of the egg cell has to take place outside the body.

1.5.1 History of IVF

For many years. The first cattle calf was born by using IVF in 1982, Brackett et al. In buffalo the birth of a live calf by using IVF technique in 1992, Totey et al. The first foal was born in 1991 by using this technique by Palmer and his associates. In goat, the first kid born through this technique was by Hanada.

1.5.2 Advantages of In Vitro Embryo Production

- Circumvention of the problem of timing ovulation for AI.
- Potential for producing more embryos.
- Make possible the use of animals suffering from certain issues of infertility such as tubal obstruction, Endometritis, etc.
- A reduced number of viable sperm needed for IVF compared to AI or natural breeding.
- By using sperm microinjection techniques, the potential of using nonviable sperm (Goto et al. 1991) and testicular or epididymal-derived sperm (Uehara and Yanagimachi 1976) for assisted fertilization.
- Potential of salvaging genetic material from female animals after death.

1.5.3 Embryo Production

In vitro production of embryo requires the recovery of oocytes and completion of three biological phases: maturation of oocytes, fertilization and developing the fertilized zygotes to the blastocyst stage, when they can be successfully frozen and subsequently transferred to recipients.

1.5.3.1 Sources of Oocytes or Donor

Any female after puberty is a potential donor of oocytes with a single requirement of presence of antral follicles on the ovaries with greater variations in the development competence of oocytes amongst donors (Lazzari and Galli 1993). Oocytes may be collected from slaughtered donor ovaries by aspirating; dissecting or slicing

(Caroan et al. 1994). Dissection technique allows the isolation of individual follicles. Sectioning of ovarian tissues gives the highest number of oocytes. Aspiration is the most efficient technique in terms of the time to obtain oocytes. Ovum pickup (OPU) by puncture of ovarian follicles in the live donor by laparoscopy or ultrasonography. OPU can provide four–eight oocytes per collection and may be an alternative to superovulation in the future. Oocytes recovery from live donors is accomplished by transvaginal ultrasound guided aspiration of follicles. This is done once or twice a week and can be repeated for many weeks (Kruip et al. 1994); resulting in average four to eight oocytes per session per donor.

1.5.3.2 Fertilization

Commercial frozen semen is used for fertilizing the oocytes. For fertilization, the motile sperms may be separated from the extender by several means. Direct washing can be used when a high percentage of sperms are progressively motile. Swim-up is also a common technique; however, it is not used with very viscous extenders. Centrifugation on Percoll gradients (45–90 %) ensures the highest recovery of motile sperms. The required number of sperms is diluted in bicarbonate buffered TALP IVF containing heparin (Parrish et al. 1989). The sperm-containing medium is dispensed in micro drops under paraffin oil or simply in wells without oil. At the same time, oocytes are matured for 22–24 h, after removal of cumulus cells. Oocytes are then placed in fertilization media and coincubated with sperm for 18–24 h at 38.5 °C in CO_2 incubator. The fertilization rate can be assessed indirectly by examination of cleavage rate 40–42 h after fertilization, but for more accurate analysis it is necessary to fix some oocytes 18–22 h after fertilization. Oocyte fixation is done in 3:1 ethanol:acetic acid for 24 h stained with lacmoid and observed under phase contrast microscope.

1.5.3.3 Embryo Culture

There are three systems for culturing the zygotes. In the first system, fertilized oocytes or cleaved embryo is transferred to ligated oviduct of recipient (sheep or rabbit). Five or 4 days later (i.e., 6 days post insemination), the embryos are recovered, graded, and frozen or transferred (Eyestone et al. 1987). In the second system, zygotes are co-cultured in vitro with somatic cells (oviductal epithelial cells, granulosal cells, buffalo liver cells, etc.) in medium TCM 199 or B_2 Menezo with 10 % serum or 1 % bovine serum albumin (BSA) at 38.5 °C in 5 % CO_2 or in medium conditioned by somatic cells (Vansteenbrugge et al. 1994). In the third system, zygotes are cultured in simple medium without any somatic cell support. A common medium for this purpose is synthetic oviductal fluid (SOF) (Tervit et al. 1972) in which serum is added (Walker et al. 1992) or BSA and amino acid addition in SOF (Gardner et al. 1994); incubation is performed at 38.5 °C in 5 % CO_2 and 5 % O_2.

1.5.3.4 Embryo Transfer

Embryo is transferred to the recipient as such or it is first cryopreserved using 10 % glycerol in phosphate buffered saline or using ethylene glycol (Voelkel and Hu 1992). Embryos that are frozen using ethylene glycol can be directly transferred after thawing without the washing procedure required to remove the cryoprotectant (Galli and Lazzari 1996).

1.5.4 Micro-Assisted Fertilization

In vitro fertilization (IVF) is mostly used while dealing with issues of male infertility but success rates are reduced either due to failure of sperm passage through zona pellucida (ZP) or due to inhibition of sperm-oocyte membrane interactions (Rogers et al. 1979; Overstreet 1980; Cohen et al. 1985). Microsurgical manipulation could assist spermatozoa which are otherwise unable to fertilize. Conventional IVF is very helpful in treating long-term and rological infertility; however, when mammalian sperms lose their ability to penetrate ova both in vivo and in vitro conditions, the capability of spermatozoa to govern the embryonic development can be assessed by the use of different micromanipulation techniques (Kyung and Koji 2006), a last option for most of the diagnosed cases of infertility of males involves the use of micromanipulators for penetration of sperm into ova.

1.5.5 Micromanipulation Techniques

Oocytes are held and micromanipulated in culture medium droplet while monitoring under an inverted microscope. The ova is kept still by the help of a holding pipette applying a gentle suction and several spermatozoa are deposited either into the perivitelline space or a single spermatozoa is directly injected into the cytoplasm of oocyte by using injection pipette. Depending on the site of sperm injections, these techniques are categorized as zonal, subzonal, and intracytoplasmic inseminations.

1.5.5.1 Partial Zona Dissection

Partial zona dissection (PZD) is technically the simplest and least traumatic to the oocyte. Partial Zona Dissection was first introduced by Gordon and Talansky (1986) in mice using digestive effect of acid Tyrode's (AT) medium on the ZP (Cohen et al. 1989). During PZD, an opening is created for ensuring sperm penetration (Malter and Cohen 1989). This procedure is performed in sucrose to induce

limited shrinkage that will help in minimizing the occyte damage. The micropipette is injected through ZP into the perivitelline space and moved out through the opposite end of ZP without disturbing the cytoplasm of ova. Then oocyte is released from holding pipette and an opening is made in the ZP overlying the micropipette by rubbing against holing pipette. Some researchers have also employed laser for PZD as it is a simple and precise technique.

Cumulus Oophorus cells of all oocytes are removed with 0.1 % hyaluronidase for 1–2 min at 37 °C, 3–9 h following egg collection. The corona radiate of oocyte is assessed for cell expansion and grouped as expanded, tight, or very tight. The oocytes are rinsed three or four times and corona cells are removed manually with hypodermic needles. When the cells are too tightly adhered to the ZP, a window is cleared for visualization of the ooplasm. The cytoplasm is reduced to facilitate needle piercing using a 0.1 M solution of sucrose in Earle's medium. The sucrose is added in one step and completely removed after micromanipulation in three or four steps. After sucrose exposure, ~50 % of the oocytes of each patient are randomly chosen for micromanipulation. All of these oocytes are checked at regular intervals 12–16 h following insemination.

1.5.5.2 Subzonal Insemination

Subzonal Insemination (SUZI) involves direct injection of spermatozoa into the perivitelline space by penetrating Zona pellucida. On the basis of morphology, 3–6 spermatozoa are aspirated into a microneedle and injected into perivitelline space. As spermatozoa are directly injected into the perivitelline space resulting in bypassing of acrosomal reaction, the techniques to artificially induce acrosomal reaction have been developed as the use of reacted sperm will decrease number of sperms required for the process, ultimately decreasing the risk of polyspermic fertilization. Two methods employed for this purpose include: Incubate the sperms in Tyrode's media+follicular fluid (50 %) and for 24 h after washing. Then electroporation is done and again incubated in 3.5 mmol/L of pentoxifylline. Although this method resulted in 54 % of sperms without acrosome, the efficacy was questionable.

1.5.5.3 Intracytoplasmic Sperm Insemination

Intracytoplasmic sperm injection (ICSI) has been described as beneficial in alleviating infertility in couples that could not be helped by standard in vitro fertilization (IVF) treatment or by subzonal insemination (SUZI) of the oocytes (Palmero et al. 1992, 1993; Van Steirteghem et al. 1993). Most of these issues of infertility are due to severe male-factor infertility (Van Steirteghem et al. 1993). The first successful use of ICSI demonstrated that freeze-dried human spermatozoa could develop into pronuclei when injected into hamster oocytes (Uehara and Yanagimachi 1976). Later, birth of normal calves after transfer of blastocysts was obtained following ICSI using bovine spermatozoa (Goto et al. 1990).

Microinjection of isolated sperm heads into oocytes is performed on a microwarm plate at 37 °C at 200X magnification using a piezomicromanipulator controller. Injection and holding pipettes used are mostly made of borosilicate glass capillary tubes. Sperm heads are aspirated into the injection pipette in a minimal amount of medium. Tip of pipette is brought in contact with zona pellucida of ova that is held by holding pipette. Zona is drilled by applying two to three pulses. Once the tip has reached the perivitelline space, it is forced onto oolemma and then into cytoplasm. After confirming that the tip is inside oocyte, a small amount of cytoplasm is withdrawn; a sperm head in a minimal amount of medium is expelled into the oocyte and pipette is withdrawn gently from oocyte. The injection procedure should be completed within 50 min after oocytes preparation. Before culturing, the ICSI oocytes are treated with ionomycin in combination with 6-dimethylaminopurine (DMAP) for activation of oocyte (Roh et al. 1998). All oocytes used in ICSI are washed thrice with TALP having 3 mg/ml BSA and cultured for 1 h in the same medium (50 ul) covered with paraffin oil in a culture dish in CO_2 incubator at 39 °C. After culture, oocytes are treated with 10 uM ionomycin in TALP containing 1 mg/ml BSA for 5 min at room temperature and then ionomycin-free TALP having 30 mg/ml BSA for 5 min to stop the activation process. The treated oocytes are washed thrice with culture medium and cultured for 3 h in the same medium at 39 °C under 5 % CO_2. Then transfer the oocytes to culture medium having 1.9 mM DMAP and culture for further 3 h. Finally, after washing thrice with culture medium, oocytes are cultured in the same medium at 39 °C under 5 % CO_2 (Kyung-Bon and Koji 2006).

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Chapter 2
Biotechnology and Animal Nutrition

Mehmet Basalan and Muhammad Abubakar

Abstract To fulfill increased world food demand and to overcome the consequences related to natural and industrial changes, scientists have been trying to select and improve both feedstuff and livestock, genetically. Biotechnology, also involving chemical and physical techniques, is applied to nutrition to increase the abundance (availability) of feed and to improve the digestibility of nutrients in those feeds. Additionally, animal nutrition studies are conducted to determine the safety of human food and modeling of some human diseases. Manipulating animal ability to absorb and utilize more nutrients starts from the plant breeders and continues until where those nutrients are utilized in the body. Microorganisms that have symbiotic life with livestock organism are transgenically manipulated to improve nutrition. Adding new genes to feedstuff gives nutritionists more applicational tools for improving nutrition and animal health through feeds. In contrast, application of recombinant DNA technology to farm animals needs more effort and may result in uncontrollable consequences.

2.1 Introduction

The success of genetic modification of livestock and their inner and outer environment depends on how scientists quantify desirable traits, select the marker genes for those traits, and redesign them in superior individuals or populations with a cheaper and more effective way than conventional systems. Despite the genetically engineered application benefits such as reducing the cost of production, scarce material and energy usage, and easing the processes by cutting the steps, etc., the public view

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toward biotechnology is always of concern and convincing them of safety may need special attention and efforts as much as efforts on DNA research. Although the current business in biotechnologically invented and sold products is around a couple of hundred billion dollars ranging from biopharmaceutical substances to enzymes, aminoacids, growth promotants, etc. (Demain 2007). In the future, using single cell protein as the only source of protein and amino acids for animals, or transferring the genes that express high amount of certain amino acids, other nutrients, or any other desired traits from various plant or forage sources to one single forage such as alfalfa (Cunnigham 1999) may solve the problem of nutrient deficiencies in livestock.

2.2 Recombinant Technology for Digestive System

2.2.1 Modification of Ruminant Digestive System

Rumino-reticulum is the main digesting environment in ruminating animals and both microbial and animal's own enzymes and chemicals work together to digest feeds and synthesize new nutrients and other substances. One of the nutrients that ruminal microorganisms digest is cellulose and its bound complexes with other substances. However, microbes and, therefore, rumen environment have limited capacity to digest cellulose substances compared to other polysaccharides such as starch or proteins. Therefore, manipulating ruminal microorganisms and the relative population of certain species might help to increase the digestibility of high woody plants or low quality feeds. In fact, ruminal fermentation is a complex system and manipulation may not always be successful although other variables of the ecosystem are closely controlled. Presumably, manipulation of the rumen system can be achieved by feed manipulation, host animal manipulation, and microbial manipulation (Nagaraja 2012). So many attempts in biotechnology have been made to manipulate the rumen environment. Since microorganisms utilize the major nutrients of plants, such as cell wall polysaccharides, their efficiency is considered manipulable. Basically, four groups of microorganisms occupy the rumen, including bacteria, methanogens, protozoa, and fungi (Nagaraja 2012). Giving one or a couple of them a chance to grow better, or in other words, decreasing the number of one or couple of them was the initial thoughts of manipulation. Lactate utilizing bacteria inoculation in rumen system had a best fit for animals prone to acidosis and such products called probiotics are produced after microbial fermentation commercially in fermenters (DiLorenzo 2011).

Rumen's ability to utilize ingested materials depends highly on how well the fermentation is constructed and how efficiently the products are removed. Some ways of manipulating rumen environment includes reduction of lactic acidosis and methanogenesis, enhancement of acetic, conjugated linoleic, or propionic acid production, manipulation of protein and fat degradation, improvement of microbial protein synthesis, and elimination of undesirable microorganisms such as protozoa and end products (Scheire and Tamminga 1996). Currently, more than 200 species of

bacteria are isolated from the rumen (McAllister et al. 2006). In spite of this diversity, only 20 of them dominate the population being 10^{15} cells/g of digestive fluid in rumen. The majority of rumen bacteria are obligate anaerobes, but the number of facultative anaerobes can reach up to 10^7 cells/g of rumen contents (Yokoyama and Johnson 1993). Initially, it was thought was to reduce some species by adding ionophores to diet and disrupt bacteria cell membrane and suppress the gram-positive bacteria population (Cunnigham 1999). In this way, volatile fatty acid production was shifted and efficiency increased. The other method of manipulating the ruminal environment was to add live microorganisms to the rumen, especially using aspergillus and saccharomyces, which resulted in a benefit for production (Gaggia et al. 2010). Rumen protozoa being larger than bacteria occupy more volume than bacteria with less numbers per gram of fluid (10^3–10^6 cells/g). When it was discovered that ruminants can survive without protozoa (Lindsay and Hogan 1972), protozoal predation of bacteria by protozoa attachment to the fiber particles from outer layers (McAllister et al. 2006) and their symbiotic living with methanogens and, therefore, decreasing ruminal protein metabolism (Finlay et al. 1994), attempts were made to decrease or get rid of ruminal protozoa (Hristov et al. 2003).

Like protozoa, yeast cells involved in plant cell wall digestion and may occupy 8 % of the rumen biomass. Methanogens attracted attention previously only for the loss of energy from the ruminant; currently, because of global warming attempts are toward reducing methane emission by culturing and trying to decrease their growth. Additionally, mycoplasmas and bacteriophages are natural residents of the rumen and may share their functions with other living organisms (McAllister et al. 2006).

Identification and determination of the function of rumen microbes is difficult being in semi-purified culture systems. Previous methods of identification and enumeration of rumen microorganisms were dependent on microscopy, substrate utilization, and defining certain parts of the microbial cells which were biased, time-consuming, needed large amount of colonies, and inaccurate. Recently, real-time PCR methods to sequence 16S rRNA gene for bacteria and 18S rRNA for protozoa have been used to determine the rumen inhabitants in their natural ecosystem (McAllister et al. 2006; Nagaraja 2012).

Based on the fact that most gram-positive microorganisms are lactate producers, ionophore antibiotics are used to change their membrane ion exchange barrier with their highly lipophylic activity. Since gram-negative bacteria have some large membrane molecules to resist ionophore actions, propionate to acetate ratio increases, and propionate used as energy source improves the performance. Additionally, ionophore antibiotics indirectly reduce ammonia accumulation and both methane emission and therefore protein efficiency are improved and energy loss is restricted (DiLorenzo 2011). However, public concern toward feed additive antibiotics claiming that they transfer the antibiotic resistance gene to human microbiota resulted in banning the use of those ionophores in animal feeds (Gaggia et al. 2010).

In rumen there are several types of microorganisms actively involved in the digestion of fibrous, starchy, protein parts of the feeds and antinutritive substances that bind the minerals and other nutrients by their exogenous enzymes. The attempt to improve this enzyme activity has focused on three areas: first, the relative

increase in the population of certain bacteria depending on the ingested materials, second determining the genes responsible for synthesizing those enzymes and manipulating them, and third, the addition of exogenous enzymes before the material is ingested. As mentioned earlier, manipulating the feed and animal has attracted less attention than manipulating the ruminal microorganisms and several ways have been attempted other than subtherapeutic antibiotics, including enzymes, direct fed microbials (DFM), or probiotics, prebiotics, and some plant extracts (Beauchemin et al. 2006; Nagaraja 2012).

Whenever a biotechnological agent was discovered and started to be used for increasing animal performance, public concern has been raised about the issue of animal health, safety and welfare, and product safety as in the case of bovine somatotropin (bST).

Direct-fed microbials, like ionophores, came into use for improving health (i.e., reducing sub-acute ruminal acidosis (SARA)) and productivity. *Saccharomyces cerevisae* was the first yeast implemented in the ruminant feed industry because of its enhancing effect on milk production and live weight gain (Beauchamin et al. 2006). Fibrolytic enzyme addition to diet to manipulate rumen environment has been the one most extensively studied in recent years (Kahi and Rewe 2008; Nagaraja 2012).

While the poultry industry is well acquainted with exogenous enzymes, especially for antinutritive factors or those not produced by the birds' own metabolism, the ruminant industry is still progressing slowly on enzyme addition to diets. Ruminant diets contain more fiberthan other animals and generally 40–70 % of the diet constitutes plant cell wall materials. Considering silages are the largest proportion of ruminant diets, even in the highly digestible ration condition it does not have total tract neutral detergent fiber (NDF) digestibility higher than 65 % (Van Soest 1994). It was also proved that low pH rumen environment decreases NDF digestibility (Basalan 2000). Therefore, scientists put their effort to improve the fiber digestibility of fiber by adding cellulose and hemicellulase, either alone or combined with DFM (Beauchemin et al. 2006). Addition of acarbose (inhibitor of alfa-amylase and glucosidase to decrease rate of starch digestion and therefore ruminal acidosis) and amylase (to increase rate of starch digestion) (DiLorenzo 2011) may both be promising applications in certain feeding conditions. Enzymes used in fiber digestion are series of endo and exo-enzymes (Beauchemin et al. 2006) usually originated from either fungi (*Trichoderma, Aspergillus*, etc.) or bacteria (*Bacillus* sp.) (Ghorai et al. 2009; Nagaraja 2012).

Effects of enzymes on ruminant performance have been studied extensively and it was found that some of the enzymes may have promising effects; however, most their mode of action is not well known yet (Beauchemin et al. 2006). For example, some fiber digesting enzymes improved the major performance parameters (dry matter intake, average daily gain, and feed efficiency) in beef cattle, but did not improve DM intake or milk production in dairy cattle. Similarly, one enzyme may improve fiber digestion in alfalfa but not in corn silage probably due to the nature of the fiber (cellulose versus hemicellulose or lignin) (Colombatto et al. 2003). Enzymes are proteins and their stability in various acidic and temperature may be questionable and may affect their activity. Therefore, contradictory results in

production trials may be attributed to the supplier or enzyme selectivity to work on rumen environment (Beauchemin et al. 2006; Bhat 2000). Beef cattle diets do not have large amounts of fiber compared to dairy cattle diets and, therefore, fiber digestibility can be undermined (Basalan 2000). However, some studies showed positive effects on increasing ADF and/or ADF digestibility and growth performance of beef calves, especially in high roughage feeding (Beauchemin et al. 2006). Gencoglu et al. (2010) found that total tract organic matter (OM) and NDF digestibility was improved by addition of exogenous amylolytic enzymes in dairy cattle. Earlier dairy cattle studies showed some improvement in milk production but not in milk constituents in different stages of lactation periods; later studies did not see any significant difference in milk production by the addition of fibrolytic enzymes (Vicini et al. 2003). Proteases and lipases can be of major interest because of the protein starch relationship in rumen (Gohari et al. 2009).

DFM can be defined as live culture of bacteria helpful to host animal when introduced in the gastrointestinal tract for improving performance (Kmet et al. 1993; Saad et al. 2012). Many single or a cocktail of microbe strains have been available for the feed industry market over the years, however, their live activity and selectivity is always questionable (Canganella et al. 1996). Most studies of probiotics focus on reducing ruminal acidosis or improving the digestion by not letting the pH to stay long under a certain level in ruminants (Beauchemin et al. 2006). *Lactobacillus* sp, *Bifidobacterium* sp, *Propionobacterium* sp, *Enterococcus faecium*, *Lactococcuslactis, Saccharomycesscerevisae, Saccharomyces bourlardii, Eschericia coli* strain nissle, etc., are more commonly used microorganisms as probiotics (Saad et al. 2012). While probiotics of fungal origin work in adult ruminants well, probiotics from bacterial origin are likely to improve health and production in young and growing calves (Nagaraja 2012). Using lactic acid producing bacteria for elevation of ruminal pH is somewhat contradictory to common beliefs, however, their effects are generally explained as the promotion of lactic acid utilizers. *Selenomonas ruminantum* and *Megaspheraeldseni* are major lactate utilizing bacteria and their numbers in population can be increased by *Aspergillus oryzae* or *Saccharamyces cerevisae* by probably producing nutrients as growth promoters for those bacteria (Nagaraja 2012).

Probiotics act in the lower or upper intestinal tract in several mechanisms to improve productivity; including competition with pathogens to exclude them from luminal cell adhesion or to limit their nutrients in the environment, stimulation of immunity, production of antimicrobial substances for antimicrobial activity, increasing the barrier integrity, and regulation of cytokine production to reduce inflammation (Saad et al. 2012). Besides defining the microorganisms in probiotics, expected characteristics such as survival, toxicity, etc., and safety criteria should be presented (Gaggia et al. 2010). DFM application in some phases of livestock production integrations is more visible than in other phases. For instance, since calves were born without ruminal population established, pre-ruminal calves are more prone to exogenous pathogen microorganisms and therefore probiotics administration to them was found to be useful. Additionally, receiving cattle passes through very stressful phases such as dehorning, castration, vaccination, transport, implant application, etc., and therefore, DFM supplementation of animals in that

phase proved to be useful. Furthermore, dairy cattle in transition period (3–4 weeks prior to parturition and 30–70 days postpartum) are susceptible to many metabolic and birth-related diseases and their consequences may require the use of DFM or yeast (Baeuchemin et al. 2006).

2.2.2 Modification of Monogastric Digestive System

Recent trends in consumer attitudes toward food producing animals, including pigs and poultry, are safe and better quality products from welfare applied animals (Adams 2006). Pigs and poultry do not have pregastric fermentation ability and therefore all feeds should be digested intestinally. Most of the developments in this area are related to the enzymes that ease the digestibility of certain feedstuff or break the cleavage of digestible nutrients with indigestible counterparts. Monogastrics lack certain enzymes that catalyze katabolic reactions. Therefore, technologically produced enzymes such as beta glucanase can be added to overcome these deficiencies. Additionally, probiotics are the other groups of substances have future in poultry industry (Smulikowska 2006).

Poultry industry currently uses biotechnologically produced aminoacids, vitamins, organic acids, other solvents, and enzymes (Demain 2007). After glutamate (used as flavor enhancer), lysine, methionine, tyriptophan, and treonine are produced in large amounts each year for the purpose of poultry diet supplementation. Additionally, chemical production of vitamin production facilitates switching their production toward fermentation processes, which involve a few bacteria and exchange genes to produce more effectively (Demain 2007). Of that produced almost half the vitamins are used for the animal feed industry and 16 % of the enzymes are used in agriculture and livestock industry (Demain 2007).

The purpose of supplying enzymes, probiotics, prebiotics, and synbiotics to poultry is to increase the animal's own supply, reduce the hazardous effects of antinutritive factors, increase the availability and absorption of certain nutrients, and modify the GI tract population (Ferket et al. 2005; Bonneau and Laarveld 1999). Although most poultry enterprises heavily depend on corn-soy-based diets, availability of other energy sources and grains and other oilseed and protein sources necessitate the use of commercially available, technologically produced enzymes, and other products. When maize is substituted with wheat or barley, endo-beta-1-4-xylanases and beta 1-3, 1-4 glucanases are used in poultry diet, otherwise "viscous grains" prevent absorption and availability of nutrients in the intestine (Cowieson et al. 2006). The purpose of addition of these enzymes is to breakdown arabinoxylans and beta glucans, which are called nonstarch polysaccharides (NSP) together with some others. If they are not supplemented, feed conversion ratio (FCR) will be poor, slow body weight gain (BWG), and sticky droppings especially in young chicks will be observed in broilers and laying hens (Bhat 2000). Additionally, clean eggs, better yolk color, uniform animals, reduced environmental waste, and increased digestion of other nutrients may be observed by the addition of single or multi-enzymes.

Enzyme amount and proper ratio may be adjusted in diets of poultry depending on the rate of use of these grains. FCR was improved by 2–9 %, BWG was improved by 4–12 %, and apparent metabolizable energy (AME) was improved by 3–10 % by the addition of xylanase and glucanase to barley, wheat, corn, rye, and triticale-based diets in broilers, ducks, turkeys, and laying hens (Cowieson et al. 2006). Multi-blend enzymes also modify the intestinal flora resulting in pathogen reduction and the non-pathogen downside effect on digestion, absorption, and metabolism and therefore improved FCR by up to 8.6 % and BWG by up to 17 % (Ferket et al. 2005). Currently, the use of relatively new by-product feedstuff such as dried distiller grains soluble (DDGS) and others may urge the supplementation of various blends of enzymes in poultry and pig diets (Cowieson et al. 2006).

Approximately 60–65 % of phosphorus in cereal grain is bound with phytic acid (Walsh et al. 1993). Presence of phytate in GI tract of birds may stimulate endogenous secretion, including mucin and, therefore, limit the digestibility of other nutrients. Addition of phytase to balanced poultry ration may increase amino acid absorption and retain more energy besides P retention to reduce environmental pollution (Cowieson et al. 2006).

After public concern and ban of antibiotic use in feeds, probiotics attracted more popularity among poultry nutritionists which is considered to decrease pathogen shedding in every aspect of production (Gaggia et al. 2010). With the concept of competitive exclusion, probiotics may have effects against common pathogens and the origin of food-borne illnesses such as *Salmonella* spp., pathogenic strains of *E.coli*, *Campylobacter jejuni, Clostridium perfiringens*, and *Listeria monocytogenes* (Gaggia et al. 2010). Although few of the culturable species can be used as probiotics, they have relatively short shelf life and their survivability is most of the time questioned during feed processing and their passage through the digestive system with various pH environments such as low pH in gizzard (Ferket et al. 2005), *Lactobacilli* spp., *Bifidobacteria* spp., *Enterococcus faecium* have proved to be successful in eliminating pathogens in the GI tract and therefore improve performance during the early ages of broilers, possibly by affecting gene expression (Gaggia et al. 2010). Similarly, some of the egg production parameters were improved by addition of a mixed culture of probiotic microorganisms (Kurtoglu et al. 2004). There are attempts to increase the efficacy of probiotic microorganisms by modifying some of their genes; however, inconsistent outcomes for improvement in performance and health has been observed (Smulikowska 2006).

Currently, prebiotics are only from oligosaccharides with a degree of polymerization and are considered to be indigestible by animal enzymes in the upper portion of the GI tract (Saad et al. 2012). These indigestible oligosaccharides are commonly found in human and animal diets, however, their selectivity of nonpathogenic bacteria is still questionable (Gaggia et al. 2010). Prebiotics have several advantages over probiotics in that they are more stable during feed manufacture, do not require culturing, and are inexpensive to be produced (Ferket et al. 2005). Currently, Mannan-oligosaccharides (MOS), Fructo-Oligosaccharides (FOS), Inulin, Trans-Galakto-Oligosaccharides (TGOS), and Xylo-Oligosaccharides (XOS) have

been used in human and animal studies for their potential for selectively providing substrate for short chain fatty acid (SCFA) producing microbiota in the intestines (Saad et al. 2012; Gaggia et al. 2010). Possible mode of action was attributed to their potential for stimulating lactic acid bacteria and SCFA in seca (Ferket et al. 2005). Bacterial and fungal organisms, which have FructosylTransferase (Ftase) enzyme, are capable of producing FOS from sucrose and, by using recombinant DNA technology, FOS can be produced in *Pichiapastoris* yeast or in *E. coli* carrying gene from *Lactobacillus reutri* (Sangeetha et al. 2005).

A mixture of probiotics and prebiotics, called synbiotics, was achieved by combining lactobacillus with lactose, FOS with *B. subtilis*, GOS with *Bifidobacteria* spp and *E. faecium*, and chicory prebiotics and they were proved to have potential for health and performance improvement (Gaggia et al. 2010).

Studies of biotechnology in pigs have been directed to the phytase activity to reduce P excretion, NSPase activity to increase nutrient availability, and porcine somatotropin (PST) to improve growth performance. Additionally, gene mapping of pigs for their potential as an organ donor is the major biotechnological investigation area. Probiotics and prebiotics commonly used for all monogastric species have been investigated and used extensively in pigs and *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces cerevisea* are proved to be useful during diseases such as diarrhea, necrotic enteritis, and other pathogen-related GI tract diseases and during weaning of piglets (Gaggia et al. 2010).

2.3 Feedstuff Development Through Biotechnology

2.3.1 Increase in Abundance of Nutrient

Livestock production cannot be separated from other fields of agriculture since feeds are mostly plant origin. Plants have short generation cycle compared to animals and it is easier and cheaper to make genetic manipulations. Biotechnological applications in plants can be classified into two groups; the first type of applications were administered through disease or insect resistance of the plants; second, to improve the nutritional contents of the plants. Removing the antinutritive factors over the years helped nutritionists to get increased amount of nutrients in feedstuff for animals. In the last two decades, global biotech crop producing lands have been growing exponentially and occupies 66 % of the world's cropland (UN 2004). Using genetic engineering, the desired characteristics can be transferred from one organism to other, which cannot be achieved with conventional breeding techniques. Public concerns about animals that are fed with genetically modified (GM) or genetically engineered (GE) crops do not have a strong base, since those animals even if they do not eat any GM feed at all, may have enzymes, vaccines, hormones, probiotics, and prebiotics from GM sources (Novoselova et al. 2007). In 2003, transgenic crops for corn, soybean, cotton, and canola were 40, 81, 73, and

70 % of total production, respectively, in the US (Gavrilescu and Chisti 2005). In 2008, these numbers reached 90+, 87, and 90+ as an acreage for soybean, cotton, and canola respectively (Hartnell 2010).

Genetic coding of plants, animals, microorganisms, and many viruses are DNA and consist of 1000 or more nucleotides in plants and animals resulting in approximately 20 and 50×10^6 different genes in a plant (Beever and Kemp 2000). Currently, genetic modification has been achieved in a way that mutation occurs and inserting the gene into a new organism has been done sequentially prior to adding to a bacteria containing Ti plasmid (Beever and Kemp 2000).

Production of crops for livestock feeds has been improved by genetically modifying crop seeds. The crops mostly modified are the energy and protein sources of animals. Major energy sources are grains including corn (maize), barley, wheat, rye, oats, and triticale. Of them, corn is modified to resist insects, to tolerate herbicides, to carry more starch, protein, certain aminoacids, fat, certain fatty acids, and finally to resist drought and harsh climatic conditions. The first GM crops commercialized for livestock feed in 1996 in the US were herbicide tolerant soybean and canola, pest protected cotton, and corn (Alexander et al. 2007). Since then the number of transgenic plants, planting area, and public concern for GE plants is increasing exponentially. Marker-assisted technology speed-up the process and made it easy to determine whether transferred gene is working; gene mapping made the trait selection appropriate and metabolomics allowed to determine the gene inacceptable in certain environment (Steering committee on global NRC 2008). Transgenic applications started with single traits, however, currently 2–3 traits crops are used extensively. Although most of the transgenic products are used in developed countries, transgenic rice was first used in Iran and largely used in China (Herdt 2006). In human nutrition, attempts have been made to decrease undesirable properties of foods such as nonallergenic peanuts or hypocholesterol fats from corn and increase the desirable traits of plants such as high amino acid or protein materials or high sugar corn. With "Golden rice" which has high beta carotene content and the color is yellowish, more than 400 million people suffering from vitamin A deficiencies will be benefitted from transgenic manipulations (Herdt 2006). Often, animal nutritionists do not seem to be interested in the agronomical traits of crops, however, environmental conditions force plant breeders to develop transgenic plants to produce in harsh environments. For example, drought has been one of the main concerns in recent years and traits are chosen to resist heat and produce in drought conditions or consume less water during growth (Federoff 2010).

Transgenic corn hybrids that are currently grown and used for grain feed and silage feed was reviewed by Owens (2005). Corn grain was in the beginning modified to be resistant to common corn insects such as corn borer or corn rootworm, and later modified for high nutrient contents, high digestibility, or certain industry needs such as waxy corn for the chocholate or food industry. In the same paper, relative feed values of transgenic versus "near-isogenic" or transgenic versus control corn hybrids were compared (Owens 2005). High starch, high protein, and high oil transgenic corns showed higher performance against isogenic or control

corn. Future prediction of the author is that fiber digestibility of corn silage, lowering the lignin content, increasing certain amino acids, fatty acids content, or increasing the bioavailability of minerals may be the aim of genetic engineering in corn plant.

2.3.2 Increment in the Proportion of Certain Nutrient

The term "functional foods" or "nutraceutics" is used to determine the technology that satisfies the needs of organisms deficient in certain enzymes or needs more of a certain nutrient to keep them healthy or use special types of therapy without any problem with the help of nutraceutics. Certain nutrients such as selenium can be enriched. Additionally, certain amino acids, fatty acids, sugars, and enzymes can be modified (Deynze et al. 2008). Decreasing the lignin contents of crops and increasing the NDF digestibility of corn and other forage-related crops is currently underway (Owens 2005).

Nutrition versus functionality of human foods was reviewed by McGloughlin (2010) and besides macronutrients micronutrients such as phytochemicals and antinutrients such as allergens and toxins have been investigated and those genes which are responsible for them have been modified to obtain functional foods.

2.4 In Vitro Gene Technologies Help Nutrition

Enzymes manufactured in certain microorganisms can help in digestion of specific nutrients. Biotechnology-related products have been investigated and approved by the regulating authorities regionally or nationally in individual countries, and the last figure was more than 75 industrial enzymes used in various industries produced using biotechnology (UN 2004). Many microorganisms exchange genes to produce one or more enzymes to be used exogenously in animal nutrition. While exogenous enzymes are widely used in the poultry industry, enzymes in ruminant animals have been directed only at polysaccharidases (Beauchemin et al. 2006). The industry has used microorganisms to produce enzymes, ferment foods and feeds, and treat to reduce waste traditionally. However, rapid expansion of the enzyme industry led to production of 20,000 tons in 2005 and an expected expansion of 5–10 % each year. For example, Trichoderma produced cellulose can be used in the textile and feed industries (Gavrilescu and Chisti 2005). In vitro methods of production are cheaper, time saving, and have highly controllable and adjustable processes. In vitro produced enzymes can be added to animal feed just prior to feeding, however, their efficiency in various environments such as low pH and high temperature should be closely monitored.

2.5 Technologies to Reduce Animal Waste

Reducing animal waste is discussed in another section of this book, however, since it is related to nutrition and certain nutrients, this topic has been the major interest of nutritionists. Choosing high protein digestibility crops as animal feed will reduce wastage of nitrogen and will not pollute the underground water. Phytase enzyme breaks down the linkages between phosphorus and phytin, which avoids phosphorus from being digested. Phosphorus release into the environment poses threat to lands that have shallow underground water drainage. Methane was thought to be only loss of energy by the ruminant animal, however, it is considered as one of the major contributors to greenhouse emission and thus reducing methane through biotechnologically engineered feeds and rumen microorganism is currently being investigated.

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Chapter 3
Vaccines and Vaccination

Hasan Tarik Atmaca

Abstract Livestock vaccines aim to increase livestock product and improve the health and welfare of livestock animals in a cost-efficient manner and prevent disease transmission. Successful livestock vaccines have been generated for pathogens including bacterial, viral, protozoan, and multicellular pathogens. These livestock vaccines have a significant effect on animal health and products and on human health through growing safe food procurement and preventing zoonotic diseases. There are successful production of biotechnological-based animal vaccines licensed for use that include virus-like particle vaccines, gene-deleted marker vaccines, subunit vaccines, DIVA vaccines, and DNA vaccines.

Keywords Vaccines · Livestock · DIVA · Marker vaccines · DNA vaccines · Subunit vaccine · VLPs · Gene-deleted vaccines · Virus-like particles

The vaccine against rinderpest is considered a milestone in the history of veterinary vaccine production (van Gelder and Makoschey 2012). In 2011, Rinderpest, a deadly animal disease, was eradicated from the world. There is only one similar example for eradicated disease, that is smallpox in humans. The World Organization for Animal Health (OIE), is currently working on strategies to control other terrible infections including peste des petits ruminants (PPR), foot-and-mouth disease (FMD), and rabies. The global eradication of rinderpest has been a remarkable achievement for veterinary science. With the eradication of rinderpest in live animals livestock production in the world has become safer and livestock industries are less at risk (World Organization for Animal Health [OIE]).

Currently, there are no vaccines for several globally significant infections. The main problem is the complexity of correlates of protection. The other is difficulty in constructing the correct presentation of antigens for many of these lacking vaccines. Luckily, molecular technology has offered many new tools for developing vaccines (Plotkin 2008). Modern vaccines are based on the scientific progress in virology,

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cell biology, and immunology (van Gelder and Makoschey 2012). These molecular equipment and knowledges may enable the scientist to make more use of cellular and humoral immune responses to antigens that are the main features for achievement of vaccines now (Plotkin 2008).

There are successful production of biotechnological-based vaccines used in protection against diseases, produced for use. These are virus-like particle, gene-deleted marker, subunit, DIVA, and DNA vaccines.

This chapter reviews the literature concerning the usefulness of livestock vaccination against and biotechnologies in vaccine development.

3.1 DNA Vaccines

DNA vaccination presents a new simple concept in vaccination strategy. It is an antigen-encoding bacterial plasmid that produces an immune response when inoculated into a suitable host (Garmory et al. 2003; Josefsberg and Barry 2012). Potential applications of plasmid DNA and other gene therapy approaches have been a matter of debate for a long time (Wolff et al. 1990). DNA vaccination elicits neutralizing antibodies and induces T helper cell response of Th1 phenotype and cytotoxic T-lymphocyte response (Donnelly et al. 1997).

Immunization is carried out via ingestion of purified plasmid in the host cells and T cell recognition of antigen-presenting cells (APC) depends on their expression of a spectrum of peptides bound to class I and II major histocompatibility complex (MHC) molecules. Subsequent protein expression results in the presentation of normal or modified forms of the protein to the immune system (World Organization for Animal Health [OIE]).

DNA vaccines have more advantages than other vaccines. The first advantage is that DNA vaccines promote humoral immune response and cell-mediated immune response. These two responses are so critical that also known DNA vaccines can induce long-term immunity in many diseases. This has become a requirement for vaccine efficacy. DNA vaccines are easy to produce and purify, and this has enabled the production and evaluation of new DNA vaccines in animal models within months. Another advantage is the long shelf life of DNA vaccines and the capability to transport (no need to cold chain). Various studies, including on humans, show the reliability of DNA vaccines (Bagarazzi et al. 1998; Kim et al. 2001). DNA vaccinations have proved in rodents as efficient. Studies on DNA vaccination are continued even in outbred species (Carvalho et al. 2009; Redding and Weiner 2009). Currently, four DNA vaccines have been licensed for veterinary use. One is for swine in Australia, for growth of hormone releasing hormone, the other is against infectious hematopoietic necrosis virus for salmon in Canada, and the others are against west nile virus (WNV) for horses and melanoma for dogs in the USA (Kutzler and Weiner 2008).

Optimization at various levels is required in large animal species to succeed for higher influence. These include vector modifications, DNA submission paths and

procedure, addition of adjuvants, and antigen targeting to APC. Inadequate benefit of DNA vaccines in large animals is likely caused by ineffective transfection of the administered plasmids. The inefficiency of the vaccine interferes with sufficient induction of development of the immunity (Rao et al. 2009).

Protective immunity has been accomplished with DNA vaccines with Pestivirus glycoprotein E2, which is the major protective antigen for bovine viral diarrhea virus (BVDV) in cattle, and classical swine fever virus (CSFV) in pigs. In poultry, the possiblity of DNA vaccination against flu using plasmids coding for hemagglutinin (HA), the main target for neutralizing antibodies, alone or in mixture with nucleoprotein (NP), the primary target of the cytotoxic lymphocyte (CTL) response, has been established by several studies (Brun et al. 2011). Vaccines induce immunogenicity and enable protection with plasmid DNA against BHV in mice (Cox et al. 1993), malaria in mice (Mor et al. 1995; Sedegah et al. 1994), hepatitis B in chimpanzees (Davis et al. 1996), human immunodeficiency virus in rhesus monkeys (Lekutis et al. 1997), influenza A virus in ferrets (Donnelly et al. 1995), *Mycobacterium tuberculosis* in mice (Lowrie et al. 1994; Lynch et al. 2011), and genital herpes simplex virus in guinea pigs (Bourne et al. 1996).

Apart from these trials, recently, DNA vaccines have entered phase I human clinical trials for protection against HIV, malaria, and influenza.

DNA vaccines against many diseases are evaluated in experimental studies and animal models.

Real-time PCR and histopathological examination confirmed that only low viral DNA loads and mild histopathological lesions appeared in pORF2-immunized mice following virus challenge to show protective immunity against porcine circovirus 2 by vaccination with ORF2-based DNA and subunit vaccines in mice (Shen et al. 2008).

Bovine leukemia virus (BLV) envelope gene encoding extracellular and transmembrane glycoprotein gp51 and gp30 was cloned into vector under Human cytomegalovirus (CMV) intermediate early promoter for DNA vaccine construction. Cellular immune response was promoted when the intramuscular administration of this plasmid vector was done (Brillowska et al. 1999). In another study, BLV transactivator Tax cloned into Mammalian cell expression plasmid pME18-Neo showed that Th1 type immune response induced by Tax DNA vaccine inhibited BLV propagation in vaccinated sheep at the early phase of infection (Usui et al. 2003).

Gogev et al. (2002) showed the efficacy of Bovine herpesvirus 1 DNA vaccine in cattle. In the study Bovine herpesvirus 1 glycoprotein C was used as antigen. In calves, the administration of recombinants Ad5CMVgD and/or Ad5CMVgC, or a composition of them, increase Bovine herpesvirus 1 neutralizing antibody responses and presented protection against challenge with BHV-1 Iowa strain (Gogev et al. 2002).

Another research made on Bovine herpesvirus 1 DNA vaccine using Vector pBISIA88 expressed glycoprotein D (gD) (Pontarollo et al. 2002) and Vector pMASIA expressed full-length BHV-1 gB (Huang et al. 2005).

Schrijver et al. (1997) prepared a DNA vaccine by which the gE gene was changed by a gene encoding the G protein of Bovine Respiratory Syncytial Virus (BRSV) in a bovine herpesvirus 1 vector. As a result of the study the G protein of BRSV can stimulate considerable protection for BRSV infection in cattle, and the BHV1/BRSV-G vaccine protects powerfully against a subsequent BRSV and Bovine herpesvirus 1 infection (Schrijver et al. 1997).

Vaccination and also poultry flock management are very important. Vaccine research and currently available commercial vaccine numbers are increasing day-by-day.

Sakaguchi et al. (1996) generated a plasmid vector expressing the Newcastle disease virus F protein (NDV-F) under the control of the human cytomegalovirus immediate early enhancer and chicken beta-actin gene promoter. Their results exhibit that the DNA vaccine is useful against the Newcastle disease (Sakaguchi et al. 1996).

Park et al. (2006) generated a chimeric Avian Influenza Virus (AIV). This virus expressed the ectodomain of the hemagglutinin-neuraminidase gene in place of the neuraminidase protein of the H5N1 AIV. A single vaccination of chickens with this improved vaccine prototype virus induced strong immunity against H7N7, highly pathogenic AIV, and against Newcastle disease Virus (Park et al. 2006). Park et al. (2006) proposed that chimeric constructs should be developed for convenient, inexpensive, and effective immunization against Newcastle disease and avian influenza in poultry (Park et al. 2006).

DNA vaccines are evaluated not only for protection against viral or bacterial diseases but also against protozoan diseases that are associated with abortions in cattle.

Various experimental studies exist for protozoan diseases, one of which is recombinant DNA vaccine with NcSAG1 and NcSRS2 proteins against *Neospora caninum* for C57BL/6 mice (Cannas et al. 2003).

Results suggested highly significant protective effect with combined DNA (based on NcSAG1)/recombinant antigen vaccine (based on NcSRS2) in cerebral neosporosis in mice (Cannas et al. 2003).

3.2 Gene-Deleted Vaccines

The availability of recombinant DNA technology and the knowledge of specific virulence factors of the pathogens have further facilitated the use of gene-deleted pathogens as vaccines. In gene-deleted pathogens, the pathogenicity and virulence are decreased without affecting immunogenicity. Gene-deleted agents cannot cause disease; however, immunogenic features are the same as in wild-type. Such agents must be genetically stable, easily reproducible, and easy to manipulate organisms. Vaccine strains of bacterial pathogens have been created that provide better protection than inactivated or killed vaccines. Gene-deleted vaccine technology is also used in marker vaccines that are used to differentiate between infected animals from

vaccinated animals. Gene-deleted Salmonella enterica serovar typhimurium and serovar enteritidis have been licensed for use in poultry (Babu et al. 2004; Meeusen et al. 2007).

In pigs, pseudorabies virus marker vaccine generated a double gene (gE and TK) deleted virus (Ferrari et al. 2000; Meeusen et al. 2007). Also in cattle, BHV-1 virus marker vaccine produced gE deleted (Meeusen et al. 2007; van Oirschot et al. 1996). Another gene-deleted vaacine is against to Streptococcus equi. In horses, Streptococcus equi vaccine generated aroA gene deleted has been licensed (Jakobs et al. 2000; Meeusen et al. 2007).

Widjojoatmodjo et al. (2000) showed the possibility of generating deletion mutant viruses lacking the entire E2 or E^{rns} for CSFV. These mutants have the ability to develop in competent cell lines. So, they are safe vaccines. The mutant viruses stimulated powerful immuno response. The immune response comprise without generating new viral progeny (Widjojoatmodjo et al. 2000). Related strategies have been used to build up several chimaeras by exchanging the E2 or the E^{rns} proteins from the attenuated C strain of CSFV with those from antigenically related pestiviruses (Van Gennip et al. 2000).

The use of conventional vaccines against infectious bovine rhinotracheitis (IBR) does not seem to have consequenced in decrease of the prevalence of the disease. BHV1 marker vaccines comprise mutant in deletion of glycoproteins included genes which is nonessential. These marker vaccines can be used as diagnostic tests to separate cattle infected or vaccinated with marker vaccine (van Oirschot et al. 1996). One of them is the Bovilis IBR Marker for cattle distributed by Intervet.

3.3 Virus-like Particles

Virus-like particles (VLPs) involve one or more recombinant proteins. They have supramolecular structures. These particles are 20–100 nm in size. They have icosahedral or rod-like structure (Jennings and Bachmann 2008). VLPs are vaccine antigens and increase immunogenicity of the vaccine via their particulate structure. This offers an advantage for VPLs (Brun et al. 2011). VLPs can be used as a vaccine or as a carrier for genetically modified antigens and have been studied for more than two decades. Hepatitis B virus (Zuckerman 2006) and human papillomavirus (Stanley 2008) vaccines are commercially presented. Studies are underway to develop vaccines against bluetongue virus, Rotavirus, and Parvovirus for veterinary application. VLPs have many advantages as a vaccine. They have high safety profile feature. Also, they have similarity to bacterial and viral structures and capability for large-scale production. The other advantage is the possibility of combining with other vaccine adjuvants.

VLPs induce strong and rapid antibody response. Induction of T-independent IgM responses activated by B cell activation via B cell receptor activation via vaccine antigens cross links (Bachmann and Zinkernagel 1996; Brun et al. 2011; Thyagarajan et al. 2003).

VLPs are also processed as if they are exogenous antigens and presented by MHC class II molecules for helper T cell activation (Pejawar-Gaddy et al. 2010; Win et al. 2011).

Thus, the complement system activates and results in elevated phagocytosis. Strong immune response is further enhanced by the uptake of the particulate structure of VLPs by APC.

It was demonstrated that particulated antigens are much efficient than soluble antigens in inducing immune response (Lenz et al. 2001).

CpG ODN and single-stranded RNA can be successfully combined with VLPs as a molecular adjuvant. Dendritic cells Stimulated by certain VLPs directly, for example, papillomavirus VLPs.

Bovine rotavirus virus has VLPs and they are highly immunogenic and has elicited protection against infections (Redmond et al. 1993).

Other examples of vaccinations with VLPs include hepatitis B surface antigen VLPs (HBsAg-VLP), HIV 1, DV (dengue virus), Norovirus, and influenza A VLPs.

Many animal virus VLPs have been derived as an immunogenic. Examples for these VLPs include NDV, Porcine parvovirus (PPV), Rift Valley fever virus (RVFV) VLPs, Porcine circovirus VLPs, AIV, and bluetongue virus (BTV) VLPs (Jeoung et al. 2011; Lynch et al. 2011).

Many of these VLPs induce neutralizing antibodies and are protective in animal models.

Roy (2004) showed the immune protective effects of high immunogenic VLPs produced against bluetongue virus in sheep (Roy 2004). Stewart et al. (2010) demonstrated that bluetongue virus VLPs were to be safe, highly effective, immunogens in sheep, reducing post-challenge viraemia to level below the threshold molecular detection limits (Stewart et al. 2010).

VLPs vaccines currently are not available in veterinary field. It is anticipated that VLP vaccines are to be developed for use in this field in the near future.

3.4 Subunit Vaccines

Two technologies used in vaccine production offer useful antigenic structures for the induction of satisfactory immune response. These include live-attenuated and inactivated or subunit vaccines. Live-attenuated vaccines are effective; however, they always possess some risks. The major advantage of inactivated and subunit vaccines are that they are safe to use (Day and Schultz 2011). Despite this advantage, limited efficacy and limited immunization limit the use of such vaccines. DNA vaccines offer a new and strong approach over subunit vaccines (den Hurk et al. 2000).

Subunit vaccines consist of semi-pure or purified proteins. Subunit vaccines have been produced by recombinant DNA technology since the 1990s and commercially available since the 1980s (Cohen 1993; Rhodes et al. 1994; Ulmer et al. 1993, 1995). Since the first bacterial genome was sequenced in 1995, genome

sequences were obtained for a number of bacterial, viral, and parasite genomes. However, the development of the bioinformatics resources and tools required to analyze these genomes allowed identification of surface exposed antigens, specific B, and T cell epitopes.

Subunit antigen productions can be obtained by both biochemical and DNA technologies. Biochemical techniques used in the production of subunit vaccines are useful where recombinant expression is not appropriate. This can be shown in fimbria of the bacteria. *Campylobacter jejuni* glycosylates many surface proteins and, therefore, they are best produced in *C. jejuni* rather than heterologous expression systems. This also applies for *Escherichia coli* that shows similar features (Wacker et al. 2002).

Escherichia coli K99 vaccine for calf scours could be one of the best examples of subunit vaccines (Acres et al. 1979).

Another example is the baculovirus used vaccine to porcine circovirus type 2 (Fachinger et al. 2008).

Subunit vaccines offer some advantages. First, they stimulate strong humoral and cell-mediated immune response. They are considerably safe. They can be combined with other subunit vaccines.

In some occasions, subunit vaccines require adjuvants to induce a better immune response. Some glycoprotein production is costly with subunit vaccines. Subunit vaccines compatible with DIVA strategies. In bovine herpes virus, glycoprotein gD has been successfully used in subunit vaccine. Although immunization with gD has proven to be partially effective, it has not reduced the prevalence of the virus in the field, thus limiting its use (Harland et al. 1992; van Drunen Littel-van den Hurk et al. 1997).

Another research for Bovine Herpes virus 1 vaccination showed the subunit gD- and tgD-vaccinated calves shed significantly lower amounts of virus than the placebo or killed virus-immunized groups throughout the follow-up period. In the gD and tgD subunit vaccine vaccinated groups only one out of eight animals shed virus (van Drunen Littel-van den Hurk et al. 1997).

A licensed porcine circovirus 2 vaccine is reported as a characteristic in inactivated baculovirus expressed PCV2 ORF2 protein in pigs by Intervet as a brand name Porcilis-PCV2 (Blanchard et al. 2003). Another licensed porcine circovirus 2 vaccine is Suvaxyn PCV2 by Fort Dodge using the Inactivated PCV1-2 chimera (Fenaux et al. 2004). Licensed vaccine against Pseudorabies virus for pigs is Suvaxyn Aujeszky distributed by Fort Dodge. The characteristic of vaccine is being a IgE- and thymidine kinase-deleted marker vaccine (Ferrari et al. 2000). CSFV infection continues to be a subject in the studies exploring successful and effective vaccines. Vaccines are commercially available. The first one is Intervet and the other is Bayer. Baculovirus recombinant E2 protein has been used in the vaccines of both companies (van Aarle 2003).

Many subunit vaccines have been tested all of which are not commercially available. These include vaccines against respiratory and enteric viruses, BVDV, BRSV, PI3, rotavirus, and coronavirus. Success of bacterial subunit vaccines over viral subunit vaccines results from recombinant and conventional production costs.

Recombinant vaccines prepared against respiratory system pathogens are commercially available. These include vaccines against *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae*. These vaccines have been formulated from leucotoxins and transferrin-binding proteins of these bacteria. Vaccine prepared for atrophic rhinitis has been formulated with nontoxic *Pasteurella multocida* dermonecrotic toxin. This toxin has been produced together with genetically modified *E. coli* strain and conventional *B. bronchiseptica* bacterin (Shewen and Wilkie 1982).

Live-attenuated vaccines for classical swine flu (CSF) have a rapid onset of immunity and are effective at preventing transmission of infection (Van Oirschot 2003). The only disadvantage is that vaccinated pigs cannot be differentiated from infected pigs. Vaccine studies following DIVA are ongoing for classical swine flu (CSF).

3.5 DIVA Vaccines (Marker Vaccines)

Vaccines have been developed for many viral diseases occurring in farm animals. However, these vaccines interfere with the surveillance of the disease in serological tests and the individual country may lose its "free from infection" status. The most prominent example is the FMD that occurs in cattle. Many vaccines are available for use against FMD. These vaccines are used in disease control (Doel 2003); however, they are not used in FMD free countries.

Marker vaccines have been developed for use in diagnostic tests by deleting a certain gene of pathogen (DIVA-differentiating infected from vaccinated animals). Antibody is not produced against the deleted gene of the pathogen. Therefore, it is possible to differentiate vaccinated from infected animals (van Oirschot 1999). Many DIVA vaccines and diagnostic test kits for these vaccines have been developed. DIVA vaccines are used and developed against many diseases important for farm animals such as IBR, pseudorabies, classical swine fever (CSF), and FMD.

Originally this term was applied to gene-deleted marker vaccines for large DNA viruses when used with their vaccine specific serological tests, but it can also apply to subunit vaccines, heterologous vaccines, or some killed whole pathogen vaccines such as the highly purified FMD vaccine, which is used in conjunction with nonstructural protein-based serological tests (Ahrens et al. 2000; Capua et al. 2003; de Smit et al. 2001; Sorensen et al. 1998).

Numerous strategies have been used to develop DIVA vaccines against CSFV, with variable achievement regarding their capacity to reduce viral replication in animals or to avoid transplacental transmission of the virus. These vaccines include full-length CSFV E2 glycoprotein or fractions, mainly (Hammond et al. 2000, 2001).

Glycoprotein E (gE)-deleted DIVA vaccine for IBR has been developed with European conventional methodology (van Oirschot et al. 1996). gE protein gene is not required for viral replication of IBR. However, this gene plays an important role

to spread across the host cells. ELISA technique and PCR amplification have been developed for diagnostic tests of gE-deleted DIVA vaccine (Perrin et al. 1996; Schynts et al. 1999). DIVA strategy developed by deletion of gE gene is also used for Aujeszky's disease (Pensaert et al. 2004).

DIVA vaccine developed for classical swine flu (CSF) has been produced in viral envelope glycoprotein E2 protein baculovirus/insect cell system and formulated with adjuvant. ELISA diagnostic kit has also been produced for this DIVA vaccine (Moormann et al. 2000; van Aarle 2003). As usual, DIVA vaccines have some disadvantages compared to conventional live or attenuated vaccines (Beer et al. 2007).

The host body must be exposed to more than one epitope of the pathogen in order to induce a strong immune response. With this purpose, studies have focused to develop vaccines combining capsid proteins (Grubman 2005).

In many diseases, DIVA vaccine is needed for surveillance or epidemiological data. These particularly include peste des petits ruminants virus, bluetongue virus in cattle, NDV and AIV in poultry, and bovine viral diarrhea virus.

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Chapter 4
Modification of Animal Products for Fat and Other Characteristics

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Abstract This chapter includes information about modification of animal products using biotechnology and the importance of different modifications on the natural composition. The species considered for modified products include beef and dairy cattle, sheep, goats, poultry, and a wide variety of fishes. Moreover, the discussion includes the importance of animal food, nongenetically engineered animal modified food products, genetically engineered animal modified food items primarily for meat, milk, or egg and genetically engineered animal food along the transgenic approach for animal welfare. Modern biotechnology can improve productivity, consistency, and quality of alter animal food, fiber, and medical products. The transgenic technology is potentially valuable to alter characters of economic importance in a rapid and precise way. The food safety issue related to genetic engineering is also included in this chapter. The harm of such modified food and transgenic strategy should also be understood by the reader along with its advantages. In this context, transgenic approaches in animal biotechnology are under discussion that ranges from animal food production to their adverse effects.

4.1 Introduction

After domestication of animals their products have been used by man for thousands of years. Animal biotechnology has been practiced in one form or another since the beginning of the domestication of animals. Previously, the tools used for

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modification of desired products in animal breeding, genetics, and nutrition have played an important role in the selection, propagation, and management of desirable and economically important characteristics in livestock. Later, it developed some breeding strategies by phenotypic characters for improvement and selection of the desired traits in animals. However, the desired characters could not have been often achieved by such conventional means of breeding and selection (Madan 2005).

Industrial scale production of proteins can be offered by milk, egg white, blood, urine, and seminal plasma of transgenic animals. In 2000, the first recombinant protein expressed in transgenic animal was marketed. A number of technical problems remain to be addressed before optimization of different systems for the production of various recombinant proteins (Houdebine 2000). However, the productions of transgenic animals became quite easy due to animal cloning technologies and other advancement in transgenic animal techniques, but the cost of production of such animals is still a major limitation. Different studies on transgenic animal production were performed in the last 15 years with limited transgene expression due to inappropriate construction of expression vectors (Houdebine 2000).

Animals were initially considered a good source of biomolecule and drug production due to a number of reasons; (1) The ability of mammary glands for the production of complex molecules, (2) engineered animals can produce high amount of protein, (3) reduced per unit protein cost in animal bioreactors, (4) finally, safer and flexible source for the production of human protein, especially vital human protein and blood products. Recently, animals are considered to develop almost all types of human proteins and proteins of industrial importance (Gottlieb and Wheeler 2011). Previously, assisted reproductive technologies (ART), such as artificial insemination (AI), superovulation (SOV), embryo transfer (ET), and in vitro embryo production (IVEP), contributed to animal breeding programs for faster development of desirable traits in a shorter period of time compared to classical approaches. The use of modern transgenic technologies along with ART to develop livestock with current genomes has played an increasing vital role in the genetic improvement and development of livestock with desired genetic materials. The stem cell technologies inclusion to the genetic "toolbox" has further improved scientists' capabilities of livestock genome desired modification and physiology (Wheeler et al. 2010).

Modern livestock production has been dependent on biotechnology for development of improved feedstuff, feed ingredients, vaccines, biological, enzymes, high quality genetics, genetic markers, and assisted reproduction. Biotechnology encompasses a variety of modern techniques including genetic engineering, genetic modification, transgenic techniques, recombinant DNA technology, and cloning to develop selected characteristics in animal food products. Some examples of these products are fat and meat content ratio, alteration of milk component, modification in hair or fiber contents, and production of targeted proteins in certain animals (Cowan 2010).

The techniques to produce genetically engineered animals have been existing for more than 20 years, but recently livestock have been produced with desirable characters to improve animal agriculture and human health (Wheeler 2003).

Commercial producers are much interested in the application of modern biotechnology to improve productivity, consistency, and quality of altered animal food, fiber, and medical products. The transgenic animal technology is potentially valuable to alter characters of economic importance in a rapid and precise way. However, this technology needs the complete information and knowledge of gene that control specific characters and their regulations. The classical program of breed improvement for desired characters does not need any specific information about genes and gene regulation (Montaldo 2006; Wheeler et al. 2010).

4.2 Modification with Modern Transgenic Techniques

4.2.1 Modification in Fat Contents

The animal fat content, as a result of modern transgenic techniques, would be the most important future strategy to improve animal dairy and meat products for safe consumption by humans. These alterations will make such products more valuable and desirable for patients suffering from cancer or heart diseases. Moreover, these products can also reduce risk of lethal diseases such as chronic heart disease and cancer with consumption of animal product lower in bad or higher in good fat contents. Some additional benefits can also be achieved along with safe consumption of fat products of transgenic animals such as increased shelf life, or storage of animal fat to avoid rancidity and oxidation. Future development may make it possible to introduce the transgenes, knockout, and knockin with more precision. Issues related to transgenic products still need attention and further improvement is required, such as large-scale production, cost-effectiveness, GMO regulation, and consumer choice.

The exogenous fats in the human diet serve as raw material for the production of indigenous fats, cholesterol, and phospholipids. Food fats contribute a greater relation to food nutritional calories; about nine calories per gram which is almost twice the energy obtained from carbohydrate and protein. Fats are a group of chemicals that belong to fatty acids. Palmitic acid, stearic acid, and oleic acid are the most common fatty acids found in animal fats. These fats can be synthesized by the human body, but another class of essential fats that cannot be produced by the body include linoleic acid, linolenic acid, and arachidonic acid; these must be from exogenous sources along with the diet (Campbell and Reece 2002).

Naturally occurring fatty acids are of two types: saturated and unsaturated. Saturated fatty acids have no double bond between the carbon atoms, all carbon chains are entirely occupied with hydrogen. Saturated fatty acids are mainly of animal origin (Keys et al. 1965). These fatty acids are considered “bad fats” because they increase both high density lipoprotein and low-density lipoprotein cholesterol level in the body of consumers. Unsaturated fatty acids mainly originate from plants. They are categorized into two major type: monounsaturated fatty acids and polyunsaturated fatty acids. Monounsaturated fatty acids have one double bond in the carbon chain;

while polyunsaturated fatty acids have two or more double bonds in the carbon chain. These fatty acid are considered "good fats" because they only increase the level of high density lipoproteins, while they decrease the level of low-density lipoproteins (Keys et al. 1965; Grundy 1986; National Research Council 1988).

Dairy milk has almost 60 % of saturated fats, as a consequence, decrease in the intake of dairy products has been observed worldwide. These saturated fats increase the plasma cholesterol level, as a result, an increased risk of coronary heart diseases (CHD) (Parodi 2004). Milk fat has a number of functional components and bioactive compounds. These functional components play a vital role in development of many diseases. It is now known fact that persons with high risk of heart-related diseases and high risk of prostate, colon and breast cancer are those who consume diet with high quantity of fatty acids, especially saturated fatty acid (Parodi 2004; Jube and Borthakur 2006). The sex, breed, and age of an animal affect the fatty acid composition but nutrition has a direct effect on the fatty acid composition in animal products. Previously, pork meat was produced with increased amounts of omega-3 with the use of 11 % flaxseeds in the diet of pork (Mavromichalis 2001).

The increase in unsaturated fatty acids components in animal products increase the risks of rancidity and reduces the shelf life of such products due to oxidation (Decker and Xu 1998). These types of undesirable sensory and health effects due to lipid oxidation can also be avoided or reduced with vitamin E supplementation in the animal diet. The animal products supplemented with vitamin E enriched diets demonstrate antioxidant activity and reduce rancidity to maintain the natural color of meat. Experimentally, beef and chicken meat and egg compositions have been altered with different feeding strategies (Decker and Xu 1998; Pszczola 1998; Sloan 2000).

Previous investigations suggest that the risk of cancers and CHD can be reduced with the modification of daily diet consumption (Parodi 2004). Health agencies including the American Diabetes Association, the American Dietetic Association, and the American Heart Association suggest that fatty acids should not contribute more than 30 % of the total daily calories consumption (Parodi 2004; Jube and Borthakur 2006). Some other studies also suggest that the risk of these diseases can also be reduced through consumption of plant rich foods. Milk saturated fatty acids are usually considered more harmful for human health. During the past decade, a lot of evidence came into consideration that many animal milk derived fatty acids can offer important human health benefits for chronic heart disease and cancer (Parodi 2004).

The expression of stearoyl-CoA desaturase in goat mammary gland and *Caenorhabditis elegans* Δ- and Ω-3 desaturase genes in transgenic mice produced milk enriched with linoleic acid contents. These approaches may also help to produce milk from transgenic animals with such value addition to reduce the cardiovascular diseases caused by milk fat. Modified milk of transgenic animals with secretion of Omega-3 lipid is another possibility to reduce the cardiovascular diseases with consumption of dairy milk (Soler et al. 2006).

Dairy milk contains approximately 3–4 % fat, depending on breed, nutrition, and stage of lactation. As previously mentioned, milk fats may increase the risk of cardiovascular disorders in humans (Soler et al. 2006). The change in major enzymes for fat production can modify the production of fat in the milk. The acetyl

coenzyme A (CoA) carboxylase is a major enzyme responsible for de novo synthesis of milk fat from acetate conversion inside the mammary gland. Almost 50 % of milk fat is synthesized by this method and modification in this enzyme can lead to alter the milk fat contents. The reduction in the quantity of this enzyme would lead to extreme reduction in the amount of fatty acids in milk. Similarly, it would reduce the requirement of dietary nutrition for the production of fatty acids in milk. However, the alteration in the α-lactalbumin would also be another strategy for modification in milk fat (Yom and Bremel 1993).

The composition of animal fat in red meat can also be managed using animal husbandry practices. The meat or beef fat composition would differ in animals feeding on feedlots and animal feeding on pasture. Fat composition depends on the genetics, nutrition of animals, meat cuts, and fat trimming influences (Scollan et al. 2006). In animals, the fat is deposited and localized intramuscularly (marbling) and subcutaneously (Scollan et al. 2006). Oleic acid contributes the most prominent part of the fatty acids present in pig, sheep, and cattle meat, while palmitic acid is the most predominant saturated fatty acid (Scollan et al. 2006). Poultry rearing in free range systems does not produce meat with high fat value, while the poultry with high energy diet can achieve a significant level of fat contents. Breast meat of free range broiler has high poly unsaturated fatty acids (PUFAs) and mono unsaturated fatty acids (MUFAs) content, while it is low in saturated fatty acids (SFAs) (Ponte et al. 2008). Poultry skin has more than 40 % fat, while dark meat has 10 %, and white meat has 4 % fat. Poultry meat contains 21 % PUFAs, 45 % MUFAs, and 30 % SFAs (Sayed et al. 1999; USDA 2008).

Conjugated linoleic acid is a naturally occurring fatty acid in animal products such as meat and dairy milk. The conjugated linoleic acid in milk has been recorded up to 2–37 mg g^{-1} , while *cis*-9 and *trans*-11 conjugated linoleic acid contents have also been recorded up to 53.7 mg g^{-1} of fatty acid and 51.5 mg g^{-1} of total milk fat (Javor et al. 2008). The concentration of conjugated linoleic acid in animal products has been improved with feed and diet such as fresh pasture, total mix ration contain fish oil or sunflower as feed additive. Furthermore, the breed, age, and lactation number also have little influence on the concentration of conjugated linoleic acid. Interestingly, the conjugated linoleic acid concentration improved after cooking or processing (Javor et al. 2008). Most recent significant discovery of animal fats and fatty acids is related to the conjugated linoleic acid (CLA) in association with ruminant milk fats and meat. It has also forced authorities to reconsider the recommendations related to impact of animal fat on public health (Javor et al.2008). Linoleic acid is also a powerful naturally occurring anticarcinogen, antiatherogenic, and immunomodulatory. The National Academy of Science report on carcinogens and anticarcinogens in food declared linoleic acid as the only anticarcinogen fatty acid present in food (Javor et al.2008). In some previous studies, conjugated linoleic acid has successfully suppressed fore-stomach tumors in mice, aberrant cryptic colonic foci in rats, and rat mammary tumors (Javor et al. 2008). Many other saturated fatty acids are neutral inside the human body and do not increase the plasma cholesterol level. Moreover, it has a significant role to alter body fat composition and as a weight reducing agent with reduction in animal fat deposition in

body by increasing the rate of lipolysis in adipocytes (National Research Council 1996; Javor et al. 2008).

Moreover, a number of saturated fatty acids present in milk have advantageous effects such as conjugated linoleic acid, vaccenic acid, sphingolipids, butyric acid, 13-methyltetradecanoic acid, and ether lipids which are mostly anticarcinogenic. Conjugated linoleic acid, oleic acid, omega-3 fatty acids are useful to avoid cardiovascular diseases. Conjugated linoleic acid is also very useful to improve the immune response and bone health (National Research Council 1996). However, dairy fats that include lauric, myristic, palmitic acid in human diet increase the blood plasma cholesterol, low and high density lipoprotein cholesterol level (Kris-Etherton and Yu 1997; National Research Council 1996). The transgenic approach to alter such fat components of dairy milk would increase the acceptability of milk along with reduction in the risk of chronic heart diseases (CHD) to consumers.

Normally, unsaturated fats present in diet are of *cis*-configuration but at the same time they are of *trans*-configuration. *Trans* fatty acids are involved in raising the blood cholesterol level. Diets containing *trans* fatty acids increase the risk of CHD. Fatty acid receives *trans* double bonds during chemical processing or partial dehydrogenation of vegetable oil. Animal products have *trans* double bond during rumen biodegradation and formation of intermediates, which is a natural source for generation of *trans* fatty acids. Unsaturated fatty acids are toxic to rumen microorganisms. The microorganisms carried out massive biodegradation, and as a result, produced 1–8 % *trans* fatty acids to the total rumen lipids. The major natural sources of *trans* fatty acids are ruminant meat and dairy products (Lock et al. 2005).

The *trans* fatty acids-related risk of CHD needs more investigation; but the *trans* fatty acids of natural or animal origin differ with industrial *trans* fatty acids with respect to chronic heart diseases. It may be due to the difference in specific amino acids and *trans* fatty acids isomers such as *trans* 9 and *trans* 10 fatty acids that mainly exist in industrial sources. The most important reason of this difference may be due to conversion of vaccenic acid (11-*trans* octadecenoic acid; VA) a major *trans* fatty acid in the fat of ruminants to rumenic acid (9-*cis*, 11-*trans* octadecenoic acid; RA) in tissues, an isomer of conjugated linoleic acid, by Δ9-desaturase. The human body can also convert this vaccenic acid to rumenic acid (Palmquist et al. 2005).

Studies have investigated the role of milk *trans* fatty acids on the blood plasma cholesterol. But interestingly, naturally enriched *trans* fatty acids animal fats have no undesirable effect on the blood plasma cholesterol even at very high intake. Vaccenic acid (VA) to rumenic acid (RA) conversion has potential benefits to control cardiovascular diseases and to reduce the adverse effects of *trans* fatty acids on human plasma cholesterol level. However, this topic needs more investigation, especially to improve human health and to enhance the use of animal natural fats (Lock 2007). Moreover, the transgenic approaches in animals to improve all adverse effects of fatty acid with substitution of useful fatty acids would be a good strategy to improve the health of consumers and consumption of animal dairy products.

Genetic manipulation in transgenic animals offers to alter the carcass lower in fat and cholesterol. In addition, modern biotechnological techniques make this possible

to put useful fatty acids in transgenic animals such as polyunsaturated fatty acids. Polyunsaturated fatty acids (PUAFs) contain 18 or more than 18 carbon atoms; and two or more than two double bonds. PUAFs two major groups are omega-6 (n-6) and omega-3 (n-3), on the basis of double bond near the methyl end of fatty acid. Polyunsaturated fatty acids n-3 and n-6 are important constituents of phospholipids in the tissues throughout the body. PUFAs n-3 is especially enriched in the retina and brain (DeFilippis and Sperling 2006; Simopoulos 2006). Mammals lacking in desaturase require the synthesis of linolenic acid (n-6) and alpha linolenic acid (n-3). Omega-3 fatty acid desaturase is also absent in mammals, as a result, n-3 and n-6 polyunsaturated fatty acids are not interconvertible in mammals. Polyunsaturated fatty acid n-3 and n-6 are essential fatty acids for mammals, which must be taken along with diet. The consumption of n-6 polyunsaturated fatty acid increased during the last one and a half century due to the use of vegetable oils of corn, sunflower seeds, cottonseed, and soybeans origin. The vegetable oil from such sources is much enriched with n-6 polyunsaturated fatty acids, but n-3 polyunsaturated fatty acids are deficient in these sources. Recently, the consumption of n-3 poly unsaturated fatty acids is much lower due to less consumption of fish meat; and consumption of meat and egg produced from commercial feed enriched with n-6 polyunsaturated fatty acids, which is deprived of n-3 polyunsaturated fatty acids (Simopoulos 2006). Previously, many potential useful effects of Omega-3 have been discovered and studied in many populations, large animals, primates, and swine. The major beneficial effects include antiatherosclerotic, reduction in the risk of cardiovascular diseases by reducing the triacylglycerol, cholesterol, and VLDL concentration; reduction in many inflammatory markers such as CRP, IL-6, E-selectin, ICAM-1, VCAM, 1IL-1β, and TNF-α; reduction in platelet accumulation and thrombosis, downregulation of PDGF and triggered mononuclear cells; preventing ventricular arrhythmias (Prather et al. 2008).

In previous studies, omega-3 polyunsaturated fatty acids desaturase gene, also called fat 1, has been successfully introduced in mice from a roundworm called *Caenorhabditis elegans* (*reference*). The product of this gene can convert n-6 into n-3 polyunsaturated fatty acids. Some later investigations revealed resistance in these transgenic mice to certain diseases such as colitis, chemically induced hepatitis, prostate cancer, and melanoma (Prather et al. 2008). Human fat 1 gene was introduced into pig with somatic cell nuclear transformation, in a result, threefold increase the level of n-3 polyunsaturated fatty acids along with 25 % decrease in n-6 polyunsaturated fatty acids by the efficient conversion of n-6 into n-3 polyunsaturated fatty acids (Lai et al. 2006; Li et al. 2006). This conversion reduced the n-6/n3 up to five folds in these transgenic pigs compared to control or wild pigs. The reduction of this ration of n-6/n-3 was observed in almost all tissues of transgenic pig in contrast to control animal (Lai et al. 2006; Li et al. 2006). Transgenic pigs were developed with plant gene insert (fatty acid desaturation 2 gene) for 12 fatty acid desaturase from spinach. In transgenic animal linoleic acid (18: 2n-6) in adipocytes was ten times more and white adipose tissue contained 20 % more linoleic acid than wild-type pig (Saeki et al. 2004). Previous findings such as expression of fatty acid desaturase gene insertion in mammals made this possible to alter the fatty acid

compositions in domestic animals with modern recombinant biotechnology for production of more useful fats of animal origin for human consumption (Montaldo 2006). Bucher et al. (2002) suggested in 11 randomized meta analysis that the intake of omega-3 reduced mortality due to myocardial infarction and death due to chronic heart disease. The people consumption of omega-3 is 8–10 % of the recommended daily intake, while of omega-6 is 1000–2000 %. This imbalance can be improved with increased intake of omega-3 enrich diet such as fishes, salmons, eel, herring, sardines, omega-3 rich eggs of hens with modified diet. The omega-3 containing transgenic approaches are under consideration of many commercial companies (Lewis et al. 2000; Pulina et al. 2006). Scheeder et al. (2001) produced cow feed rich with omega-3 without any unpleasant effect on meat texture. Furthermore, omega-3 fatty acids gene can be introduced from fish or other sources to livestock to make transgenic animals rich in omega-3. Transgenic animals with omega-3 transgene or the transgene to convert omega-6 to omega-3 would produce valuable diets to balance the omega-6 in daily intake (Wheeler 2012).

Potential other genes can also be targeted from cholesterol and fat biosynthesis pathways to develop transgenic animal for modification in fat contents such as cholesterol 7-alpha hydroxylase, hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, fatty acid synthase, and lipoprotein lipase. Furthermore, leptin hormone gene and the low-density lipoprotein (LDL) receptor gene can be possible targets in transgenic approaches. The products of these transgenes could decrease fat and cholesterol in dairy and other products of transgenic animals (Wheeler 2012).

Cholesterol 7-alpha hydroxylase (CYP7A1) protein controls the pathway for conversion of cholesterol to bile. Bile acids have a significant role in the digestion of fat soluble vitamins and triglycerides. In addition, bile acids provide a metabolic pathway to reduce excessive cholesterol from the body. Bile acids also have some toxic effects such as farnesoid X receptor (FXR)/small heterodimer partner (SHR) dependent and independent reduction in the expression of cholesterol 7-alpha hydroxylase (CYP7A1) gene (Davis et al. 2002). In a recent study, transgenic C57BL/6 mice produced with CYP7A1 expression in the liver increased fivefold the enzyme activity and twice the bile acid pool size. Instead of toxic effects, the transgenic mice with overexpression of CYP7A1 in the liver clogged the diet-mediated atherosclerosis and gallstone formation. These studies propose that CYP7A1 may be a better therapeutic target in the future to control these diseases (Davis et al. 2002).

Similarly, hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase is microsomal enzyme in the de novo synthesis of cholesterol in liver. More than 50 % of the cholesterol in humans comes from de novo synthesis in liver. The cholesterol level in the body can be modified with alteration in the expression of HMG-CoA gene. Furthermore, fatty acid synthase is a vital dimer protein in fatty acid synthesis. The dimer subunits alter confirmation at two acyl carrier protein center to generate fatty acids. The malonyl-CoA in mammals to palmitate conversion can be catalyzed with this single dimer multifunctional protein, which can be a better target in transgenic approach to modify the fat content in animal products (Smith et al. 2003).

A single lipoprotein lipase (LPL) gene is also a better target for production of transgenic animals with modified fat components. Lipoprotein lipase is also an

important protein in mammals for fat metabolism and transportation. Lipoprotein lipase can catalyze the hydrolysis of fatty acids components of circulating chylomicrons and LDL. This catalysis provides nonesterified triglycerides and 2-monoacyl glycerol for tissue consumption. Previous studies reveal that this protein can bind simultaneously to low-density lipoprotein and cell surface receptors such as proteoglycans, which enhances the intracellular uptake of low-density lipoprotein with non-catalytic bridge function. Abnormal expression of LPL and function is directly or indirectly associated with physiological and pathological consequences such as diabetes, Alzheimer's disease, atherosclerosis, obesity, chylomicronemia, and cachexia (Mead et al. 2002).

Previously, many other studies were conducted and successfully produced transgenic animals with lower in fat contents. Ward et al. (1989) developed transgenic lamb with ovine GH insert; the transgene insert was successfully expressed and controlled by ovine metallothionein IA (oMT-IA) promoter in transgenic animals. Transgenic animal reduced fat components up to five times compared to control animals. Similarly, Pursel et al. (1989) developed transgenic pigs with human GH and bovine GH gene inserts, which successfully produced transgenic animals with reduction in carcass fat but with little effect on growth. The transgene expression in transgenic animals was controlled with mouse metallothionein IA (MT-I) promotor. Wieghart et al. (1990) developed transgenic pig with bovine GH gene insertion and expression was controlled with rat phosphoenolpyruvate carboxykinase (PEPCK) promoter. The back fat depth was reduced by up to 41 % in the transgenic pig.

4.3 Modification in Animals for Meat and Carcass

The development of transgenic animals using modern biotechnology has been put great insight into the action of gene regulation to control growth and development. This technology made it possible to manipulate and evaluate the different growth factor receptors, growth factors, and growth modulators (Rexroad et al. 1991; Seidel 1999). Initially, scientists put great emphasis on the production of livestock with simple or transgenic approach to alter carcass composition for meat purpose with direct or foreign gene incorporation.

Previously, metabolic modifiers were also used to increase productivity of animals such as modification of carcass composition (meat-fat ratio), increase in milk yield, and decrease in animal fat. Most common modifiers are hormones of biological origin. The recombinant bovine somatotropin (rBST) with modern biotechnological tool in dairy cows has been successfully used to increase both milk production efficiency and decrease animal fat. The rBST typically increased milk yield by 10–15 %. However, this increase is not significant because of low milk yields and the high cost–benefit ratio. Recently, rBST is used commercially in 19 different countries where the economic returns make its use worthwhile. A porcine somatotropin has been developed and used to increase muscle ratio over fat deposition, which is produced in transgenic pigs at greater market value (Wheeler et al. 2010).

Transgenic mice have been produced in different studies with tremendous increase in growth characteristics. Previously, the transgenic mice have developed successfully for transgene expression for growth factors such as growth hormone (GH), growth hormone releasing factor (GRF), and insulin-like growth factor I (IGF-I) (Palmiter et al. 1982, 1983; Hammer et al. 1885; Mathews et al. 1988). Further successes along the lines of mice and sheep were achieved with independent expression of the foreign genes encoding growth hormone-releasing factor (GRF) or insulin-like growth factor I (IGF-I) (Cameron et al. 1994; Murray et al. 1999; Montaldo 2006). Details of some important studies related to transgenic technology for the improvement in meat and growth are given in Table 4.1.

Table 4.1 Transgenic technology application to improve meat production and growth rate (Modified from Wall et al. 2009)

Introduced	Modification	Application species	References
Insulin-like growth factor 1	Increased meat production	Pig	Pursel et al. (1999)
Human and porcine growth hormone releasing factor	Increased meat production	Pig	Draghia-Akli et al. (1999), Pursel et al. (1990)
Human growth hormone releasing factor	Increased meat production	Pig	Rexroad et al. (1989)
Bovine, human, and porcine growth hormone	Increased meat production	Sheep	Nottle et al. (1999), Pursel et al. (1989, 1990)
Ovine growth hormone	Increased meat production	Pig	Adams et al. (2002); Ward and Brown (1998)
Inducible myostatin knock out	Increased postnatal muscle growth	Mouse	Grobet et al. (2003)
Myostatin disruption	Increased meat production	Mouse	Yang et al. (2001)
Sex-specific disruption of myostatin	Efficient cattle production system for dairy cows and superior beef bulls	Mouse	Pirottin et al. (2005)
Chicken c-ski oncogene	Primarily involve in hypertrophy of muscles	Mice, sheep, pig and cattle	Palmiter et al. (1982), Pursel et al. (1990), Bowen et al. (1994), Cameron et al. (1994), Murray et al. (1999)
Growth hormone releasing factors and insulin-like growth factor	Increase the diameter of muscles	Pig	Neimann (1998)
Piscine growth hormone	Shorter time to market	Fish	Devlin et al. (1994), Du et al. (1992)

In some other transgenic animal trials, growth hormone expression was effectively increased in domestic animals such as pig, sheep, and rabbit (Prather et al. 2008). A number of genes have been used to make transgenic pig for growth and modified carcass products such as growth hormone (GH1), insulin-like growth factor 1 (IGF1), delta 12 fatty acid desaturase, B-cell lymphoma 2 (Bcl-2), omega-3 desaturase (hfat-1), and bovine alpha-lactalbumin (LALBA), phytase (Prather et al. 2008). These modifications improved the body weight and growth rate by increasing the meat quantity, while some modifications only altered the meat composition such as delta 12 fatty acid desaturase and omega-3 desaturase (hfat-1) (Prather et al. 2008). Pig was a suitable candidate for production of modified body composition with increase in carcass quantity. In the first study on transgenic pigs, Pursel et al. (1989) successfully developed transgenic pigs with unsatisfied results. The growth hormone (GH) transgene was inserted in transgenic pig, and little effect was achieved on the growth. The transcription was not efficiently regulated in these transgenic pigs 'as a result' the high level of growth hormone expression produced side effects such as lameness, reduction in fertility, and vulnerability to stress (Pursel and Rexroad 1993). Later, "super pigs" were also developed with ovine growth hormone (oGH) transgene into pig. The transgenic pigs clearly gained more muscle content compared to wild or nontransgenic pigs. Unfortunately, super pigs suffered from arthritis and bone thickness (Pursel et al. 1997).

High level of serum growth hormone was observed in transgenic pigs and sheep with GRF and IGF-I, which incremented with increase in protein diet. However, transgenic animals reduce fat content on body but no serious effect was observed on the growth rate of the animals. Additionally, transgenic animals developed pathological conditions such as severe reduction in reproduction (Murray et al. 1999). In two other studies, transgenic pigs were produced with IGF-I and GRF, which expressed and incremented the IGF-I and growth hormone level. Transgenic pigs developed increased diameter of muscles and reduced fat contents without any serious undesirable or pathological effects (Neimann 1998). Some other genes were also attempted in the development of transgenic pig to change the meat or muscle growth along with metallothionein promoter to control their expressions. A chicken oncogene "c-ski" was introduced into transgenic sheep, mice, pigs, and cattle. This gene is primarily involved in hypertrophy of several muscles, while decreasing the fat deposition on the body. This approach has resulted in partial success although muscular hypertrophy has been obtained in a few pigs and cattle (Palmiter et al. 1982; Cameron et al. 1994; Murray et al. 1999; Wheeler 2012).

The acid meat gene or Rendement Napole gene has been found associated with lower processing output in many Hampshire pig lines and crossbreds. Pork with low pH is produced in these lines and can be distinguished in different qualities such as firmness, processing yield, shearing force, marbling, color, and water holding capacity. Modern biotechnological tools such as "knockout technology" may be a better approach to modify the postmortem pH, glycolytic potential, and pork meat quality (Wheeler 2012). The growth pattern can also be affected by other loci such as ryanodine receptor, *myo-D*, growth hormone release factor, sheep callipyge, high affinity insulin-like growth factor binding proteins, and myostatin

gene (Wheeler 2012). The differentiation factor 8 (DF-8) or myostatin "a member of transforming growth factor β (TGF-β) family" gene has been found to have high suitable locus for the development of transgenic animals for the increase in carcass.

Some cattle breeds such as Belgian Blue and Piedmontese developed double muscle mass as a result of mutation in myostatin gene. The myostatin gene is highly conserved in other species of animals such as human, ovine, rat, murine, zebrafish, chicken, and turkey, which may also suggest to conserve functional role. Myostatin knockout mice has been developed with increased lean muscles mass, which enlarged the hip and shoulder of transgenic mice. Moreover, transgenic mice achieved incremental skeletal muscles mass all over the body without any gross abnormality. The homozygous animals of such knockout animals have achieved 2–3 times more muscle weight and 30 % more body weight compared to normal animals. This increase in the muscular growth was due to hyperplasia of muscles (Mcpherron et al. 1997). The myostatin knockout animals have some adverse effects on reproduction on transgenic animals such as dystocia. The animal welfare concern forced the researchers to overcome this health issue with postnatal inhibition of myostatin gene in transgenic animal (Grobet et al. 2003).

The prodomain of myostatin could bind to mature myostatin, which would inhibit biological activity of myostatin (Yang et al. 2001). The increase in muscle growth and body weight has been observed in experimental mouse with transgene over expression of prodomain segment. The transgenic mouse with overexpression of prodomain segment presented 17–30 % more body weight and 22–44 % total carcass weight compared to wild or control animal. The pMEX-NMCS2 vector with a rat myosin light chain 1 (MLC1) promoter, an SV40 polyadenylation sequence, and an MLC enhancer were used for overexpression of prodomain segment into transgenic animal. In addition, the transgenic animals were not presented any defective phenotype, reproductive, and other undesirable health effects (Yang et al. 2001). The RNAi is a more suitable tool for inhibition of myostatin pathway. The site-specific RNAi molecule could provide more flexible, controlled, and site directed inhibition of this gene expression (Yang et al. 2001). Pirottin et al. (2005) has developed transgenic mice with site-specific Y-chromosome associated expression of such competitive inhibitor molecules, which successfully produced 5–20 % more carcass weight or skeleton muscles in male animals. The females were lacking transgene, transgene expression, and also growth characteristics due to absence of Y-chromosome. The inducible or postnatal expression combine approach would be more suitable to make transgenic bull for meat and elite dairy cow simultaneously. Moreover, the sex-sorting technology would enhance the application of this approach, because only bull contain such transgene and expression (Wall et al. 2009).

There are many important genes associated with growth rate, growth factors, growth receptor, and modulators, which can be considered for the future animal transgenic approach for increased growth rate and feed conversion efficiency. Indirectly, muscle growth or carcass can be achieved with cloning of different genes to make transgenic animals for fat reduction such as hydroxymethylglutaryl Coenzyme A (HMG-CoA) reductase, lipoprotein lipase, lipoprotein synthetase and

cholesterol 7-alpha hydroxlyase (Wheeler 2012). Previously, the transgenic pigs were also made with certain transgenes such as Bcl-2 and LALBA to gain indirect carcass benefits. The transgenic pig with Bcl-2 transgene produced more number of viable eggs in females, and transgenic pig with LALBA transgene made possible to massive increase in weaning weight (Prather et al. 2008). Indirectly, more pork could be produced with more number of animals and increased body weight at time of weaning or marketing.

Certainly, many potential target genes can also be used to increase the feed efficiency, feed digestion, and appetite to animals. The increase in feed efficiency and appetite has a profound effect on the animal production for meat purpose. The alteration in digestive enzymes profile can increase the feed efficiency and nutrient up take from the digestive tract. The phosphorous bioavailability can be increased from phytic acid in corn and soybean feed with introduction of digestive enzyme such as xylanase phytase transgene into digestive system of other species such as pig and poultry, which naturally lack this enzyme in their digestive systems (Wheeler 2012).

The enzyme isocitrate lyase glyoxylate cycle can catalyze the conversion of isocitrate to citrate and glyoxylate. The isocitrate lyase and malate synthase are the enzyme of glucose metabolism in bacteria, fungi, and plants, while the placental mammals missing these enzymes. As result of combine effects of both enzymes solicit to evade the two carboxylation process in tricarboxylic acid cycle. The glyoxylate cycle generates intermediates for the glucose synthesis and other biosynthetic precursors. In addition, greater expression of isocitrate lyase in a microorganism such as fungus could increase the pathogenicity of disease in plant, animals, and humans (Dunn et al. 2009).

In higher plants, these enzymes have a most significant role in germination of plants from oil seeds, which provide the acetyl-CoA for glyoxylate cycle to deliver the primary nutrients and other metabolic intermediate prior to photosynthesis (Eastmond and Graham 2001). The organisms could produce the acetyl-CoA derived glucose and other metabolic intermediates from energy sources such as acetate, fatty acids or poly-β-hydroxybutyrate, and ethanol (Dunn et al. 2009). Ward (2000) has been reported sheep with transgene expression of bacterial isocitrate lyase and malate synthase. The expression of both genes in transgenic sheep the successful expression of these transgenes solicited to increase the glucose supply, better feed conversion into energy, and indirectly better growth rate. The transgenic pig has been produced with phytase transgene. The phytase expression was detected in saliva at day 7 of transgenic pig, the salivary phytase made this possible to digest the phytate completely in the diet of transgenic animals. Furthermore, this alteration reduced the need for inorganic phosphorous supplementation and environmental pollution due to animal production (Golovan 2001).

Cellulose is a major component in plant materials and foods. Ruminants have cellulolytic enzyme for degradation and consumption of plant food. Mono stomach or nonruminants are deficient in such enzyme and are incapable to digest plant food. The transgenic nonruminants with transgenes of cellulolytic enzymes and successful expression into digestive system could allow these animals to digest the

plant material with cellulose. Fibrous feedstuff could be more frequently used for nonruminants such as poultry and pigs for livestock production. In addition, the competition could be reduced between human and animals for grains and cereals as food. These would be beneficial in the future in the time of more food competition and shortage (Wheeler 2012).

Increased carcass growth and quality would be solicited to overcome the future food shortage challenges. The control of undesirable effects in genetic modification needs more work of researchers for production of meat for safe human consumption. Similarly, commercial producers of meat have great advantage with rapid growth of transgenic animals in rearing to reduce the cost of production. In addition, transgenes with additional properties of animal welfare such as harboring harsh environment would encourage economical and more sustainable farming in different regions.

4.4 Genetically Modified Fish for Increased Growth Rate and Other Traits

Fish are considered the healthiest food for human consumption, and they are good candidates to be the first transgenic animal to be approved. The release of transgenic animal in wild condition could be a good option, but a reliable technique for 100 % sterilization of transgenic fish has not yet been established (Jube and Borthakur 2006). However, the transgenic technology solicited to develop large size fish with more than 11 times as control. In addition, transgenic fish has no major side effects and health problems. These "super fish" are more resistant to disease, more adaptable to cold water, more sustainable in the wild condition compared to nontransgenic fish (Devlin et al. 1994; Du et al. 1992).

The additional characteristics along with growth rate can also be achieved such as antifreeze protein expression into plasma of transgenic fish. The ocean pout (*Macrozoarces americanus*) and winter flounder (*Pleuronectes americanus*) has been previously developed with such proteins and successfully tolerated the freezing temperature of Atlantic Ocean (Davies and Hew 1990). The antifreezing proteins are characterized into two major types such as antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGs). Antifreezing proteins and antifreezing glycoprotein could lower the freezing temperature of fish serum by attaching to ice surface, to avoid crystal formation. Four type of AFPs (AFP I, AFPII, AFP III, AFP IV) has been discovered, while only one type of AFGs has been identified (Davies and Hew 1990; Jube and Borthakur 2006). The species of fish with aquaculture importance, such as tilapia and Atlantic salmon, do not survive in the freezing temperature especially in Northern Atlantic coast regions. Therefore, the cage forming of such species are not possible in these regions of subzero temperature. The production and use of transgenic fish, especially salmon with antifreezing protein transgenes would expand the fish farming in the regions of subzero

temperature along with reduction farming cost and price for consumers (Jube and Borthakur 2006). The flounder AFPs belongs to AFPs type I with two isoforms such as skin type and liver type. Skin type AFPs intracellular protein produced into several peripheral tissues in the form of mature protein, while liver AFPs produce in liver in immature form and need further modification before release (Hew et al. 1986; Jube and Borthakur 2006). Hew et al. (1999) reported transgenic salmons (*Salmo salar*) to introduce piscine antifreeze protein transgene, which produced liver-specific winter seasonal antifreeze protein. Initially, a single copy of liver type AFPs was injected into fertilized salmon egg, and a stable expression of antifreezing protein was achieved. The consistent expression was observed up to three generation with low concentration of antifreeze protein (250 μg/ml), which was normally present up to 10–20 mg/ml in winter flounders. This expression was insufficient to harbor the freezing temperature in transgenic salmon. Later, the high concentration was achieved with increase in transgenes copy number. This modification made capable to transgenic salmon in successfully harboring the winter freezing temperature. Similar study was performed on goldfish (*Carassius auratus*), and transgenic goldfish was successfully harboring the freezing water due to inference of piscine antifreeze protein transgene expression (Wang et al. 1995). Previously, the transgenic tilapia fish (*Oreochromis niloticus*) have been established with several salmonids genes constructs, but better results achieved with the *Chinook salmon* GH gene under the control expression of ocean pout antifreeze promoter (Rahman et al. 1998). The insertions of transgenes were achieved with fertilized egg cytoplasmic microinjection technique. The researchers achieved a successful genomic integration of transgene into founder (G_0) tilapia, as well as in subsequent generations G1 and G2 of transgenic tilapia. *Chinook salmon* GH transgene expression into transgenic fish presented three times more growth rate compared to wild tilapia and 33 % more feed conversion ratio (Rahman et al. 1998). The farmer would be benefited with more body weight and lower feed consumption. In addition, the expression of this gene would also cause reproductive infertility, which is high desirable trait for the environmental release of transgenic fish (Rahman et al. 1998).

Human glucose transporter 1 protein is encoded by *SLC2A1* gene in human and first categorized transporter protein. It belongs to solute carrier protein family 2, which is involved in glucose transport across the cell plasma membrane (Mueckler et al. 1985). The constant glucose supply from blood plasma is required to erythrocyte for energy yielding. The human glucose transporter 1 protein increases the glucose supply to erythrocytes with facilitated diffusion, which is almost 50,000 times higher than translated transmembrane diffusion (Nelson and Cox 2008; Montel-hangen et al. 2008). The expression of human transporter 1 would increase with decrease in glucose level in blood and decrease with increase glucose supply. This transporter protein is very important for the uptake of basal glucose at very low level of glucose for sustainable respiration in all cells (Montel-hangen et al. 2008). In addition, this transported protein is also responsible for the vitamin C transport along with glucose supply in nonvitamin C producing mammals (Montel-hangen et al. 2008).

Hexokinase is an enzyme for the phosphorylation of hexoses and converting into hexoses phosphate. The most commonly glucose is converted into glucose-6-phasphate, and the genes encoded hexokinase are found in different species domains such as bacteria, plants, animals, and human. Hexokinase II and glucose transporter protein 4 are most significant protein isoforms involved in the glucose phosphorylation and transport in glucose sensitive tissues such as heart, skeleton muscles, and adipose tissues (Burcelin et al. 1993). Hexokinase I and II can be associated with mitochondria outside of exterior membrane with specific binding of porin or voltage dependent anion channel. Mitochondrial attachments make this possible to gain ATP directly after synthesis, which is one of the two substrates for hexokinase. The tumor cell have almost 200 times higher or elevated level of hexokinases, compared to normal cells (Bustamante and Pedersen 1977). Krasnov et al. (1999a, b) has been reported the transgenic fishes with transgenes human glucose transporter 1 (GLUT 1) and rat hexokinase II. The successful expression of both transgenes improved glucose consumption in transgenic fish, which indirectly solicited in growth and performance of transgenic fish compared to control or wild animals. Similarly, many other enzymes genes from the glucose metabolism and other pathways may be future strategies to improve productivity of farm animals.

Transgenic approaches in fish with growth-related transgene have been more applicable to increase growth rate of nondomestic fish species, such as salmon, while less applicable to domestic species of fish, such as trout, already selected on base of growth rate. Some hurdles related to transgenic fish massive production and consumption may be included; (1) effect on wild population of fish (2) doubts related to health effects, and (3) whether these will be available soon. The creation and release of animals into environments have two constrains; (1) the issues related to release of such genetically modified organism and (2) controlled production of the transgene expression, which need the production of more complex constructs (Montaldo 2006). Because some studies has not revealed consistent results related to the effect of growth. However, the safe genetic modification in fish such as lacking any undesirable effect during wild culturing on other population would be the next challenge for the researchers. Furthermore, the genetic modification in aquaculture for desirable trait such as medicinal and nutritional benefits would also be under the consideration of researchers.

4.5 Modification in Milk Contents

Milk is almost considered a perfect food in many societies due to its balanced protein, fat, carbohydrates, mineral and ash contents. Milk has been used for the nourishment of suckling animals for a long time due to complete and hygienic source of food. The ruminant milk and dairy products have been accepted as significant human foods from as early as 4000 B.C. The dairy industry has made tremendous progress over the years and develops a variety of new dairy products for human use (Lock 2007; National Research Council 2002). Moreover, dairy

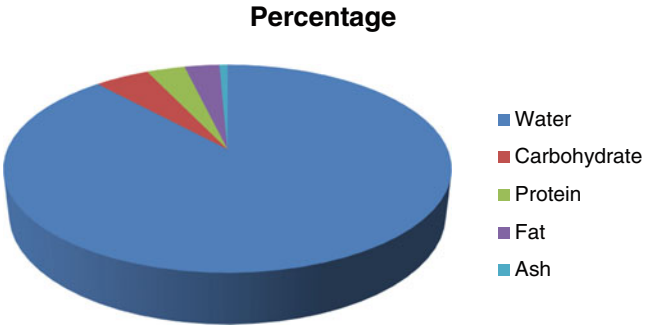

Fig. 4.1 Composition of cow milk (Lock 2007)

industry helps to meet the dietary requirements of human beings for energy, great quality of desire proteins, minerals and vitamins. In the last quarter-century, the nutritional quality of food has become increasingly important as a result of emerging buyer awareness related to strong relation of food and health. Many dairy foods have been identified or developed that contain specific components with potential benefit to human being along with traditional nourishment (Karatzas 2003; Lock 2007).

Cow milk constitutes 88 % water, 4.7 % carbohydrate, 3 % fat, 3.3 % protein, and 0.7 % ash, which contains mineral and vitamin contents. Recent research has illustrated that milk comprises a variety of bioactive constituents such as protein, peptides, and fatty acids. The growth and quality of health in newborns can be improved by increasing the level of any nutrient major nutrient such as protein, fat, or lactose. Moreover, fermented milk products with desirable traits have the potential to elicit addition health benefits (Lock 2007) (Fig. 4.1).

Bovine caseins can bind to bivalent cations due to phosphorylation; so this milk is a good source of dietary calcium. Caseins can bind other bivalent cations such as magnesium, iron, and zinc more in bovine milk than caseins in human milk. Casein in bovine milk binds almost 65 % of calcium, while human milk binds only 5 % calcium (Karatzas and Jeffrey 1997).

4.5.1 Milk Modification and Value Additions for Human Consumption

Large scale sequencing and genome mapping techniques improved the knowledge of animal genetics. A number of gene sequences or gene elements information is available for desire effects (Karatzas and Jeffrey 1997). The conventional cross-breeding of livestock, improvement through nutritional managements, and other methods of quantitative genetics have very slow change in milk yield improvement and now change in protein composition of milk. Milk with alter composition from

transgenic animals are now possible with modern technology. The modified milk can be produced with high amount of proteins and all other growth factors naturally deficient in milk (Bremel 1989). The modification in the composition of milk is not limited to milk proteins; but can be extended to the manipulation in the lactose, metabolic enzymes, milk fat, and the minerals in milk (Yom and Bremel 1993).

Modification of milk composition through genetic engineering can also improve the health of animals and supplementation of other components along with milk such as growth hormones, bioactive reagents, and other growth factors. In addition to human, these components can also help to improve the immune status, growth, development of gut and endocrine system of neonates (Wall et al. 1991). The genetically modified animals can produce; (1) high quantity of milk, (2) greater quantity of nutrient in milk, (3) milk with beneficial value additions such as nutraceutical proteins in the milk (Hartmann 1984) (4) milk without the undesired components (e.g., lactose) (5) modified milk with the composition to make more suitable for human consumption especially in neonates (Karatzas and Jeffrey 1997). The number of such proteins like epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-I), transforming growth factor beta (TGF-β) and lactoferrin in the newborns milk can increase the growth, health, and mental status (Konakci et al. 2005; Malcarney et al. 2005; Zhang et al. 2008).

Previously, only pharmaceutical proteins were produced from transgenic dairy animals (reference). Now, milk designed with special compositions or milk with potential human benefits may be competed for next a few years to capture the global marketing worth almost $400 billion annually (Karatzas 2003). In addition, the modified milk for nutrients and therapeutic value can be very beneficial for the health of newborns. However, the concept has been postulated for many years that milk protein contents can be incremented to the natural milk composition with the help of modification in casein gene quantity (Table 4.2).

Milk composition can be altered by changing the specific and unique copy of different milk protein genes. Milk composition modifications in transgenic animals have different applications. Modified milk composition can make more suitable milk for infants to drink. In composition human milk is deficient with β-lactoglobulin, which has higher relationship of serum protein to caseins. Human milk has more lactoferrin and lysozyme compared to bovine milk. These are involved in transportation of iron and inhibition of bacterial growth in milk (Van-Berkel et al. 2002). Previously, transgenic cow was successfully developed with human lactoferrin gene and bovine milk with lactoferrin expression for human consumption (Reference). In addition, human lipase can also be expressed in the milk of the transgenic animals. Milk with human lipase is capable to digest the lipid content of milk more efficiently, especially in neonates. Lipase can stimulate the bile salt to increase the conversion of milk triglycerides into fatty acid and glycerol (Houdebine 1995).

Instead of value addition to the components in bovine milk, the removal of undesirable components such as β-lactoglobulin is also interesting subject among to major bovine milk allergens (Wheeler 2003). The bovine milk is directly dependent on the lactose production; lactose is prepared inside the Golgi complex of the mammary secretory epithelial cells with lactose synthase complex. This complex

Table 4.2 Potential modifications of milk composition by gene addition, with expected functional outcome (Karatzas 2003)

S.No.	Modification functional consequence	Modification functional consequence
1.	*Introduction of casein genes*	
	Increase ratio of κ-casein to β-casein or concomitant increase of all caseins by transferring casein locus	Increase in protein and calcium content. Reduction in micelle size, enhancement of heat stability
2.	*Modification of casein genes*	
	Add phosphorylation sites	Increase in calcium content, micelle size, and stability of milk. Enhanced amphiphilicity of β-casein increases its emulsifying and foaming properties
3.	Introduction of protease (chymosin) cleavage sites	Increase in rate of cheese ripening
4.	Deletion of protease (plasmin) site from β-casein	Increase in emulsifying properties. Elimination of bitter flavor in cheese
5.	*Introduction of other functional proteins*	
	Add lysozyme, lactoferrin, or lysostaphin	Milk with antimicrobial activity
6.	Add reversibly inactive lactase that is activated in gastrointestinal tract upon ingestion of milk	Elimination of sweet taste of lactose hydrolyzed milk and alleviation of lactose intolerance symptoms

comprises on the α-lactalbumin and the enzyme β1,4 galactosyltransferase and a catalyst of reaction lactose synthase. Lactose is released from Golgi complex in secretory vesicles and transported to apical of epithelial cell and secreted into the lumen. First lactose attracts water inside the vesicle by osmosis, because it cannot diffuse out from vesicles without this process. The lactose synthase have vital role in lactose synthesis, influx of water into secretory vesicles and finally lactose release into the lumen. The α-lactalbumine have vital role in osmoregulation of milk (Wheeler 2003).

A milk modifications have been made this possible to consume the milk by patients suffering with metabolic disorders such as phenylketonuria. The patient suffering with phenylketonuria lacks the enzyme that metabolizes phenylalanine and needs diet with lower phenylalanine contents. Otherwise, the modification in milk proteins such as α-lactalbumine without disturbing the structure of protein can be used in diet of the patient suffering with phenylketonuria as supplementary food without undesired components (Colman et al. 1995). The reduction of lactose in the milk is also among the major desirable characters related to the consumption of bovine milk in people with lactose intolerance. According to an estimate, 70 % of people worldwide are suffering with lactose intolerance due to deficient level of lactase in their intestine. The lactose can be reduced into milk by expressing β-glactosidase into bovine milk or with removal of α-lactalbumin components from milk with modern biotechnological techniques (Montaldo 2006). Stinnakre et al. (1994) were reported α-lactalbumine gene inactivation from mouse. Transgenic

mouse has been produced milk without lactose with a serious disadvantage that milk of such animal was very viscous in consistency and unable to secrete from mammary glands of mouse, because lactose is an important protein in osmoregulation of milk.

One glass of milk comprises almost 8 g of protein with 78–80 % caseins contents of this amount. Casein along with milk fat gives white chalky color to milk. In cow milk, four types of casein proteins such as αS1- and β-casein (10 g/l each), αS2-casein (3.7 g/l), and κ-casein (3.5 g/l). The sequestering and binding of calcium and magnesium with in a spherical particle (micelle) is one of the major functions of the caseins. The outer surface of micelle is made up of κ-casein, which can easily destabilize by chymosin enzyme to the form the curd and finally cheese from milk. The modification in chymosin cleavage sites of aS1-CN to peptide linkages for more efficient hydrolysis can also reduce curd formation time of cheese. Moreover, casein has little sulfur containing amino acids; the alteration in milk by increasing Met contents can improve the nutritional value of such milk. So, any alteration in casein in milk can directly affect the cheese yield from milk (Bawden 1994; Karatzas 2003). Therefore, modification in casein gene is also very helpful to alter and increase nutritive value, cheese yield from milk and other components of milk processing (Table 4.2). Previously, efforts were made to increase the copies of the κ-casein gene into transgenic animals to decrease the size of micelles and to make more susceptible modified κ-casein (References). These studies successfully demonstrated that extra copies of casein gene in mouse model successfully encoding two type of casein: bovine β-casein and κ-casein. Modified milk collected from cloned transgenic animals with extra copies of casein genes were enriched with β- and κ-casein, which increased the 30 % total milk casein and 13 % milk protein (Gutiérrez-Adan et al. 1996; Brophy et al. 2003) with such alterations change the physical properties of protein (Brophy et al. 2003). In another study, the transgenic cow was developed with modification in κ-casein and an overexpression of this gene decrease the size of micelles in milk, which favor the cheese industry; while this milk was slightly more yellow in color and lower β-casein contents. Economic value of this milk still needs to be proven (Soler et al. 2006).

In addition, casein solubility could increase only with increasing in glycosylation, which could reduce the time for coagulation of rennet and expulsion of whey. This process would yield the firm curds from milk and would improve in cheese making. Alteration in other physical properties could improve the quality of food produce from milk, such as cheese with low fat and better taste. The production of milk along with processing and transportation cost of milk can be reduced with genetic engineering techniques. These techniques made this possible to use more milk and milk products with better value addition. These methods offered a better opportunity for the production of protein milk to improve animal agriculture and creation of new medicines in milk (Gottlieb and Wheeler 2011).

Mastitis is a major factor on the milk quality deterioration. It is most devastating disease of dairy industry with reduction or termination of milk. This disease leads to undesirable consequence of remaining milk such inclusion of bacteria and inflammatory cells in the milk, unpleasant taste and undesirable effects. Mastitis

causes of 53 billion dollars loss per annum worldwide (Ratafia 1987; Miller 1993). Some specific antibodies can be produced in the mammary gland in genetically engineered animals which can protect animals from specific problems such mastitis. The antibodies secreted through the milk can protect our domestic livestock and humans from diseases (Zang et al. 2008). Mastitis resistant animal is possible by inclusion of certain genes in animals which are expressing antibacterial substances into milk such as nutriceuticals, proteases, lysozyme, and transferrin (Houdebine 2000; Kerr and Wellnitz 2003; Felmer 2004). Cow's milk with some antibacterial proteins such as lysostaphin has great antibacterial activity. These animals have been recognized very resistant to *Staphylococcus aureus* mammary infection. The milk of these animals was also resistant to spontaneous bacterial infections arised from environmental contamination. However, the acceptability of lysostaphin containing milk for humans was unexplored, whether this milk would alter the composition of human intestinal microflora (Soler et al. 2006).

The bacterial systems cannot glycosylate the mammalian protein, while the fungal system although have glycosylation ability with addition of many undesirable groups on the proteins. So, the functional properties of such proteins are strongly affected by this alteration. Other techniques of protein production such as baculovirus system and mammalian cell culture techniques are suitable for the production of proteins along with specific problems. Instead of all, the genetically engineered animals are very suitable alternative for the production of complex mammalian proteins (Rudolph 1999).

The 5′ flanking end of the genes for milk proteins, which normally have regulatory role for their expression, can help to express foreign genes in mammary epithelia cells of genetically engineered animals. Previously, transgenic animals were developed for the expression of different proteins along with these regulator genes; almost have similar observations of expression as in the case of transgenic mouse (Wheeler 2003). Interestingly in the case of complex process of milk production, the expression of one protein is indigenously counterbalanced by the expression of other protein. So, it is quite difficult to alter the milk production with single gene transgenic approach. It is very necessary to understand the biological control for the production of milk before the remarkable investment to make transgenic animals of dairy importance (Wheeler 2003).

With a biotechnological approach, many proteins of human therapeutic importance were expressed in the milk of transgenic animals. Even human collagen protein has been produced into the milk of transgenic animals, which is a very large and complex protein human protein. Modern technologies such as bacterial fermentation and cell culture can produce human proteins e.g., human plasminogen activator (htPA) more economically into transgenic animal milk. In other proteins of human industrial interest were successfully expressed and isolated from the milk of transgenic domestic animals include antitrypsin and human antithrombin III. Industrial research is focused on the production of the human industrial proteins with greater value and low volume human beneficial protein in milk of the transgenic domestic animals (Karatzas and Jeffrey 1997).

The previous studies clearly revealed the importance of modern biotechnological tools for synthesis of modified milk with value addition human. Considerable time and resources are required for the production of dairy animals with new genetics and commercial values. Such modifications in the milk would be made animals of low value to animals of higher commercial importance. Other policies related to consumption of genetically novel milk of modified dairy animals will be overcome soon in future by the drug and food administrative authorities of developing countries. Modified animals with desire characters will be soon in the breeding programs of developing nations to improve the livestock.

However, the production of modified milk with wanted character or component is an excellent source for the production of protein at a large quantity. Milk transgenic technology can also exploit the economical productivity of mammary glands. In major problems include the accurate production of transgenic animals along with regulatory issues. Additionally, the issue to address is the large scale economical production of milk and to overcome the public concern.

4.6 Modification of Hairs or Fibers and Wool Production

The biotechnological modifications of wool and fiber contents were relatively slow compared to other characteristics. The skin biology and development in cellular level has been understood since 1950s. However, most of the studies were focused on the wool follicles and growth of wool fibers from wool follicles; while other higher organization of mechanisms in this process such as trio groups of primary and secondary follicles received little attention of scientific investigations (Fraser and Short 1960; Purvis and Franklin 2005). The development of wool follicles start at early stage of fetus development and continue through the lifespan of the animals (Fraser and Short 1960; Purvis and Franklin 2005).

Animal hairs, fibers and wool are used in fabric and yarn production. Transgenic manipulation in livestock for modification in fabric and yarn production has been recognized another application area of modern animal biotechnology. The genetic manipulation has been focused for the modification in hairs, fibers and wool for color, quality, yield, harvesting ease (Wheeler 2010). The different transgenic method has also been solicited to examine the wool and hair fibers refinement, excellence and crimp. The modification in the keratin and keratin associated protein (KAP) genes of sheep have been improved the processing and wearing quality of wool fibers. The cortical-specific high expression of intermediate wool filament type II keratin gene (K2.10) produced the modified microstructures and macrostructures of wool filaments with higher luster and lower crimps. Several modifications in genome of transgenic animals have been demonstrated with more elastic and strong fibers, which would reduce the shrinkage quality of garments (Bawden et al. 1998; Wheeler 2010).

The efforts were made to increase the growth of wool yield in sheep. The insulin growth factor-1 (IGF-1) gene expression in the follicle was used to increase the wool

production. The expression of IGF-1 gene in transgenic sheep produced 6 % increase growth of wool at year 1 compared to wild type sheep. Moreover, the wool was poor in quality wool with lower stapled strength and coarse in texture. In addition, the transgenic sheep produced higher wool content only at first year and no significant improvement was observed in next year (Damak et al. 1996; Su et al. 1998).

The wool production was boosted with over expression of ovine growth hormone, while the higher plasma concentration produced some harmful effects (Ward and Brown 1998). The transgenic merino sheep was produced with improve construct, resulted in 12 % improvement in fleece production. In Poll Dorset crossbreed sheep, opposite results were observed such as reduction in wool contents in transgenic sheep contain ovine GH gene construct. The results explained a significant association of breed type with transgene expression and response (Adam et al. 2002; Wall et al. 2009). The wool produce from transgenic animal with ovine GH transgene expression were poor in quality such as larger but less desirable diameter of fibers. The ovine intermediate filament keratin transgene in wool follicle has also been expressed to improve the quality less desirable fibers (Adam et al. 2002; Wall et al. 2009). In sheep, harvesting at specific time is another problem for the farmers and labor intensive. The modern methods of transgenic technologies made this possible that sheep shed their wool fiber at specific time period (Hollis et al. 1983). The mouse epidermal growth factor (EGF) has been introduced in transgenic sheep under the influence of inducible promoter (Hollis et al. 1983). The infusion of Mouse epidermal growth factor (mEGF) induced follicular regression and fleece harvesting more rapid than previous depilatory agents without any anomaly, apart from sustained epidermal thickening. A spot in the wool fibers can be produced with successful expression of such a transgene and endorsed fleece to remove with hand pressure. This modification reduced the cost of metal shearers or clippers during wool harvesting. This cost effectiveness is really an advantageous for wool producers or farmers (Hollis et al. 1983; Wheeler 2012). This modification can also be applied to other wool producers such as camels, mohair goat, alpacas, and other wool producers (Wheeler 2012).

The single transgene expression in transgenic animals to improve the wool and fiber content was forced to utilize some novel transgenic strategies such as bacterial derived biosynthetic pathway genes for synthesis of amino acids such as cysteine. Cysteine is a wool growth regulating element in livestock rear for wool or fiber production. The approach was successfully demonstrated with two different bacterial genes in transgenic mice, while this approach was unsuccessful in sheep due to low level of unsustainable biosynthetic enzyme expression (Ward et al. 1994; Bawden et al. 1995; Ward 2000).

Animal wool production and properties of fibers can be altered with transgenic technology. Sheep wool contains limited cysteine fibers contents, with increase in this amino acid may increase the production and properties of wool in transgenic animals. Some previous studies have been performed to introduce the cysteine biosynthesis genes from bacteria to the genome of sheep. This strategy did not allow to produce and efficient expression of these enzymes in the rumen of transgenic sheep (Murray et al. 1999; Montaldo 2006).

Transgenic approach has been used for the expression of very valuable fiber in the milk of transgenic goat (Karatzas et al. 1999). The seven different types of silk are used by the spider in the synthesis of orb webs. The unique mechanical properties of each fiber differentiate these fibers from other natural and synthetic fibers (Karatzas et al. 1999). The dragline silk is the most robust variety of silk, which has properties of 35 % elongation from original material and high tensile strength. In addition, the capability of energy absorption before cracking is more than steel (Wheeler 2010). Breed Early Lactate Early (BELE®) goat system has been used for expression of protein monomers gather to develop the spider silk fiber in the milk of transgenic goat. The fibers assemble from protein monomers of transgenic goat were obtained similar quality of spider silk. These fibers have different applications such as medical devices, aircrafts, sutures, automotive composite, ballistic protection, and special clothing (Wheeler 2012).

Further investigations are required in addition to rate limiting amino acid modification such as cysteine, protein modification like wool intermediate filament keratin, wool-related amino acid enrichment of keratin and Insulin growth factor 1 overexpression in wool follicle; which can increase the wool fiber growth or harvesting ease. The consideration of wool and fiber growth and quality-related QTLs could also be included in future animal breeding strategies for the production of more suitable breed with better growth and quality of wool. The human future demand for better cloth may be emphasized researcher to produce the better wool and fiber contents from transgenic animals with different and unique transgenic approaches. The transgenic animal biotechnology can play a vital role for the production of fine fiber and wool to meet the incremental future demands of garments with better processing and wearing quality.

4.7 Modification for Infectious Disease Resistance

Genetic engineering related to animal biotechnology should primarily apply on the animal welfare and health. The interesting application of modern animal biotechnology related to animal welfare is to make the animal resistance to different diseases. The genetic information has been made this possible to make transgenic resistant animals to various diseases. The disease resistance in animals is polygenic trait and a few loci are identified related to disease resistance against particular disease. The transgenic approaches related to animal disease resistance include the transfer of major histocompatibility complex, immunoglobulin genes, T cell receptor gene, gene affect lymphokines/specific disease resistance. The genes involve in the histocompatibility complex (MHC) during immune response can be used as major instrument for the animal disease resistance and transgenic animal with better disease resistance (Wheeler 2012).

Many genetic aspects for the disease resistance in livestock have been determined, while the role of specific genes in immune response and disease development still need keen intentions of researchers (Ebert and Selgrath 1991). Previous

study results showed that, the genetic manipulation for disease resistance revealed that the embryonic cells are very useful to modify such genes. The germ line transfer has been possible up to >100 kb with pronuclear injection, while >400 kb has been transformed into transgenic animals with yeast artificial chromosome vector. Therefore, the disease resistant livestock could easily be produced with embryonic stem cell transgenic approach (Wheeler 2012).

Haller et al. (1981) has been produced newborn transgenic swine containing the Mx1 protein gene and high expression has been made resistance to lethal infection of influenza virus. The MxcDNA transformation and expression in 3T3 cell lines produce resistance against the lethal influenza virus infection (Staeheli et al. 1986). The transgenic piglets have been developed with cDNA construct encoding Murine Mx1 protein. The transgenic piglets were successfully developed resistance against lethal influenza virus infection (Muller et al. 1992). The transgenic piglet with over expression of Mx1 protein might have much detrimental effect on the piglets. Furthermore, the expression of Mx1 protein was eliminated with rearrangement of transgene insert in transgenic piglets (Muller et al. 1992). Mastitis is udder inflammatory disease of cattle 'as a result' animal loss productivity and milk yield. Previously, the transgenic cattle resistant to *Staphylococcus aureus* infection has been produced with transgene expression of lysostaphin, an antimicrobial peptide secreted in milk. Lysostaphin killed the bacteria with in a dose dependent manner (Donovan et al. 2005). Moreover, the mammary gland associated expression of lysozyme in goat inhibited the growth and development of bacteria involve in mastitis and cold spoilage of milk (Maga et al. 2006).

The transgenic technology has been proved a vital tool to produce transgenic animals resistant to different diseases in above mentioned studies. Furthermore, the transgenic technology made this possible to enhance the useful allele inside transgenic animals and to knockout the harmful genes from these animals. Previously, the knockout of *Escherichia coli* K88 receptor inside transgenic experimental swine has been ascertained completely resistant to *E. coli* positive with K88 compared to wild animal (Edfors-Lilia et al. 1986). Moreover, the similar investigations should be applied on other animal health issues including parasitic infections such as trypanosomes and nematodes, viral infections such as foot and mouth disease, bovine leukemia virus, pseudorabies virus, bacterial infection such as clostridium infection, streptococcus infection and deficiency disorder such as deficiency of UMP synthase, mule foot and bovine leukocyte adhesion deficiency (Wheeler 2012).

The transgenic pig, sheep and mice were successfully developed with the mouse immunoglobulin A (IgA) transgene construct and expression (Lo et al. 1991). The transgene of murine IgA was successfully introduced in two transgenic pig lines with only light chain expression of IgA molecules (Lo et al. 1991), while a transgenic pig has been developed with successful expression of murine monoclonal antibodies with great binding ability to specific antigen (Weidle et al. 1991). The resistance against the ovine Visna virus has been developed in transgenic sheep. Transgenic sheep was developed with Visna virus envelop transgene and expression without any harmful or pathological effects (Clements et al. 1994). The transgene of viral envelop was not expressed in monocytes, while antibodies were developed after

experimental infection to transgenic sheep (Clements et al. 1994). The virus neutralizing antibodies were produced in the transgenic mice model milk against a coronavirus responsible for economically important transmissible gastroenteritis (TGEV) disease (Castilla et al. 1998). The transgene expression and engineered passive immunity was achieved throughout the lactation. In near future, this work would also be demonstrated and verified in transgenic pig (Castilla et al. 1998).

The self-immunization against pathogenic organisms is also a great application of this exciting technology. The construction and transformation of transgenes under the influence of physiological or specific stimulus to express could be produced antigen at specific time or with specific response to stimulus with this tremendous technology. This expression would immunize the transgenic animals against particular disease in response of specific stimulus or physiological condition (Wheeler 2012). The transgenic animals will be developed with transgene construct under the control of stimuli zinc in feeding or specific antibiotic to express the antigen at specific time period to increase the specific antibiotic level in serum (Wheeler 2012).

Scrapie and bovine spongiform encephalopathy free or naturally resistant animals may be produced in future with technology. The transgenic technology is a vital tool to develop animal model in epidemiological investigation of such disease and eradication from farm animals. The experimental production of either human or bovine prion proteins in transgenic mice is a great example of such application (Bishop et al. 2006). In addition, knockout is only a successful of approach to avoid the infection and transmission of spongiform encephalopathies like scrapie orBSE. Denning et al. (2001) has first time targeted the ovine prion (PrP) gene locus, while the clone lamb died short after birth. The transgenic cattle cloned with knockout of prion locus have also been developed (Cyranoski 2003).

Similarly, the resistance against brucellosis has been successfully established in fetus of transgenic bull develop from somatic cell line nuclear transfer, which is highly contagious disease of zoonotic importance, and caused high fever, muscular pain, and reproductive disorder in females (Shin et al. 1999). It is still a very short and selective discussion related to application of transgenic technology on the animal and human welfare. It can be applied on a number of organisms related to animal health and productivity. Healthy animals would be more productive and useful for farmer and less effort would be required in rearing. In future, the use of animal biotechnology especially transgenic technology will be increased in future to improve the animal health and production. Some ethical issue related to transgenic animals and very high cost associated to animal transgenic technology are still major constraints in application of this technology for animal welfare.

4.8 Harmful Aftermaths of Different Animal Modifications

The transgenic animal approach is very useful to understand the function of different proteins and secondary gene products related to these proteins. The transgenic research related to allied fields of nutrition is benefiting to understand the normal and

alter metabolism effects (Prieto et al. 1999). The food ingredients, food nutrients and food products from transgenic animals have not yet extended to consumers reach. This technology is maturing and producing thrilling results in model and experimental farm animals. The advent transgenic food products emphasized the need of guidelines and legislation, which has been developed in many countries to anticipate such products in market for consumer (Prieto et al. 1999).

Practically, the transgene expression is not always useful for the animal health. The transgene unusually expressed in the nonspecific tissue and biological fluid in which they not normally expressed. This nonspecific expression was caused some adverse and undesirable effects; for example, the transgenic mice with GH have elevated level of GH compared to wild-type littermates. This high expression of GH elevated the level of insulin, as result, the transgenic animals died due to liver and kidney impairment (Chen et al. 1997). The products of transgenic plants such as some fruits and vegetables are already available in the market, while animal functional foods or modified foods are not yet reached to consumer market. The animal modified food derived from predictable or nonpredictable animal modification methods and food safety consequences, which may be a major reason for animal derived food unavailability in the market (Prieto et al. 1999).

The undesirable effects observed in transgenic animals emphasize the need for further research related to genes/genes expression control and physiological consequences due to chronic expression in case of certain proteins. Furthermore, it is clearly demonstrated from previous studies that the fetus is unable to develop, when transgenes used the systemic regulatory elements during gene expression. In this regard, the expressions of glycosyltransferases and enzymes' general effect on glycosylation are most significant (Metzler et al. 1994; Prieto et al. 1999), while the early development is arrested due to a sialic acid-specific esterase under the metallothionein transcription regulatory element (Varki et al. 1991). Similarly, the development of mammary gland morphogenesis of transgenic mice was impaired and inhibited due to overexpression of cell surface β1,4-galactosyltransferase, under the influence of metallothione in transcription regulatory element (Hathaway and Shur 1996).

Furthermore, the genetically engineered mice has been developed with transgene expression of human α1-3/4-FT glycosyltransferase for carbohydrate biosynthesis pathway under the control of rat intestinal fatty acid binding protein transcription regulatory element or the whey acidic protein promoter without any harmful effect. The secondary gene product and tissue-specific expression of transgene was localized into tissue, but the outcome of the expression may be specific to species (Bry et al. 1996; Prieto et al. 1999). The transgenic rabbits with transgene expression of fusion protein were demonstrated harmful effect to milk production and lactose free milk was produced from transgenic animals. The transgenic animals were studied up to many generations to understand the harmful of enzyme effect on the lactation. These studies demonstrated many factors effect on final result including the capability of transgenic animal species, even when the same gene construct or primary gene product is expressed (Prieto et al. 1999). In other factors included the limitation of tissues for the expression of specific protein such as lactating mammary gland (Bleck et al. 1995).

The transgenic rabbits developed from human erythropoietin gene under the influence of whey acidic protein gene promoter, and the ectopic expression of transgene produced some deleterious effects (Massoud et al. 1996). Moreover, the transgenic pig was developed with transgenic extemporaneous expression of whey acidic protein. The mammary glands development was impaired in transgenic pig (Shammay et al. 1992). These studies are remarkable related to protein function, development and morphogenesis along with species-specific response. Wall et al. (1996) demonstrated that the transgenic sheep expressed the mouse whey acidic protein in all tissue except into lactating mammary glands. Some of the harmful effects are mentioned in Table 4.3.

Future Constrains and Prospective

The green revolution has brought about reasonable prosperity in the life of farmers with land, but the majority of landless farmers remain poor. Poverty, malnutrition, disease, poor hygiene, and unemployment is widespread in the developing world and biotechnologies can play a vital role in this context. Some modified animal food products are developed with modern biotechnological tools. Such animal food items are economical and more useful in respect to health. The modern biotechnology implementation to alter animal products for fat and other characteristics is constrained by a number of factors. One of the major constraints on applying biotechnologies is the economic and wealth status of animal farmers. The major constrains to adopt modified food products with new technology in developing nations include the absence of an accurate database on livestock, biodiversity with animals, disease resistance, and nutrient utilization characteristics.

Table 4.3 The undesirable and unpredictable effects from transgenic animals (Modified from Prieto et al. 1999)

S.No.	Transgenic animal	Trasngene	Effect	References
1.	Pig	MTh-bovine GH	Gastric ulcer, cardiomegaly, arthritis	Pursel et al. (1989)
2.	Pig	mWAP	Failure to lactate (agalactia; characteristic mammary gland phenotype)	Shamay et al. (1992)
3.	Mice	baLac-bbcasein	Short lactation	Bleck et al. (1995)
4.	Rabbit	rWAP-EPO	Infertility, agalactia, premature death	Massoud et al. (1996)
5.	Mice	MTh-b-Gal-transferase	Impaired mammary gland development	Hathaway and Shur (1996)
6.	Rabbit	mWAP-a1-2FUT I	Lactose free milk (changes in milk protein quality and content)	Prieto, unpublished results

MTh mouse metallothionein promoter. *mWAP* mouse whey acidic protein promoter. *rWAP* rabbit whey acidic protein promoter. *EPO* human erythropoietin. *baLac-bbcasein* bovine alactalbumin promoter-bovine b-casein. *α1-2FUT* human α1-2 fucosyltransferase "H"

Moreover, in other challenges to adopt such products in developing nations include the lack of trained manpower both in the government and in the private sectors for the development of modified animal food products along with the absence of an interface between industry, universities and institutions, the high cost of technological inputs such as materials, biological and equipment, the failure to address issues of biosafety and to conduct risk analyses of new modified animal food products, the negligible investment in animal biotechnology. Criticisms of such technologies and applications involve issues ranging from food safety and social resistance to potential negative impacts on animal safety and on ecosystems along with the current regulatory structure to assess and manage risks created by these technologies.

This technology has massive expectations in the future for production of transgenic livestock with desired modification for food production. Furthermore, this technology still needs to improve related to modified animal food composition and production capability such as growth rate, disease resistance, and reproductive efficiency. These modifications need improvement in the desirable traits quantity, identification of markers linked to certain genes with specific characters or traits, selection of better animals, propagation of superior animals, practical and economically viable methods. The four things to be considered for animals in the transgenic approach for desirable traits are time period for commercial development, regulatory concerns and related issues, economic feasibility, and adoptability of these production methods (Prieto et al. 1999). Transgenic animal technology is a powerful tool to improve food production and to study nutrition effects in health and disease development.

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Chapter 5
The Adverse Impact of Modern Biotechnology on the Environment

Mirza Muhammad Fahd Qadir, Attya Bhatti and Peter John

5.1 Introduction

5.1.1 Importance of Biotechnology

It has been the requirement of the human population to obtain greater supplies of food items for consumption. This is due to the extensive consumption as well as the growing population worldwide (Naylor et al. 2000). This increase in production has been deemed possible by use of new biotechnological advances shifting production to low priced and widely available products (Quinn 1992). This increase has been brought about by the increase in use of genetically modified organisms (GMOs). Generally, there exist only two broad instances that will allow food production to match the overall production requirements of the growing population. They include utilizing alternative food products whether based in the sea, or as single cell products (Ratledge and Kristiansen 2006; Iibery and Kneafsey et al. 2000). Secondly, enhanced plant as well as animal breeding efficiency can allow for enhanced production output to be achieved. At the advent of the millennia, exciting new prospects like that of genome sequencing and molecular-based research on a multitude of proteins and compounds allowed for various advances in the field of biotechnology to take place (Pisano 2006). However, the implications that these changes have toward the environment have not been studied extensively. It is hypothesized that intervening with the natural course of nature will indeed lead to altering of various factors within the environment, particularly biodiversity (Wilson and Salyers 2002; Sharma and Vrendenburg 1998; Kaplan 1987).

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It is notable that since the usage of recombinant DNA (rDNA) technology, many ethical as well as biological concerns have originated which have shown to oppose the use of biotechnology in our daily lives (Thompson 2007; Comstock 2000; Blank et al. 1987). Since rDNA represents a quantum jump in the possibility of inculcating novel and unique traits in organisms, it has now become the hallmark of most biotechnology research globally (Diem-Lane 2008). The ability to develop new and improved strains which have the ability to withstand harsh environment, like low moisture content of soil or high climatic temperature, has caused the inclusion of new genetically unique organisms in the market and environment (Moorhead 2009; Kimsky 1996). The question that, however, looms over every researcher is the fact that a duality exists when discussing the role of rDNA organisms (McCance 2013). These organisms have the ability to pose a unique and tangible risk to the environment; these include gene transference and toxicity over other organisms.

5.1.2 Biodiversity and Biotechnology

The greatest cause for alarm, however, is the manifestations of anomalies in soil and water ionic concentrations. Due to the altered genome of many plants and animals alike, there has been an altered state of utilization of mineral resources (Suzuki 2007). This altered state of utilization could have a negative impact on the environment particularly in case of biodiversity, which is at greatest risk (Jones et al. 1996). Plants having more resistant and dominant genes tend to repress their less resistant wild-type counterparts. This has lead to the rDNA plants not only asserting a more dominant role in that ecological niche but also modifying that position in the environment by removing the element of adaptation and evolution (Skippington and Ragan 2011; Prakash 2007). Since the dynamic state of the environment has been changed, hence the exact equilibrium between plant, animal, and the environment has also been altered in this regard (West-Eberhard 2003; MacArthur 1967). This alteration in the complex and the little understood environmental phenomena we observe as biodiversity, may have repercussions we fail to observe at this time and can be a cause for alarm (Blaustein et al. 1994).

Due to the ever-increasing global population, and increased food demand globally, it is notable that biotechnology may be the knight in shining armor policy makers and scientists are looking for (Jay 2013). Biotechnological advancement is an invaluable tool and could have a positive outcome on a sustained managerial system of both biological as well as physicochemical resources (Brayton 2013). What is important to realize, however, is that like any other technology it has the potential to be used for short-term gain, without any regard for the environmental outcome (Hannan and Freeman 1977). Therefore, it is empirical for population geneticists, conservational biologists, ecobiologists, and policy makers to closely monitor and be involved in the development and release of transgenic organisms into the market or environment. This is how the overall risk to biodiversity is minimized and the possible benefits made a realization (Diem-Lane 2008).

5.2 Genetically Modified Organisms (GMOs)

Rapid and hasty developments in modern biotechnology have left many governments with the advertent challenge to develop an efficient food production system within their country (Koehler 1996). This can be seen by some as a means for development; however, by some it can be seen just as a means to put others out of business by monopolization. At this moment, it is difficult to gauge whether GMOs will be continued to be used as sources of food for the population, even though its disadvantages have been emphasized by numerous scientists from the scientific community at large (Roman-Alcala 2013; Chen 2009).

Some examples of GMOs nowadays being used by the public, especially in case of drugs, is the use of Atryn (recombinant human antithrombin) produced in genetically modified goats has been approved by the FDA (Kling 2009) as well as Rhucin® (recombinant C1-inhibitor) produced by a genetically modified rabbit (Schmidt 2006). However, there is no research on the long-term effect of making these recombinant proteins (Laible 2009). Although many countries like China and the United States are looking into the process of legalizing GMOs and are accepting the fact that they may be required for human use, many oppositions are still in place particularly in the European Union against the use of genetically modified animals as well as crops for use. A particular example is the use of GMOs to produce recombinant bovine somatotrophin hormone (also known as recombinant bovine growth hormone rBST/rBGH) has shown to not only affect the health of the animals consuming it but also to have drastic effects on the individuals consuming milk from rBST-treated cows, as they produce insulin-like growth factor-1 (IGF-1) in their milk and can lead to uterine cancer (HPBH Canada 1998). This has lead to rBST being banned in over 27 European countries including countries like Australia, New Zealand, and Japan.

5.3 Agriculture and Biotechnology

5.3.1 Agricultural Biotechnology: Adverse Impacts

Agriculture has been regarded as the modulation of the environment to produce concentrated sources of energy and fiber. It is also pertinent to mention that historically cultivated land has been a source of habitation for many adapted and naturalized species of plants (Angus et al. 2009). It has been observed that some species range back over 3,000 years. Some agricultural systems may give rise to novel ecological life cycles, of the crops cultivated that now depend on the cultivation cycle as a part of their normalized life cycle (Wenzel et al. 2000). Similarly, it can be also be argued that many agricultural regions are now remnants of the original ecosystems that once originated in those regions (Dean et al. 2011; Haila 2002). It is all too unfortunate that numerous species of plant and animal life have come under serious threat due to the constant pressure of deforestation as well as habitat destruction.

However, some instances of organisms existing in ecological niches present within agricultural farmland have also been observed, though it is incomparable to the rich biodiversity present in natural habitat (Council 1991; Hekstra 1981).

It is notable that there is no agricultural program that provides a complete solution to the issue of ecosystem equilibrium. There is no regulated, soil fertility, crop pollination, or pest biology application during the process of cultivation in agriculture (Scialabba and Hattam 2002; Pretty 1995). It has been generally observed that greater crop biomass results in lesser nutrients as well as reduction in soil fertility. Also, it is a well-known fact that during crop cultivation, greater biomass of weeds results in less nutrients available for the crop itself (Fisher and Mexal 1984). Hence, agriculturists have used the general principle to eradicate all pests and weeds that affect the performance of their primary product. This has led to an overall depletion in the ecosystem present in the area under cultivation (Radosevich et al. 2007; Radosevich 1997). There has been a significant success of this technique resulting in enhanced crop yield and production. This, however, has come at a high price. Sustainability in the long run has been compromised, as it is the diverse ecobiological milieu that results in the maintenance of soil fertility (Labrada and Officer 2008). As farmers have started using crop varieties that drain more and more nutrients from the soil, it has resulted in significant soil damage by a reduction in the overall soil fertility (Magdoff and Van 2000; Bot and Benites 2005). Another reason for decreased soil potential is the use of fertilizers to combat the issue of fertility reduction. This has caused an overall damaging effect on the long-term sustainability of the soil fertility and maintenance of the nutrient content in the soil (Tilman et al. 2002). Furthermore, the usage of genetically-enhanced varieties which have increased ability of soil utilization and conversion rates to biomass, have resulted in a damaging scenario for crop overall production.

5.3.2 Genetic Modifications in Crops

Primary goals in case of crop breeding are to establish novel more assiduous varieties. Those varieties are preferred which have a higher potential to convert matter into biomass and hence help in the overall output generated from a hectare of land (Smil 2004). It is important for the reader to grasp that previous concepts of biotechnology are redundant in a way. For example, the usage of certain fungi like *Penicillium* cannot be compared to genetic recombination in a species genome (Lynd et al. 2002). Hence the concerns relating to biological impact are far greater than they used to be 20 or so years ago.

Modifications and forced, in breeding of crop varieties has lead to the establishment of new crop varieties that are very different in morphology and biological characteristics in both phenotypes and genotype (Burdon 1987). Human plant breeders have selected those plants having superior genetic characteristics in terms of nutrition, easier management, enhanced productivity as well as easier crop collection and processing (Acquaah 2009). There has been significantly enhanced

success in crop breeding which has maintained its popularity amongst breeders. There is, however, the impeding issue of crops still having many flaws like, their low efficiencies relative to their desired traits, along with their varying tolerance to pests and weeds (Rissler and Mellon 1996). A utopian crop would have higher efficiency as well as an enhanced ability to withstand harsh conditions like the environment and pests. Similarly, a ecobiologist would like to view these crops as requiring little or no management and not being a cause for alarm when the plant is analyzed for its involvement in the biodiversity of a region (Clayton and Radcliffe 1996). Therefore, an efficient crop or ideal crop should not adversely affect the environment. It should in fact support the diverse ecology of plants, insect, and animal life.

Natural genetic variation has allowed the survival of the fittest to allow for the establishment and progression of competent plant species. The diversity in the gene pool is accompanied by natural adaptation resulting mutagenesis, results in random but substantially significant evolutionary changes to persist and occur in an area (Morris 1974). Plant breeders attempt to isolate those characteristic properties of plants which give them uniqueness in terms of desired traits. Furthermore, it has been of considerable interest for plant breeders and geneticists alike to establish competent and novel varieties of plants which have been used in case of harsh environmental conditions (Chaleff 1981). Additionally, plant breeding has been observed to be a market-driven enterprise, which depends on demand of a particular variety with respect to a trait. Geneticists and breeders attempt to isolate this type of trait, and then use the corresponding genes responsible for the phenotype in question (Stewart 2004). This exploitation of genes with relevance to phenotypic performance is the gold standard that all geneticists attempt at locating and identifying as possible targets for recombination. This has been the hallmark of genetic engineering in crops (Hardy and Segelken 1998).

The involvement of recombination in plant genes has resulted in altercations resulting in variations in the gene pool to rise up. The greatest challenge is the homogenization of the gene pools of such genetically modified or altered plant types (Frankel 1995). This alteration in the gene pool of plants results in lesser options available for geneticists to isolate and utilize as a part of their genetic recombination of genes associated with specific traits. The possible options for most geneticists appears to be increasing day by day, this is simply because of the pre existing gene pool that is very diverse. Some may argue that a stringent selection mechanism may not be enough to eradicate or influence a gene pool due to the preexisting variety in case of a region where that plant species is indigenous to (Creech and Reitz 1971). However, this selection pressure has resulted in depletion in the overall gene pool and diversity of plant life.

5.3.3 Genetically Modified Crops: Sociocultural Impacts

Numerous articles have been published elaborating on the use of genetically modified crops and their deleterious impact on the environment. One example is the

use of genetically modified corn's pollen which is applied on milkweed consumed by monarch butterflies resulted in adverse impacts to the butterfly (Losey et al. 1999). Another instance of the adverse effects of biotechnology is the effect of the usage of genetically modified corn in Mexico. This resulted in contamination of the gene pool in the Mexican wheat with genetically modified wheat variety's DNA. This was also published in nature and due to this various government laws were passed to prevent the usage of genetically modified crops in the country of Mexico (Quist and Chapela 2001). The greatest concern featuring genetically modified crops has always been the fact that they might have significant impacts on the environment at large. Hence the inherent behavior of these organisms and their genetic composition in relation to the environment and naturally occurring predecessors is of grave concern (Muir and Howard 1999). Due to this fact, the unpredictability as well as irreversible risk with reference to genetic poisoning is what is of grave concern (Wickson 2005).

Since crops cover a large portion of the earth's surface, their characteristic features as well as their management techniques are essential factors which affect biodiversity in various regions of the world (Stewart 2010). The primary challenge for the future remains to meet human consumption and demand by the overall production capability of the earth. Until and unless a stable ecological culture in various regions is not maintained, degradation of biodiversity across the world will have adverse effects on the ecological status of the world (Raudsepp-Hearne 2010; Nellemann 2009). A tightly monitored as well as regulated agricultural policy is required, in order to support the maintenance of biodiversity in various regions in the world. Only then can we hope for a successful biome.

5.4 Animal Biotechnology

5.4.1 Role of Biological Interventions in Animal Biotechnology

Many advances have been made in the field of animal sciences. Particularly in case of genetics research it has been seen that substantial breakthroughs have been achieved in isolating and identifying genes which are responsible for vigor and weight gain. This is the result of greater and more stringent techniques associated in animal genetics (Boyd 2001). It is pertinent to note however that like every type of technology, animal biotechnology also has its adverse effects. Since biotechnology has been associated with the alteration of animals for human benefit and usage, many techniques have been applied for the enhancement of animal characteristics for human usage (Falk et al. 2002; Seidel 1998; Dresser 1988). Rapid changes have been made in the artificial selection of animals particularly genetics. Novel techniques have been involved, particularly the insertion of novel genes and their manipulation for the expression of desirable traits (Bruce and Bruce 2014; Yom and Bremel 1993).

Important considerations when using recombination and gene insertions are the escaping of these genes into the environment (Altman and Hasegawa 2012). The genetic and physical altercations have not been able to be categorized. It is also very difficult to predict the possible physical and environmental outcome of these genes (Linard 2012). Hence predicting the hazardous nature of escaped genes is unknown, and their interactions with the environment are not understood fully. Animal feeds and vaccines are also bioengineered when it comes to commercialized animal farming (Lappe and Bailey 2014). The question arises what the result of these interventions will be on the outcome and propensity of genetic variations occurring due to modifications if any as a result of these technologies (Nicholson and Wilson 2003). Amongst other technological interventions that may cause variations in the subject phenotype, it is pertinent to visualize and note that changes in the genome are reflections of both external and internal changes that occur as a result of both environmental and biological stressors (Adelman 2005). The key is to identify these changes and then extrapolate possible effects or hazardous implications on the environment (Levins 1985).

5.4.2 Applications of Biotechnological Techniques: Their Adverse Effects

During the past couple of years the science of engineering and modifying organism to meet and comply with requirements have been quite significant. The commercial applications of these animals have been quite significant; however, the role of these interventions have been under considerable scrutiny (Serrelli 2013). The insertion of genes has been used in mice and numerous other transgenic animals which have been used for animal experimentation. Genetically engineered mice have become the hallmark of vivisection and research especially in case of animal studies (Singleton 2000; Moore 2004; Curtin 2009). Hence, genetically altered mice have become widespread in their usage in biomedical research. The question arises, however, if these mice were allowed to interact with other mice in the environment, then what would the resulting interactions result in (Barbaric et al. 2007).

Since it is now possible to generate animals which have important functions in biotechnology and biomedical research, similar techniques are being adopted to engineer animals having superior weight gain and physical characteristics which is what farmers and animal breeders are looking for (Kulseth 1990; Straughan 1999). Methods that originally originated from mice as well as *Drosophila melanogaster* have been adapted to be used in other animals as well (Aravin and Tuschi 2005). Manipulation of the fertilized egg, and in vivo implantation are a part of the biotechnological techniques used. Sperm manipulation before it reaches the egg, also the usage of embryonic stem cells post manipulation ex vivo, somatic cell culture as well as the usage of embryonic stem cells are a part of the techniques used to manipulate the animal's characteristics (Bobbert 2006; Okere and Nelson

2002). The nuclei of these animals can be used to produce generic transgenic animals, which are then used in various frontiers of biotechnology. Some of these methods have the advantage of the geneticist to incorporate particular characteristics into the transgenic animal of interest which is engineered as a result of manipulation (Smith 2001, 2002).

The selection of certain genes that have characteristic features of particular interest to the animal breeder will be incorporated or selected for the production of these transgenic animals. The implications, however, will result in the formation of animals which possess a certain set of "selected genes," these selected genes are a representation of the breeder/geneticist's interpretation of characteristic traits and may not be the appropriate controller (Lieschke and Currie 2007). The greatest issue relating to this method is depletion in the gene pool. Due to increased artificial selection and prevention of appropriate natural selection, genes continue to be lost from the abundant gene pool (Frankham et al. 2002). This can be seen in animal species, particularly in case of cattle. Here simple inbreeding or forced selection will result in the prevention of appropriate genetic diversity within a species. Variations in the genome are what give a species its various traits, particularly in case of animals where traits are dependent on genes for their phenotypic expression (Jefferson et al. 1999). These expressed traits are selected on the basis of required characteristics enhancing the overall feed conversion and efficiency in case of animals. However, it has been noticed that when genes are introduced then genetic instability is noted in the genome. This instability may have adverse effects on the overall health status of the animal in question (Ruden et al. 2005).

The biggest question that looms over animal breeders and scientists is the use of transgenic animals for human use. The reason being that the incorporation of recombinant animals may have inadvertent adverse effects on the human population which is in direct contact with these animals (Crews and McLachlan 2006). Many chemicals and growth promoters used to enhance the vigor and muscle to fat ratio in bulls may have effects on the physiology of the bull. When that bull is sacrificed for human consumption, the resulting accumulation of toxic biotransformed products present in various tissues will result in negative effects on humans on consumption (Ruden et al. 2008; Tartari et al. 2008). A novel inclusion of certain genes in animals used for formation of transgenic animals may cause the production of protein structures that can induce allergenicity and toxicity in the consumer (Lapeña 2010). These variations in proteins that are produced as a result of the toxicity of the included genes or chemicals used to enhance the vigor of animals, and the sheer variations in the expression of proteins, will result in complex immune responses to the change occurring (Burns-Naas et al. 2006).

Dead carcasses when consumed by other animals also may result in the transference of these toxic compounds to other animals resulting in the death of animals in the ecosystem. When certain essential animals in the ecosystem are affected, the delicate fabric of biodiversity is affected and the equilibrium is lost (Hubbs 2010). This loss of equilibrium will result in a failure of the complex ecological system in place; a failure that can be translated as disastrous for the environment (Skies and Gannon 2011).

5.5 Environmental Concerns as a Result of Genetic Intervention

The segregation of animals into those that are to be released into the environment intentionally and those that are to be bred in captivity requires careful categorizing and establishing (Estrada 2014). This is important since animals having transgenic properties have different effects on the ecosystem when they are confined or released into the environment where they are causing the negative change that we are trying so hard to prevent from progressing. Questions that are important for us to ask ourselves here is whether we truly consider the transgenic animals to be released either intentionally or unintentionally into the environment. As there is the possibility that some animals that are kept in confinement may escape and then cause a variation in the environment that is uncalled for and adversely affect the ecological balance in which they involve themselves (Lezaun and Porter 2014; Snow et al. 2005). Of particular importance is the spread of the transgene to wild-type variants of the same species. Although the transgene may not appear to have any deleterious effect, in fact it may even increase the overall phenotypic outlook of an animal; however, the long-term effects of genetic transference cannot be ruled out. It is important to elucidate whether a gene is 'leaking' into the environment, and if it is present in the environment then what should be done to isolate and prevent its further spread (Bisbee 1992; Tiedje et al. 1989). As we do not have an advanced outlook or idea as to the potential of these transgenes, we cannot predict or evaluate their outcome or change in the long run. Hence physiological traits may vary at an unprecedented rate, and this needs to be checked in case of genetic leakage.

Another issue of relevance to genetically modified or transgenic animals is the ability of transgenic animals to proceed with mutations causing unknown changes in their genome. The implications for these changes due to mutations, or a variation at this unprecedented rate, may significantly alter the overall physiological outlook of the animal in the environment as well as those reared in captivity (Bailey et al. 2006; Palmiter and Brinster 1986). This is often one of the primary reasons for adverse and suppressive mutations. As recombination is a highly invasive process, loss of certain genes or inclusions of genes in regions which are tumor suppressor genes may result in the potentiation as well as expression of a disease phenotype (Ruiz 2006; Lian and Lane 2005). This is the reason that many transgenic animals are susceptible to cancers at higher rates with respect to their unaltered counterparts that exist in the wild.

Enhanced immunity is an evolutionary process, and results from numerous genetic interactions and adaptations that occur at the cellular and tissue level. However, due to the inclusion of various transgenes this ability of wild-type animals to combat disease is dampened (Klasing 2007; Correa et al. 2007). This occurs as the inclusion of certain genes in the genomes of animals may affect certain vital regulatory genes which are involved in immunomodulatory function. Genes responsible to T cell maturation or B cell maturation may be affected and this

variation then may result in lower effective T cell counts (Kundu and Thompson 2008). Sometimes, T cell populations may be reduced along with T cells; B cells may also be inhibited as genes controlling their maturation and progression are either deleted or mutated due to insertions of the particular genes required for the transgenic animal in question (Grazia et al. 2006). This is therefore an important functional variation that results due to inclusion of genes in transgenic animals.

5.6 Genetic Interventions in Animal Biotechnology

There are many techniques being used in animal biotechnology, both for manipulation of animal growth and for regulation of their immune system to fight off diseases. These interventions have negative impacts as they inadvertently affect the genetic response to various stresses in the environment (Sandhu et al. 2012; Donnelley et al. 1994). As previously discussed, loss in genes which are involved in immunological processes as well as genetic interventions can cause changes in the way the animal responds to the environment. These changes can be deleterious to the long-term health of the animal as well as the environment (Raberg et al. 2009; Frankham et al. 2002). Some genetic interventions are discussed in detail to complement the changes that result in the genetic interventions used in animal biotechnology.

5.6.1 Transfection

Direct microinjection of segments of DNA into the nuclei, electroporation of DNA regions inside induced pores, using polycations to insert genomic segments, lipofection, sperm-mediated transfection are all methods of including segments of selected DNA sequences into the ovum to form a zygote (Glover et al. 2007). The method which is used is dependent on the actual recombination events that occur and then result in the growth of the zygote in vivo. However, the DNA is not as well understood in its interactions with the host and the environment (Maulik 1997). Simple manipulations can have a long standing and adverse effect on the overall outcome and expression of important proteins required as a part of cellular growth and differentiation. These changes although might not be deleterious in the short term, may have repercussions which are both harmful as well as regressive in the future (Purnick and Weiss 2009; Foyer and Noctor 2009). Altering genomes, particularly in case of animal genomes is hence very tricky, as the sheer advanced set up of complex interactions between the genome and the environment itself prevent the association of the technique used and the application outcome (Primrose and Twyman 2009; Garfinkel et al. 2007).

The adverse changes if released into the environment can result in an animal becoming a ‘carrier’ for disturbed genes. These disturbed genes then when

transferred from animal to animal will result in the modulation of existing genes into a dynamic unstable state (Hawkins et al. 2010; Robertson et al. 2008). The unstable set of genes will inadvertently affect the overall stability of the genome, possibly affecting the overall physiological appearance of the animal population, particularly in the wild. Changes in wild as previously discussed can have adverse effects on the environment. These changes can be disastrous when it comes to the entire ecological system involved in the maintenance of a biodiversity and ecological harmony.

5.6.2 Retrovirus Vectors

Infectious elements are also sometimes used for transference of infectious elements into the genome of subject animals. These particles or retroviruses have the ability to reverse transcribe RNA into DNA and incorporate this DNA segment into the genome of the host in question. The integrated DNA can then go into a quiescent state, or it can start replicating and producing mRNA for insertion into viral structures (Bushman et al. 2005; Smith 1995). Hence retroviral transfection mechanisms have been used to introduce genes into susceptible species and incorporate genes into their genomic structures. This can be done by using inactivated viruses, which lack the sequences required for viral replication (Tomanin and Scarpa 2004). Since the virus cannot replicate, they only insert the target sequences into the host genome and allow for replicative processes to occur and allow for a varied expression pattern (Miller and Vile 1995). These vectors have been used for the insertion of genes in many biomedical research studies and are a part of use in biotechnology around the globe. However, the ethical and hazardous outcome of using retroviruses is under considerable debate (Eisenberg and Schenker 1997). The reason for this is the randomized insertion of the DNA sequences into the genome of the host. These randomized insertions can alter the genome and add to instability. Instability in the genome can alter the rates as well as the dynamic expression patterns of proteins. Alterations in the expression of genes can then result in the formation of an abnormal phenotype. This phenotype can then result in altered environmental effects. These effects can have disastrous implications on the ecosystem (Kimmelman 2009; Pappas 1994).

5.6.3 Transposons

Transposons are genetic elements that have the ability to transfer genetic information from one site to another. They are able to perform this in the presence of *transposases.* Although mammals do not have active transposon systems, they do contain sequences that are involved in transposition (Behura 2006; Shapiro 1993). This is notable since the sequencing of the genome allowed for the similarity to

exist between insect and mammalian genomes. However, this is an example of some common heritage within the two types of species (Feldhamer 2007; Hughes 1999). A unique usage of transposable elements is the transposition of genes into regions of interest during genetic engineering. These techniques, however, are performed in primitive organisms, particularly in bacteria (Doyle et al. 2012; Yergeau and Mead 2007). In order for us to eliminate the required genes from organismal DNA sequences, transposon systems like the *sleeping beauty* transposon system can be used to target certain genes or genetic sequences (Ivics et al. 2009). Although transposition does not occur in mammalian systems, the argument remains that there could be a possible genetic influence of biotechnological research involving transposon systems. In order to eliminate this risk it is imperative that all research involving transposon systems be regulated. It has already been evaluated that certain methods for acquiring new strategies concerning the usage of transposons are already under the consideration of various research groups (Horie et al. 2001). Using these transposable elements and their incorporation into the genome may result in instability later on during mutagenesis. The repercussions are endless and can be disastrous if those genes are passed down to subsequent generations (Miskey et al. 2005; Ivics et al. 2004).

5.6.4 Techniques Involving Direct Genetic Manipulation

Transgenic technology involves a crucial and major part of animal biotechnology. Manipulation of certain genes in animals and their regulatory sequences can greatly affect and modulate their function and phenotypic outcome in an animal (Van Reenen et al. 2001; Boverhof et al. 2011). The techniques most commonly used, however, include animals being engineered in such a way that they lack certain genes, (knock out) or possess certain genes having certain characteristic features. These changes in genomic structures and their outcomes are quite favorable for an animal breeder (Spier 2002; Renaud 2006). From an animal breeder's perspective, certain modulatory functions especially in genes being expressed due to their presence can result in the higher productivity in an animal (Chapman and Frankel 2003). Certain proteins like PrP responsible for Bovine Spongiform Encephalopathy (BSE) can be eliminated from cattle populations by simply deleting the genes responsible for expressing PrP (Ersdal et al. 2009). Mice models which were knockouts for PrP expressing genes showed that there was a significant decrease in their ability to get scrapie or mad cow's disease (Creurtzfeldt Jacob's syndrome). However, such interventions may have an outstanding effect on the animal population as the normal function or PrP which may be imperative in genomic stability and may be lost forever if this deletion occurs. However most animal breeders would argue that this would result in the elimination of BSE and higher productivity of their product (Watts et al. 2006). This again is an example of the excessive misunderstanding of the environment and how genes will interact and possibly react to changes incurred by human intervention (Bradley 2004). This intervention will

cause deleterious effects. Although elimination of genes that code for proteins which are of harmful propensity, is a cause for application, the long-term effects of these interventions are unknown. One cannot predict the changes that will occur on both the genomic and organismal levels (Warnecke and Hurst 2011).

5.6.5 *Genetic Propagation Using Nuclear Transference and Selection*

After the advent of cloning, particularly therapeutic cloning, it has become quite clear that biotechnologists are concentrating on the use of entire genomes for production of animals. Although there are numerous ethical concerns as well as implications scientifically due to the use of these techniques, they are still under considerable scrutiny for success (Twine 2010; Tudge 2002; Greely 1998). The possibility of reproducing entire genomes and preserving favorable traits for future use is both alluring and desirable for scientists and commercial enterprises. So far, at present the propagation of animals using nuclear transfer is highly inefficient, and this is observed to be less than 10 % successful (Acquaah 2009). By successful, it is drawn to represent live offspring. It has been observed that most failures occur during development, as it is very difficult to correctly infer upon the genes and the types of genetic interactions that result due to the complex genome in higher mammals (Allendorf et al. 2012; West-Eberhard 2003). There is therefore a very high rate of prenatal death, particularly during the development at later stages of gestation. Many theoretical applications have been thought of to be applied using this methodology. Better sires, dams, pets, conservation of species and breeding are common applications of cloning (Betteridge and Reiger 1993). The sheer vastness of the applications in this technique; has not only increased its practical applicability but also have increased the risk involved to the environment.

Modulating changes in the genome is one thing, but incorporating an entire genome into a host cellular structure is another. It is not only hazardous as a stable phenotype may be passed down, but the possibility of destabilizing at any generation is a risk that cannot be taken for granted (Dale et al. 2012). Simple techniques like artificial insemination are now widespread all across the world. It is a simple and effective method of transferring semen of high quality in close proximity of surrogate ova. In this way 'low grade' females having fewer phenotypic characteristics will inadvertently be used to simply womb the higher more viable and phenotypically advanced variant (Tan 2013; Mellor et al. 2009). The question remains, however, whether this 'forced selection' is truly a correct and accurate method of enhancing the quality of a species or not? Artificial selection may have its positives, but the question remains whether eons of evolutionary can be augmented with a few years of forced selection. This is a cause for considerable debate (van der Helm 2002; Dugatkin 2000; Noss and Cooperrider 1994). As it is not possible to replace the mechanisms that nature has in place and which has

maintained so many years of equilibrium on this planet for so long, and replace it with an augmented and highly theoretical method which could in the end go either way (Williams 2008; Wackernagel and Rees 1998). Although the immediate gain may be quite encouraging; however, the outcomes are what matters the most, in fact it is the biological implications in subsequent generations and their interaction or absence in or with the environment, which may result in a breakdown of the complex ecological stability that we observe today.

5.7 Conclusion

The deleterious effects of biotechnology are not only numerous, but the extent of damage to the environment as a result of biotechnological intervention is notable. It is highly likely that the interventions we perform today might have repercussions in the times to come. These changes may be physically affecting the environment or biochemically, based on the epigenetic or genomic variations that we are causing in the genome. It is obvious now to scientists around the world, who hold skeptic ideologies concerning biotechnology, that variations in the genome at any level in the environment can have effects that will eventually be disastrous to the biodiversity of this planet. Genetic manipulation and controlled genetic influence has only short-term benefits but cannot be a supplement for good old-fashioned adaptive evolution.

Although the benefits may outweigh the probable disadvantages at this time, and many scientists may argue it to be true, the hypothetical preamble many present itself in their argument for rebuttal of the effects of biotechnology. There is a vast uncharted frontier in biological science that exists and the ecological outcomes are still unknown, mainly because of our little knowledge of the environment and how it responds to our interventions. As biotechnologists, we require more stringency while promoting biotechnological techniques involving genetic intervention. Although we may feel that synthetic biotechnology is the next frontier of biology that will supplement our immense need for resources, it is however a Pandora's box. We will have to tread carefully, as we are charting the unknown.

Conflict of Interest Statement

The authors have no conflict of interest with any scientist or scientific group. The ideas portrayed in this chapter are merely a representation of the state of biotechnology as a whole, and we do not wish to single out any individual or group.

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Chapter 6
Reduction in Animal Waste

Ali Saeed, Ali H. Sayyed, Sohail Safdar and Shumaila Manzoor

6.1 Introduction

The term "animal waste" is used for the dung or fecal material of livestock such as ruminants, poultry, swine, horse, birds, and other mammals with or without litter. The livestock and poultry manure is mixed with urine components along with sticky components from animal carcasses such as tendons, hide, and other collagenous materials. Animal manure is used as land fertilizer and its proper application provides nitrogen nutrients to soil, while inappropriate use or overuse can result in release of animal waste into surface waters such as rivers and watercourses. Excessive nutrients or "nutrient-pollution" is called eutrophication and increases unnecessary algal growth on surface of water. Reduction in the fish population is also observed due to depletion of oxygen, with overgrowth of aquatic algae against the surface of water beyond its capacity to support. Water pollution with animal manure is also associated with waterborne infections such as cryptosporidiosis and giardiasis. Water pollution is among the several adverse consequences of improper disposal of animal waste and further advocates to us about the proper disposal of this biological waste.

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6.2 Livestock Waste

Farmyard manure (FYM) and farm slurry or liquid manure are the most common animal wastes from excreta of animals. FYM is composed of fecal material along with remains of plants such as straws from animal bedding and fodder particles from animal food. Liquid farm slurry or "liquid form of agriculture manure" is produced from intensive livestock farming on slats or concrete floor instead of plant straw beddings. Farm slurry is also called agriculture manure.

Different types of animal wastes have various qualities and can be used for diverse applications such as fertilizer for agriculture land. The waste and subsequently the manure from different sources such as chicken, horses, sheep, cattle, pigs, rabbits, guano, turkeys, bats, humans (sewage), and seabirds have different properties. Sheep manure contains high nitrogen and potassium content, while pig has relatively less content of N_2 and K. Horses largely consume green grasses and weeds although horses cannot digest weeds completely compared to cattle. Therefore, manure from horses mainly comprises grasses and seeds of weeds. Poultry farm or bird manure is enriched with both nitrogen and protein.

Animal waste is mostly mixed or adulterated with other animal products, such as wool or shedding hairs, plumages, bones, and blood. The dung is often mixed with fodder or animal feed due to spillage. Chicken litter has mixed animal manure because poultry feed contains different animal wastes and products such as meat and bone meal, an animal product. Presently, animal manure is not used as a valuable fertilizer due to loss of potential nutrients during return to agricultural systems. Millions of tons of livestock waste is produced with a number of associated problems such as unpleasant odor (Table 6.1).

Therefore, there is a need for modern biotechnological methods and techniques to remove the problem of animal waste handling and recycling for better purposes.

Table 6.1 Livestock population and amount of waste produced in India

S. No	Animal species	Population (in million)	Daily average excreta animal-1 wet weight (kg)
1.	Cow	185.18	11.6
2.	Buffalo	97.92	12.2
3.	Horse	0.75	–
4.	Donkey	0.65	–
5.	Sheep	61.47	0.76
6.	Goat	124.35	0.70
7.	Camel	10.63	–

Source Livestock Census Report 2003, Directorate of Economics and Statistics, Ministry of Agriculture, Government of India

6.3 Poultry Waste

Poultry meat and eggs are consumed by most ethnic populations worldwide, which are affordable and better quality food products. Intensive poultry production and growth has increased in developing countries during recent decades with the advancement of modern knowledge. Recently, poultry waste management operations have gained enormous importance related to poultry production due to a number of issues such as environmental pollution, public health, and quality of life near poultry rearing areas. Critical attention is required on waste management related to sustainable poultry production in intense poultry farming near urban areas along with small poultry production houses and backyard poultry of rural areas (McCaskey 1995).

The production of poultry has produced different types of wastes such as bird manure of excreta, wastes from hatcheries, bedding material, or litter wastes such as straw, wood shavings, sawdust, and hulls of peanuts or rice along with mortalities at farm. Poultry and poultry processing products generate additional waste such as feathers and slaughtering wastes of organs/entrails, bio-solids, and water used in processing. Poultry waste can generate both organic and inorganic nutrients regardless of flock size, which can be converted into valuable products, if managed and recycled properly. Poultry waste increases the potential risks to the environment and to human health due to presence of animal medicine, insects or parasites, and pathogenic microorganisms. Environmental and health concerns are also associated with small or domestic backyard poultry, usually raised in contaminated surroundings (FAO 2008).

6.4 Fish Waste

The fishing industry also generates a significant amount of waste; according to an estimate 43 % or 359,964 tons fish is used for human consumption and another 492,020 tons is wasted from a resource of 851,984 tons in the UK, shellfish and fish. Fish waste majority of 35 % is generated during onshore processing, whereas smaller quantities of waste are processed and discarded into the sea. The quantities of waste produced during catering and retail sectors are difficult to estimate, which ends up with minor contribution of domestic waste (Archer et al. 2001).

6.5 Hazardous Effects of Animal Wastes

6.5.1 Environmental Pollution

Feedlots are also called factory farms or massive livestock farms, which can house thousands of animal such as chickens, pigs, and cows and can generate a staggering

quantity of animal waste. Human health and environment can suffere seriously due to improper animal waste management. In these dense farms, animal wastes are channeled into big lagoons containing a combination of animal manure and urine. The breakage or leakage into cesspools along with the problem of overflow of big collection can increase the chance of exposure to microorganisms and drug-resistant bacteria via water supplies. Nitrate pollution is also a problem related to intensive farming. Additionally, the emission of noxious gases such as hydrogen sulfide, ammonia, and methane can generate an unhealthy environment. Manure is also applied to soil or "spray fields" as fertilizer, which increases the risk of these harmful substances mixing with air (NARD 2005).

The huge amount of animal waste from factories or farms that escape to harm humans or other living organisms is due to a major gap in legislation and implementation law related to animal waste management. Previously, the natural resource defense council filed a number of cases over the years against factory farms to enforce the authorities to deal with waste management problems in the United States. The release of pathogens into the environment by factory farms is controlled under the court order and release of animal waste is under great oversight. "Pollution reduction plans" are necessary and ensure public health along with farming of animals. The developing nations have more adverse and discouraging situations to control such deleterious substances into the environment (NARD 2005).

"Farm factory" odor is unpleasant, unbearable, and contains harmful gases and toxic chemicals. Hydrogen sulfide can induce coma, seizures, sore throat, and death in humans even at very low dosages. Noxious gases produced by factory farms can also induce shortness of breath, headaches, coughing, wheezing, and diarrhea (NARD 2005).

Farm manure and urine can easily pollute drinking water. As a result, serious health issues can be caused. Nitrate-contaminated water can induce "baby blue syndrome", which can easily cause death in the newborn. Spontaneous abortions have been linked to high level of nitrates in drinking water supplies near "farm factories" (NARD 2005).

However, the use of antibiotics for growth and animal infection in large-scale animal farms is an additional threat for public health concerns related to antibiotic-resistant bacteria. Antibiotic residues can enter into the food, environment, and food chain to generate more resistant bacteria, which are difficult to treat. The natural environment can also be damaged in several ways from farming practices. Occasionally, adverse effects on the environment can appear abrupt and disastrous, such as cracked lagoons can causes massive deaths in fish. Overapplcation of farm manure in agriculture lands also accumulates due to nutrient pollution in water-courses (NARD 2005). Excessive contents of phosphorus and nitrogen in animal wastes can cause explosion of algae in water and kill other aquatic animals due to reduction of water oxygen. For instance, in North Carolina billions of fish were killed due to toxic contamination by the microorganism "*Pfiesteria piscicida*" in coastal water. Animal waste with heavy metals can come up in the food chain via water contamination. Secondly, animal farms use groundwater for cleaning,

drinking, and cooling purpose, which can also reduce the groundwater reservoir (Ferket 1994; NARD 2005).

Specific apprehensions are documented related to farming such as reduction of surface groundwater, and increased deposition of nitrogen and phosphorus. Air pollution is less understood such as concerns related to hydrogen sulfide, ammonia, and volatile compounds along with dust from poultry farms. Other issues such as bad odor near farming areas, climate changes, and dense population in close proximity of farming still need to be addressed (FAO 2008; Nahm and Nahm 2004; Williams et al. 1999).

Soil receiving farm manure increases nutrient load in a geological region, because the farm yard manure is applied near the poultry farm areas to avoid transport cost, especially in developing countries. Proper distribution of nutrients is important for ecological sustainability and diversity of soils along with maintenance of naturally occurring living organisms within a region (Gundersen 1992).

Meat and egg production in dense poultry farming systems is gaining importance to meet the food requirements. The application of excessive manure on lands leads to nutrient imbalances and adverse environmental effects. Poor management of animal waste increases soil erosion and nutrient loss during rainfall along with contamination of groundwater with manure (Donham and Thelin 2006).

Nitrogen compounds are the most dynamic part of animal manure and are taken up from the soil by plants during growth. Grasses can also release nitrogen directly into the atmosphere in volatile form (ammonia gas) and harmless di-nitrogen (nitrous oxides). Nitrogen can also move to groundwater or nearby shallow groundwater. Phosphorus in litter or manure unlike nitrogen is immobile, but can easily leach into surface water, or move to shallow water via erosion of land. Groundwater adulteration requires a certain amount of nitrogen and phosphorus in drinking water due to public health concerns. Excess of certain metals such as zinc and copper in poultry excreta along with nitrogen and phosphorus can also be detrimental to plant growth. So, these metals should also be considered during long-term planning of sustainable poultry and other animals production (Zublena 1994).

Emission of ammonia can cause significant aerial pollution and can affect the life of people in their surroundings. Assessment of air pollution from poultry production facilities is also important and needs the attention of authorities (FAO 2006). Ammonia emission into the atmosphere may not be well understood but local ecosystem and human health can be affected with high concentration of ammonia into the atmosphere. The assessment of environmental release of ammonia especially in water and air from poultry waste should be considered for sustainable and long-term poultry production.

Nitrogen from poultry operations generates ammonia, as it is an essential part of poultry feed such as amino acids, other dietary proteins, and essential biomolecules. Poultry excreta contains organic nitrogen, because dietary nitrogen cannot be converted into tissue, egg, or meat. Most of the excreted nitrogen is converted into ammonia. The release of ammonia into the atmosphere depends on different factors such as treatment practices, litter storage, manure, poultry house design, and climate.

Metabolic breakdown of poultry waste also generates hydrogen sulfide and other volatile organic compounds. Usually, poultry waste needs low oxygen level for breakdown (fermentations) in a pit under the birds or in earthen lagoons or open air operations. Waste management operations are more common in dairy livestock and swine waste than poultry. Layer poultry waste sometimes controls these operations. Hydrogen sulfide and volatile organic compounds released from open air fermentation can generate unpleasant smell and lead to human health concerns above a certain concentration. Donham and Thelin (2006) reported ambient hydrogen sulfide production to lethal concentration from manure slurry pits present under the animals. Air quality guidelines of the World Health Organization state that hydrogen sulfide levels should not be higher than 0.15 mg/m^3 within 24 h (WHO 2000).

Along with hydrogen sulfide and volatile organic compounds from poultry waste, dust pollution is also produced from all poultry production units, where a considerable number of birds are confined. Dust particles contain insect fragments, mites, bacteria, feathers, dried fecal matter, molds, and endotoxins (Clark et al. 1983). Production of dust pollution is dependent on a number of factors such as type of feed (pellet or dry), climate, control mechanisms, and building. Recently, low price dust barriers have been developed and used to avoid the spread of poultry large dust particles (Poultry Science Association 2009). However, the conversion of ammonia gas can also generate very fine dust particles, which cannot be controlled by dust barriers and can produce more serious human health concerns. These factors further advocate the importance of monitoring and control of ammonia emission into the atmosphere. Climate can affect animals' productivity regardless of flock size; excessive dryness can produce respiratory problems in animals and very wet or humid condition can produce excessive ammonia and pathogens. Excessive fish waste in marina basins can reduce oxygen level in water and waste decomposition can produce foul smell and death of marine animals. Fish waste floating on water surface is an additional problem.

6.5.2 Pathogens Causing Health Problems

Humans can be infected with a number of zoonotic protozoa, viruses, and bacteria, which can cause serious disease and death. Microorganisms from animals can infect during occupational exposure of contaminated soil, water, air, and food. Animal and human pathogens can be present in various types of agricultural and wild animal wastes such as skin, feathers, fur, urine, excreta, and respiratory secretions. The level of some pathogenic microorganisms can be in millions or billions per gram of animal waste. Additionally, rearing of thousands of animals in small vicinities can generate very large quantities of different animal waste, which in turn increases load of zoonotic microorganisms and pubic health concerns (Sobsey et al. 2006).

Table 6.2 Existence of potential human pathogens in animal wastes

S. No	Organism groups	Organisms
1.	Viruses/ groups	Influenza viruses (Orthomyxoviruses)[a], Hepatitis E virus (swine), Reoviruses, Adenoviruses[a], Rotaviruses, Caliciviruses[a]
2.	Bacterium/ group	*Listeria* spp, *Salmonella* spp., *Aeromonas*, *hydrophila*[b], *Leptospira* spp., *Yersinia enterocolitica*, *Vibrio* spp., *Escherichia coli*[b], *Campylobacter* spp
3.	Parasites	*Giardia lamblia*, and *Balantidium coli*, (*Protozoans*), *Cryptosporidium parvum*

[a]Humans and animals (including swine) usually have distinct strains of these viruses, but not always

[b]Some strains of these bacteria are nonpathogenic and others are pathogenic. The extent to which pathogenic strains occur in animal wastes varies with the animal species and other factors

Veterinary services both in urban and rural areas can increase the risk of pathogenic microorganism spread from different types of animal waste. Veterinary animal waste produced during these practices, especially in developing countries, is not properly disposed off and can cause serious epidemics in vast areas. Veterinary hospital wastes should be categorized according to consistency of waste, density of waste, and weight (Nowlan 1997).

Human health concern zoonotic animal pathogens with potential risks include a number of viruses and parasites. The outbreaks of these organisms on commercial livestock are difficult to control and eradicate from animals and animal facilities. Presence of these animals in animal waste is an additional threat and concern (Table 6.2). The presence of antibiotic-resistant bacteria in animal waste due to excessive use of antibiotics for growth and production is also a very important public concern (Sobsey et al. 2006).

Inadequate animal waste management can contaminate air, land, and water. Pathogens can survive for a long time in this environment depending on the conditions and nature of pathogens. The release of discharges and recycling of farm manure is avoided in waste management programs. However, the transport and movement of animal waste can spread pathogens via water, air, and soils. Potential risk of infection to farmworkers and possible transmission to healthy humans especially to their family members cannot be neglected in animal farming systems.

Human-to-human transmission and disease capacity is considered in pathogenic viruses, bacteria, and parasites present in feces. Various degrees of treatment for raw sewage is required before distribution to soil or discharge. Inappropriate treatment can lead to serious public health concerns. Mostly, the contamination of water and food with pathogens is difficult to identify. Similarly, animal waste also contains pathogenic microorganisms, which are lethal for both animals and humans. Therefore, animals have been involved in many previous foodborne and waterborne outbreaks. The food and water supplies should be protected from mixing of animal feces and it is the most critical measure for animal and human health along with sustainable agriculture (Sobsey et al. 2006) (Table 6.3).

Table 6.3 Prevalence of cattle, pigs, poultry, and human enteric pathogens

S. No	Name of organisms	Cattle (%)	Pigs (%)	Poultry (%)	Human (%)
1.	*Campylobacter jejuni*	1	2	100	1
2.	*Cryptosporidium* spp	1–100	0–10	0	1
3.	*E. coli* 0157:H	16	0.4	1.3	7 1
4.	*Giardia lamblia*	10–100	1–20	0	1–5
5.	*Salmonella* spp	0–13	0–38	10–100	1
6.	*Yersinia enterocolitica*	<1	18	0	0.002

It is often quite difficult to determine the source of microorganisms involved in an outbreak. However, some evidence have been recorded previously about the involvement of animal manure in disease outbreaks. An outbreak in Walkerton, Ontario has been associated with well-water contamination with nearby animal manure washed due to heavy rainfall. The outbreak affected over two thousand people along with seven mortalities. Similar outbreaks of Campylobacter and *E. coli* were also recorded in Washington County, New York; animal manure contaminated the untreated well-water due to heavy rainfall (Himathongkham et al. 1999).

An outbreak of *E. coli* O157:H7 was recorded in the 1990s due to contaminated apples with animal manure from the ground of apple orchards previously fertilized with cow manure or land used for grazing of cows. Now, it is compulsory in the United States to pasteurize apple cider. According to an estimate, 403,000 people were affected in Milwaukee, Wisconsin, in an outbreak of *Cryptosporidium.* This outbreak was also primarily caused due to animal manure runoff, although later evidence revealed an uncertain origin of the causative agent. Similarly, animal waste was associated with *Cryptosporidium* outbreak in Carrollton, Georgia and affected over 13,000 people in 1989 (Morse et al. 1997).

6.6 Animal Waste Management

Animal feedlots and feeding processes are very concentrated for confine animals or poultry for egg, milk, or meat production. Animal waste produced from such procedures should be considered for proper waste disposal. A pig can produce 2–4 time more manure than a human. According to an estimate, hog waste produced is around 9.5 million tons annually in North Carolina. Mostly, “wet animal waste procedure” is used to dispose hog waste. Such slurry is buried into a lagoon or pit for decomposition and then applied to land as fertilizers for crops; this spraying is also called “land application of manure.” Land application is most common and a good source of nitrogen for crops, but improper application leads to serious public health concerns (Sobsey et al. 2006).

Following are the suggested "best management practices," which can further decrease the risks of food and water supplies contamination with animal waste.

1. Animal manure should not be applied on saturated or wet soils.
2. Mechanically mix the solid manure on the same day of application.
3. Animal manure should only be applied on the land approved with manure management plan or frozen lands, for example, land with proper erosion control program, no flooding area, and land with less than 2 % slope.
4. Avoid the use of liquid manure on karst areas.
5. Animal manure should be used after proper erosion control program.
6. Cover crops and proper erosion control strategy should be applied to land which is prone to erosion.
7. The storage of animal manure should be perfectly managed and maintained.
   "Paramount management strategies for fish waste disposal are";

   A. Fish waste should not be dumped into marina basin.
   B. Fish cleaning should be avoided at floats and docks.
   C. Fish cleaning should be done at your marina.
   D. Properly enlist the rules and regulations for fish cleaning.
   E. Rinsed water from cleaning of fish should be directed to sanitary sewer or sand filters.
      It should be free from solids.

   The following disposal methods should be used:

- Composting fish wastes can be used and appropriated for landscaping.
- Fishermen should be encouraged to freeze the fish parts as bait for the next trip.
- When composting and baiting are not possible from some fish waste materials, encourage the boaters to dump them in a double bag into regular trash.
- Try to clean the fish at the place of catching and discard the waste in unrestricted waters, unless length limits the processing and cleaning at the place of catching.

6.7 Methods to Reduce Animal Waste

Animal waste management is now a rising global issue as it has hazardous impacts on human health and environment (Ritter 1989). Human life remains at risk during animal handling and care, especially animal wastes like urine, feces/dung, and carcasses, which are the carriers of pathogens. On exposure to these excretes, the pathogens are transmitted to humans through touch, inhalation, or drinking water, thereby showing serious impacts on the health of both animals and farmworkers in particular and the common man in general. Sometimes complicated diseases like Listeriosis, Leptospirosis, Salmonellosis, Brucellosis, and Vibriosis can be transmitted to humans from the excreta of animals (Korner et al. 2003). The other hazard of animal excrete is environmental pollution and its degradation. Other than

environmental pollution, there is a loss of 1.5 mt of nutrients, which are available in farm yard manure and 101 mt of nitrogen from poultry manure (FAO 2000). Proper utilization of animal waste could produce a healthy environment and save expenditure on fertilizers. Awareness should be created among masses through training, workshops, and seminars about the effectiveness of composting, especially for managing animal waste.

In order for a sustainable solution for animal waste, appropriate strategies should be adopted. There are many animal waste management (AWM) techniques and tools to design the exact amount of diet for a particular animal according to its weight and the right amount of waste disposed by it is calculated and managed through this machine designed by Clint (2004). However, AWM demands high precision in the operation process. Moreover, it is an expensive tool and developing countries can hardly afford it. Therefore, relatively cheaper strategies affordable by an ordinary farmer should be adopted. As the animal population like humans is expanding, disposing wastes and at the same time composting remains the most appropriate process to cope with the problem of waste. Long before people inhabited the planet, composting was just something that happened. In every swamp, forest, and meadow, wherever there was vegetation, there was composting. In the past decades, it was noticed that crops grew better near piles of rotting manure and vegetation.

6.8 Classical Method

6.8.1 Composting

Composting is a naturally occurring phenomenon that works under controlled conditions (air, temperature, moisture content) in which organic material is converted into more usable form of organic matter with the help of microorganisms (Chapman 2005). The most appropriate AWM, i.e. composting requires animal waste material (Teira-Esmatges and Flotats 2003) and other than animal waste, leaves, yard and garden debris, grass or cereal straw, food waste sewage sludge can also be used as an organic material for composting (Su et al. 2003). Composting begins as soon as a pile of waste is made. Microorganisms start decomposing by utilization of oxygen and converting it into CO_2, water vapors, and heat (González 1982). The following objectives should be considered:

- To determine the appropriate animal waste management strategy
- To analyze the importance of composting
- To study the steps of composting method
- To identify the advantages and disadvantages of composting.

Composting Methods

It may be on a farm or in a building at commercial level. Composting can use dead animals and biofilter the animals from pathogens in high temperature (Keener et al. 2000). Two types of composting systems are used (1) open and (2) close; organic compounds are decomposed either in open pile or rows or in a closed container or closed reactors (Jones 2000).

The following steps are involved in composting:

- Animal waste such as animal dead bodies, animal manure, clean wood waste, crop residues, bedding, and urine should be piled up. However, dead animals are assimilated within 24 h of death; put a cover of 3 ft with solid manure. The process of composting is executed to prevent formation, leach outs, release of runoff, rodents, files vermin, and odors (Morrow 2001).
- The pile should be mixed up and down regularly to control aeration.
- Dead animals should not be composted until all external and internal organic soft tissues get decomposed (Su et al. 2003).
- Composting process should be away from wetlands or 100 yards from flood plains and a minimum of 100 ft away from private wells.
- Permanent structures for composting should be able to utilize weather and consist of rot-resistant materials. These structures should be resistant to damage during composting.
- Maintain moisture contents like silage damp, not wet. Add manure solids or water when needed, its temperature should be 40 °C for 3 days to kill pathogens. It should be turned 5 times for a complete process of 180 days and first turned after 1 month (Chapman 2005).
- It can spread on crop field as well as bedding material being odorless sterile, weed-free related, and dried (Daniel et al. 1987). However, it is a risk to compost dead bodies of animals (Fig. 6.1).

Cautions

Dead animal bodies with neurological diseases should be disposed off according to recommendations, because prion decomposition during composting process is unclear. Prion is a causative agent for Mad Cow Disease or Bovine Spongiform, Encephalitis. Animal dead bodies from neurological diseases, anthrax, or those under quarantine should not be composted (Bonhotal 2003).

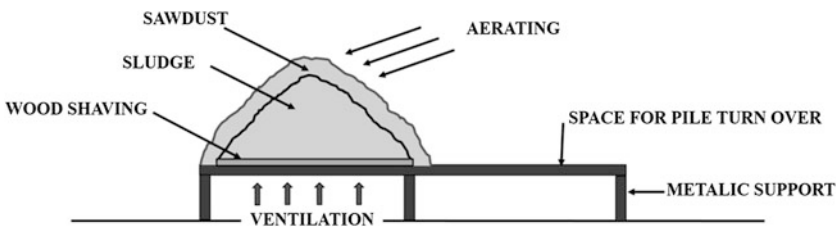

Fig. 6.1 A pilot system diagram for composting (Buyukgungor and Gurel 2009)

Factors affecting composting

There are five major factors that affect the composting process. These factors coordinate with each other, and also with organic materials and begin the decaying process by the action of decomposers.

Air Control

Composting is an aerobic process (in the presence of oxygen). Air should be regularly provided by exhausts, fans, and blower or by continuous stirring or mixing the organic material (Chapman 2005).

Nutritional Traffic

Compost should have a definite ratio of incoming and outgoing nutrient traffic in order to maintain balance. This process is most successful when the pile contains 20–40 parts of carbon to oxygen (Eldridge 1995), i.e., C/N ratio as 20/1 and varies to 40/1 (Korner et al. 2003).

If nitrogen is too low, excess nitrogen is converted into ammonia and escapes into the air causing odor and air pollution. On the other hand, if this ratio is too high, the process reduces.

Suitable Temperature

Temperature is an integral factor of every decomposing process like composting in order to regulate the breakdown of organic material by microbial activity. The process begins when the outer temperature is up to 45 °C for 2 days (Morrow 2001). The optimum temperature to maximize composting is between 35 and 45 °C and for global market production, 40 °C for 3 days to destroy all weed seeds, parasites, and unnecessary microbes (Mahfooz et al. 2006).

Moisture Contents

Moisture content is essential to integrate the composting process; however, it also depends on continuous mixing. The stabilizing rate for moisture content is between 40 and 60 %. Below 40 %, the process reduces and beyond 60 %, it becomes more anaerobic. Overall, 50 % moisture content can be maintained by adding water in case of dryness (Bonhotal 2003).

Physical Characteristics

The physiochemical properties of components should also be considered during the process of composting. Physiochemical properties of compost can affect the amount of decomposition, aeration, and sustainability of aerobic condition into pile. Mostly, structure, texture, and porosity are considered for the process of composting.

6.8.2 *Modern Techniques of Biotechnology to Reduce Animal Waste*

Biotechnological treatments to reduce animal wastes have gained enormous attention among the other modern treatment technologies since the 1970s. Biotechnology has been used and has influenced a number of sectors related to the economy such as chemical production, manufacturing of goods, protection of environment, production, processing of food and agriculture, and forestry (Gavrilescu and Chisti 2005). Biotechnological treatment or reduction of waste is executed with fermenters or bioreactors, and different terms can be used for waste reduction with this technology such as bioleaching, bioprocessing, bioremediation, bio-pulping, biofiltration, phytoremediation, and biodesulphurization (OECD 2005).

6.8.2.1 Anaerobic Digestion of Animal Wastes

Organic waste can produce biogas with well-recognized biotechnological methods of anaerobic fermentations. These modern techniques reduce the pollution load by agricultural or animal slurries in addition to use for plants as fertilizers. The use of natural biogas can help us to produce electricity from cheap and alternative energy sources other than fossil fuels and reduce production of carbon dioxide. Carbon dioxide production from fossil fuels is a major event in global warming. Carbon dioxide and methane can be produced via a series of anaerobic reactions on organic materials catalyzed by a mixed population of bacteria. Briefly, monomers and oligomers are produced from polymers such as starch, pectin, hemicellulose, and cellulose. Fermentative bacteria further metabolize these into carbon dioxide, hydrogen, and volatile compounds such as butyrate, propionate, and acetate. Volatile organic compounds further converte into methanogenic precursors such as CO_2, H_2 and acetate with syntrophic acetogens. Finally, methane is produced from CO_2, H_2 and acetate by methanogenic bacteria (Wilkie and Colleran 1987). Dynamic equilibrium in bacterial groups is required for stable production and operation of biogas. Final products from anaerobic digestion of carbonaceous material are carbon dioxide and methane; both gases are odorless. Fermentation waste can be stored during the process in the stage of acid formation. The accumulation of acid forming products can easily be prevented because of slow rate of reaction between acid forming products and methane production. Thus, inappropriate degradation of organic matters occurs during acid forming and methanogenic steps of microbial degradation, which also explains the accumulation of volatile malodorous substances (Spoelstra 1978). Such degradation of volatile products generates carbon dioxide and methane. However, the conversion of animal manure into methane in control digesters of both anaerobic processes are well balanced and less odorants are effluents are produced or degraded during control processing. Anaerobic digestion needs compulsory control on odor management along with

animal operations. Moreover, anaerobic digestion in confined surroundings can be used even to reduce the spread of odorants from animal manure (Wilkie et al. 1995). Additionally, liquefaction and waste stabilization can also be achieved from anaerobic digestion for biogas production for other purposes of farm or domestic use. Further, fertilizer quality and value of raw manure also remain conserved in digested effluents (Field et al. 1984; Dahlberg et al. 1988) along with significant reduction of pathogenic organisms (Demuynck et al. 1985; Bendixen 1994).

Odor Impact

Unpleasantness of animal manure and odorant spread has been significantly reduced with anaerobic digestion (Welsh et al. 1977; Pain et al. 1990; Powers et al. 1997). Anaerobic digester systems reduce significantly the presence of unpleasant odor from swine manure. High temperature (35 °C) can induce early control on odorants than low temperature (25 °C) (Welsh et al. 1977).

Pig digested slurry used on land for fertilizer has unpleasant odor for 3 months, but it is much less than the odor produced and retained from application of raw or undigested pig slurry (Pain et al. 1990). Maximum odor can be generated in the initial 6 h of spreading, which can be reduced until 79 % with anaerobic digestion of pig slurry. However, storage does not give additional benefit of reducing this unpleasant odor. Anaerobic digester effluent of dairy manure was less odorous compared to fresh slurry effluent. Separation of most fibers from manure and preserving longer hydraulic retention time can further reduce unpleasant odor from manure (Powers et al. 1997). Anaerobic digester system was established initially to eradicate and control the unpleasant sludge produced from domestic or urban areas (Noone 1990). So, farmers can also benefit from the system to avoid environmental pollution pressure associated with animal production. Presently, aerobic processes are the only main operations of choice for animal production units, although complications and cost associated with aerobic systems are greater compared to anaerobic systems. Aerobic conventional methods produce a large quantity of sludge and consume energy, whereas anaerobic processes produce energy along with very less quantity of sludge productions (Evans 1986).

Digester Design

Digester design should be suitable for producers and waste management individual operation systems at a specific site. Usually, animal waste systems are digested or degraded anaerobically in lagoons. Lagoon digestion can also serve as good storage facility with substantial degradation of solids with still some smell. Hydraulic retention time can exceed 60 days with proper planning and design of lagoon and can solve the problem of unpleasant odor for the rest of the year; sometimes, rise in lagoon temperature during spring can increase bacterial action and odor production. Mostly, overloading of lagoons is associated with unpleasant odor (Miner 1981; Ritter 1989). Manure load on lagoons can easily increase due to increase in volume of animal waste and animal number as estimated at the time of lagoon construction without affecting lagoon capacity.

Biogas production is variable with temperature and seasons, but the collection of biogas can easily increase with gas-tight covers. Grounds with high water table should not be used for anaerobic open or close lagoons to avoid the risk of groundwater contamination. More unpleasant compounds can be converted to less odorant in closed anaerobic systems, which increase the digestion and conversion of organic odorous compounds into less smelly compounds. The open lagoon operations are more concerned with unpleasant smell. Plug flow digesters and continuously stirred tank reactors are most common digester systems used in the United States. Continuously stirred tank reactors increase mixing and digestion process of waste during anaerobic digestion.

Generally, digested waste can overflow from the area of storage due to intermittent or continuous feeding of digester with fresh waste. Total solid should be present around 5–14 % in digester with mechanical mixing. Hydraulic retention time of more than 15–30 days is required for stable continuously stirred tank reactors. Plug flow digester is a horizontal reactor and non-stirring system and waste pass through semi-continuous underground tubular tank reactors or connected concrete channels. Hydraulic retention time is more than 30 days and more solid waste can be used in this system compared to continuously stirred tank reactors. Effective treatment requires long retention time and is unsuitable to flush the manure system due to high capital cost of production and large amount of waste.

Hydraulic retention time is the same as solid retention time of continuously stirred tank reactors. The risk of active biomass washout increases due to steady growth of methanogenic and syntrophic bacteria and reduction of hydraulic retention time, which can lead to complete system failure. Microbial population should be maintained in continuously stirred tank reactors to address this issue at low hydraulic retention time in the digester. The retention of activated microflora in digester is the main focus of research to reduce animal waste. New research is mainly focused on the capacity of bacteria to form a biofilm by attachment to inert surfaces, ability to settle down, or accumulate in the form of granules and process stability with better control (Wilkie and Colleran 1989).

Hydraulic retention time increases from 20 to 30 days with anaerobic reactors in a conventional design due to active biomass, which can further increase from several hours to days. Secondly, initial cost required for digester can reduce with reduction in hydraulic retention time. However, microbial biomass washout can be prevented with anaerobic fixed bacterial film bioreactors, which provide an immobilized surface for the growth of bacteria. In anaerobic digesters, wastewater can pass through inert packing material channel or media, which also provides a surface for the attachment of microorganisms and traps free microflora. Organic particulates and soluble waste are converted into carbon dioxide and methane as flow passes through this column by both entrapped and attached anaerobic microflora. Wastewater can flow downward or upward in the media, which is completely submerged (Dagnall 1995).

6.8.2.2 Nitrification

Increase in animal production in confinement and at large scale has caused a major environmental issue due to animal waste management and disposal in the United States such as fish killing due to surface and groundwater contamination and other concerns, including ammonia release. Modern pit discharge and flush system is used to dispose pig waste in southeast United States, which is mostly treated anaerobically in lagoons before land application. Large-scale concentrated pig herds produce large amounts of manure for land application in small regions, which can produce imbalance among nitrogen availability and absorption (Barker and Zublena 1995; Gollehon et al. 2001). Overload of land with animal waste nutrients leads to water and air pollution. According to an estimate, anaerobic lagoons produce 0.6–104 kg NH_3/ha/day in North Carolina (Arogo et al. 2003).

Plants can absorb a significant amount of ammonia from the atmosphere, but atmospheric deposition of ammonia emission in animal production areas exceeds absorption (Hutchinson et al. 1972; Walker et al. 2000). Therefore, it is important to develop an inexpensive and practical functional method for animal operation areas to manage or maintain ammonia emission in the atmosphere. The removal of nitrogen from atmosphere is also called nitrification, which is considered an important component in animal farm management operations. Nitrification effectiveness depends on microorganisms or nitrifying bacteria with ability to oxidize or fix atmospheric nitrogen (Martinez 1997).

Animal waste is treated with denitrification or biological nitrification to remove large amounts of nitrogen, which is associated with specific conditions (Loehr et al. 1973). Temperature and pH can control the equilibrium among two forms of ammonia such as free ammonia (NH_3) and ammonium ions (NH_4^+). Ammonium ions (NH_4^+) can be oxidized into nitrite (NO_2^-) and nitrates (NO_3^-) by nitrifiers (Anthonisen et al. 1976). Additionally, denitrification or conversion of N into N2 needs further two control conditions such as anaerobic environment and carbon, which is specifically found in liquid manure storage operations or wetlands. Denitrification in pig waste has been successfully evaluated for the removal of nitrogen previously (Szögi et al. 2003). The process was able to remove a huge quantity of nitrogen from animal waste, but it was limited to availability of nitrates (NO_3^-). Pretreatment of wetlands increased the five-time potential of nitrogen removal through the process of nitrification compared to removal without pretreatment (14,000 kg/ha) (Humenik et al. 1999).

Nitrifying bacteria competes poorly in carbonaceous enriched waste material with heterotrophic microorganisms. Effective nitrification needs sufficient number of nitrifiers in growth phase and a surface area with lower organic carbon and oxygen. Pig ammonium enriched waste was not oxidized even after 49 days of incubation (Blouin et al. 1989), while enriched nitrifying population (10^6–10^7 most probable number/milliliter) did a complete nitrification in this waste. Vanotti and Hunt (2000) also reported inefficient nitrification of wastewater from pig lagoon using encapsulated nitrifiers without the addition of nitrifiers, and mostly ammonia was stripped in air during this process. However, fast nitrification rate can be

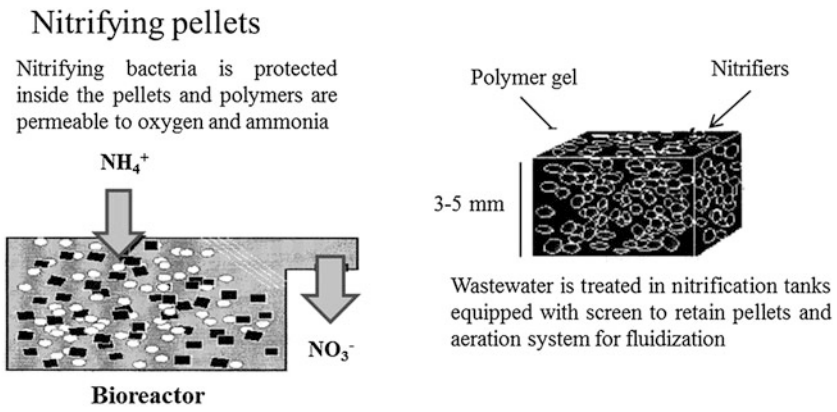

Fig. 6.2 A schematic diagram of wastewater nitrification using polyethylene glycol immobilization of nitrifying bacteria (Vanotti and Hunt 2000)

achieved by using huge number of nitrifying bacteria trapped in resin polymers to avoid the problem of volatilization of NH_3 (Fig. 6.2).

6.8.2.3 Vermicomposting

According to Aristotle, worms are "intestines of the earth." Worms have played a very significant part in the history of life on earth. Earthworms constitute a large part of biomass (living bodies) inhabiting soil. In recent years, efforts have been made to use the potential of earthworms in recycling of nutrients, waste management, and development of vermicomposting systems at commercial scale. Earthworms increase the specific type and number of microbes in different soils to create an ideal environment for its own growth and development.

Potential Benefits of Vermicomposting

Vermicomposting is associated with a number of benefits compared to orthodoxly produced compost in different important aspects; for instance, compost produced through vermicomposting is considered better for compost inoculation for the production of compost, worms used in vermicomposting increase the quality of animal feed on form, and vermicomposting also increases the potential of additional income for organic farmers.

Moreover, compost produced from vermicomposting is considered a better fertilizer compared to chemical fertilizers, because it can restore the microorganism population of phosphate solubilizer and nitrogen fixer bacteria. Additionally, it can increase the availability of micronutrients for plants along with better texture of soils and increase the waterholding capacity of the soil. Vermicomposting process also can improve the growth of plant roots due to better aeration in soil along with better growth of soil beneficial microorganisms. Soil erosion can also be avoided

due to the process of vermicomposting due to increase in the stability of soil structure and less use of pesticides to control plant pathogens. Plant grains gain more sugar content and increase their fruit quality due to vermicomposting process in soil.

However, the vermicomposting process is more complicated than the conventional process. For example, vermicomposting is labor-intensive so as to make the process quicker. Worms cannot feed under one meter in depth due to surface feeding nature of earthworms and need a lot of space for operations. Earthworms are vulnerable to severe environmental conditions such as drought, freezing, and high temperature conditions. The vermicomposting process is an expensive process to start and execute, especially related to purchase of worms and labor. Following the epigeic species of earthworms is suitable for vermicomposting such as *Perionyx excavates, Eisenia foetida, and Eudrilus eugeniae.*

Vermicomposting Process

Vermicomposting is aerobically earthworm mediated bio-oxidation and decomposition to mix organic waste, to make fragment of large particle present in organic waste, and to increase the microbial activity for further microbial decomposition. Following are some basic necessary conditions required for vermicomposting: suitable pH of organic waste, suitable temperature during decompositions, adequate aeration during process, moisture control, type of food source, and suitable bedding.

1. pH Control

Optimum pH is required for proper growth and development of worms, pH range for suitable growth of worms is 5–9. However, pH range from 7.5 to 8 is considered optimum for the growth of worms. pH normally depends on type of food or waste used for vermicomposting, but pH can also drop during the process of decomposition and fragmentation of organic matters due to a series of chemical reactions. However, alkaline food or waste can only avoid the major shift in pH and the process can complete at neutral or slightly acidic pH. Acidic foods such as peat moss and coffee grounds can drop pH below 7. The acidic conditions are really suitable for the growth of mites. Acidic pH can be controlled with application of calcium carbonate.

2. Temperature Control

Temperature can influence the reproduction, growth, metabolism, and activates of earthworms. Moderate temperature for vermicomposting for most earthworm species is around 10–35 °C, but temperature requirement for can vary among different species. Worms cannot tolerate dry and hot temperature, while they can survive in moist and cold temperature. Vermiculture for *Eisenia foetida* is preferred at the temperature range from 15 °C minimum to 20 °C maximum, while vermicomposting can effectively be performed at temperature range from 10 °C minimum to 15 °C maximum. Higher motility is recorded at temperatures >35 °C, but favorable temperature again redistributes worms to windows or bed within piles.

3. Aeration
Vermicomposting can be rendered anaerobic due to many factors such as higher moisture contents and higher levels of oily or fatty substances into feedstock. Anaerobic condition can induce high mortality in worms due to production of toxic products such as ammonia; this condition has less availability of oxygen for worms to consume. So, feedstock with oily or fatty meat should not be used in vermicomposting system or without pretreatment for breakdown of these products.

4. Moisture
Worms need moisture for appropriate growth and development. Adequate moisture should be applied for vermicomposting, which ranges from 60 to 70 %. Humidity or moisture more than the recommended range can be lethal for growth and function of earthworms.

5. Food Source
The most important consideration in vermicomposting is the consistent input of food for the growth and function of earthworms. Worms can survive by extracting nutrients from soil under adverse conditions. However, earthworms like to feed on decomposing organic wastes, active soil microflora, and funa. In the ideal environment, food consumed by worms is more than their body weight, and worms consume food usually half of their body weights per day. Feedstock with higher nitrogen contents are used for feeding of worms such as livestock manure from pigs, cattle, and goats. Carbon and nitrogen ration should not exceed 40:1, otherwise supplementation with nitrogen is required from proper decomposition. Heat can be generated from excessive use of waste, so waste should be used in limited layers. Vermicast is produce from undigested food from worms or excreta of worms, and 5–10 % ingested food is used or assimilated for body functions.

6. Bedding
Worms need a comparatively stable environment in the form of bedding. The bedding should fulfill the following criteria for vermicomposting:

- *Higher absorbance*: Bedding should be able to retain some moisture, because earthworms can breathe through skin.
- *Good potential of bulking*: Bedding should have such a bulking potential that worms can easily breathe.
- *Adequate nitrogen and carbon ratio in bedding*: Bedding is used by worms for food, but nitrogen and protein content should be adequate or less in bedding material. Bedding with higher nitrogen and protein contents can produce a lot of heat during degradation and may produce a lethal effect on worms.

7. Other Important Parameters
Vermicomposting and vermiculture need a number of essential parameters for proper execution.

7.1. Organic waste precomposting: Previous studies report the loss of *Eisenia foetida* worms after 2 weeks on cattle fresh solids, although all other development and growth factors were appropriate such as NO_3 contents, electric conductivity, pH, carbon and nitrogen ratio, moisture contents, and NH_4. The reason for death was the anaerobic conditions developed after 2 week growth on fresh cattle solids. Worm mortality can easily be avoided in this system by pretreatment or precomposting of organic wastes.

7.2. Salt contents: Earthworms cannot survive at higher salt content; salt content should not increase from 0.5 % in feed. Worms cannot use all type of seaweeds until they are rinsed with water before use as feed for worms. Worm food sometimes has more than 8 % soluble salt content. Usually, manure from such high salt food cannot produce any problems for worms, because these soluble contents normally present on surface and worms wait until leaching out of salts from manure. Salt should be leached out before the use of such manure for bedding to reduce the salt content, which can be achieved by simple running of water through such materials for some time. Salt cannot produce any problem if manure is precomposted outside.

7.3. Urine contents: Manure produce on concrete feed offs or farms has excessive urine contents, because concrete grounds do not absorb urine. Leaching of urine from such manure is required before use as feeding of worms, because urine mix manure can produce toxic gases such as ammonia in worm bedding materials.

7.4. Additional lethal component: Animal manure can have many potential toxic components for worms, some of them areas follows:

- *Wormicide medicines*: Wormicides are commonly used in animals for deworming or to kill intestinal worms. Fresh manure just after deworming can induce harmful effects on vermicomposting or vermiculturing. Normally, modern dewormers can easily and quickly degrade into manure, especially in horse manure.
- *Pesticides, cleansers, detergents, and other industrial chemicals*: Such a product can be mixed into waste generated from septic sludge or sewage, paper mills sludge, and wastes from processing of foods.
- *Tannins*: Some waste of trees can produce this sort of waste such as from fir and cedar, which can harm worms and bring them away from feeding beds. Precomposting can reduce or completely remove these harmful threats in worm feed, although nutrient value also reduces during the precomposting process.

7.5. Pests and Diseases: Vermicomposting systems can also be affected from mole predators on earthworms, especially in open air processing. Barriers such as paying, wire mesh, and clay under windrows can prevent moles and other rodents from inducing damage. Windrow cover with old gunny bags can help to avoid damage from flying birds and unnecessary leaching due to rainfall; additionally, it can also help in retention of moisture contents in worm feed. Windrows can also avoid to a great extent centipedes eating compost worms and cocoons. Profound wetting of worm beds can reduce their number significantly, hence flooding of

worm beds should be avoided. Water in feeding beds forces centipedes, pests, and other insects to the surface without affecting worms, so using propane torch they can be exterminated the surface.

Worm feed can also be consumed by ants, which is an additional problem. The consumption of worm feeds by ants can be avoided by maintaining pH at 7 or slightly higher and by avoiding sweet feed contents. Brown and white mites can compete for food with worms also, but red mite is parasitic for these earthworms. They can feed on body fluids and the blood of cocoons and worms. Prevention of red mite can also be achieved by maintaining pH near neutral or marginally alkaline. pH can be maintained near neutral or alkaline by retaining moisture content under 85 % in worm beds or with mixing of calcium carbonate. Soup crops such as crops with high protein content can also induce toxicity due to accumulation of gases and toxic acids produce due to protein breakdown. But better management to maintain a better microenvironment and feed quality can help to avoid the toxicity and adverse effects of soup crops in worm feedings.

Vermicompost

It has been estimated that earthworms add 230 kg N/ha/year in grasslands and 165 kg N/ha/year in woodland sites. Earthworms increase the nitrate production by stimulating bacterial activity and through their own decomposition. There are reports that concentrations of exchangeable cations such as Ca, Mg, Na, K, available P, and Mo in worm casts are higher than those in the surrounding soil. Vermicompost is considered more valuable related to nutrient value than other manures of organic origin for soil. It is a unique way of manure production Garg et al. (2006).

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Chapter 7
Future Challenges Related to Animal Biotechnology

Ali Saeed, Muhammad Abubakar and Sehrish Kanwal

Abstract Consumers and society may be least willing to accept the modern practices for genetic modification of animals. Public and animal welfare organizations criticism on animal cloning and transgenic modification is a hurdle in production of modified animal food. Ethical and moral concerns are also reduced the acceptability of such technology for the production of cheap and better animal food. Moreover, food industries are also very sensitive about the acceptance of consumer's towards the products from genetically modified animals. Consumer is concern about the safety of such a food as compare to naturally produce animal food. Segregation and compulsory labeling about GMO's food products by regulatory authorities also produce assumption of less safe and inferior food as compare to convention animal food products.

The history of biotechnology is as old as civilization since man learned and practiced the art of "planting crops" and "selective breeding of domesticated animals." This was the same time when they started to learn how to ferment various fruit juices into wines, beer, and cheese. Humans learned to prepare foods in a short time and also to convert milk into yoghurt and to make spongy-bread using bacteria and yeasts. The core objective of biotechnology is to adopt new ways to produce sufficient amounts of foods for the increasing world population and to find ways to overcome food shortage in the coming decades, which are the results of global warming, increasing pollution, increasing human population, and decrease in cultivation lands.

Animal biotechnology has emerged from the wide range of recently developed techniques aimed at the genetic improvement of domesticated animals of interest, though this term is increasingly concerned with such technologies that became controversial since their advent, for example genetic engineering (GE) and cloning

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(Eenennaam 2006). Though both these biotechnologies bear so many potential applications, yet no organization from the government or private sector has marketed a genetically engineered food-animal product in the market, in advanced European countries as well as in Asian countries with high population growth rates. Even in advanced countries like the USA the sale of such foods for example, milk and meat products, originating from genetically cloned animals, is subject to voluntary moratorium.

The industry of animal biotechnology is facing a wide range of issues such as scientific regulations, ethical bindings, political and, most importantly, issues concerned with social acceptance. More effective, prompt, and responsible communication is required among stakeholders of the concerned scientific communities, general public, relevant industry, and government to gain a societal consensus regarding the acceptance issues of cloning of animals and GE.

Despite both these technologies that bear the potential, undoubtedly, to deliver such benefits for humans, no genetically engineered livestock are being marketed currently and the Food and Drug Administration (FDA) of the United states of America continues to call for the voluntary prohibition on the marketing of the products originated from genetically engineered clones and also from their offspring, such as meats and milk products.

Biotechnology may be defined as "technology based upon biology." From the perspective of this definition, it becomes clear that animal biotechnology is being practiced by animal breeders for many years. So the history of the animal biotechnology is old. Traditional selective methods include information through observation regarding the biological attributes and other physical characteristics for the selection of parents of an animal of new generation.

Traditional dog breeding provides us with so many dog breeds to understand the influence of breeding on the physical appearance of the particular breed and also the biological attributes of their offspring from the parents of a specific species.

Various kinds of biotechnologies have been employed in livestock breeding programs for the purpose of genetic improvement of specific animal species of interest. These may include insemination by nontraditional means such as artificial insemination (AI), use of data obtained from thousands of offspring for "sire testing programs," estrus synchronization, embryo transferring, cryopreservation of embryos and gametes, and marker-based selection such as DNA-based marker-assisted selection using genetically more suitable animals. Before their application and eventual broad spectrum adoption, a few of these newly developed technologies still had related controversies and faced resistance at the time of their introduction (NRC 2002).

Earlier, AI was thought to be "against the laws of God, a repugnant practice that would lead to abnormal outcomes" but today this technology is being utilized in agriculture and livestock (NRC 2002). Use of AI veterinary and human medicine is also widespread. Traditional animal breeding for genetic improvement, which we practice today, has not emerged without paying a price because there have been some associated concerns regarding the health and welfare of these animals of high productivity. These include gait abnormalities in the case of broiler chickens as well

as high yielding dairy cattle that face fertility problems. Selective breeding of domesticated dogs has also given rise to such a questionable legacy, which are affected with more than 200 known genetic diseases. Before the appearance of Dolly sheep, considered as the first cloned complete animal, cloning was actually being practiced a long time back. In the 1980s, making of identical twins followed by bisection or splitting of an embryo was practiced in livestock breeding programs in which cells from developing embryos were split and transferred among different recipient mothers. Technically, identical twins may also be referred to as clones but presently this term is increasingly being used to refer to an individual that originates after transplantation of the genetic material from a single somatic cell (non-egg) obtained from an adult healthy organism and then reintroduced into an un-nucleated egg whose DNA has been previously removed, called enucleated oocyte. This phenomenon is termed "somatic cell nuclear transfer (SCNT) cloning" and is performed in many livestock species with good success rates such as cows, buffaloes, goats, and sheep.

Owing to the prospective of breeding of animals, the significance of SCNT cloning is that this method involves replication of healthy adult animals having visible extraordinary performance characteristics over others with inferior attributes or biological performance. In the United States some commercial companies have emerged that provide cattle cloning facilities and hence offer cloned offspring at a rate of $8000–$15,000 each. From the perspective of agricultural operations, prospective uses of the cloned animals and their products at commercial level are limited.

A specific genotype of livestock, which is specifically well adapted to a particular environment, can also be generated using these clones. The significance of this technology is that any genotype that is proven to perform and behave reasonably well in a specific location can indefinitely be maintained without having genetic shuffle, which usually takes place in each generation followed by traditional means of reproduction. But this technique does have some drawbacks as well, such as this approach freezes genetic progress ultimately pointing toward desirable attributes, including milk production and resistance against different diseases at a point in time.

Since the population of clones does not bear genetic variability, hence within-herd selection there does not exist further chance for genetic improvements. Moreover, lowering of chances of genetic variation among the herd or flock can make that specific herd or flock more susceptible to any disease outbreaks occurring in that area, or poor adaptability, or not suited to the environmental changes at individual level.

The clones may still differ from one another even though all of them have the same genetic information in their chromosomal DNA, this is very much the same as identical twins who do not behave alike even though they might look like one another. These clones only have identical nuclear DNA but they do not share identical cytoplasmic DNA, for example mitochondrial DNA, which comes from the donor egg. Also, because they are frequently and traditionally born and raised by different parents so they do not even have the similar gestational environment. A

study has shown that SCNT clones are more different more from each other compared to their contemporary half-siblings (Lee et al. 2004). Cloning is still in its infancy stage so this cloning technique is obviously currently inefficient and hardly 1–3 % of the transformed egg cells are actually able to develop into live offspring. Additionally, pregnancy loss rate is seen to be very high at different stages after the egg, having nuclei of somatic cells of an adult animal, has been placed into different recipient individuals. However, these issues may not be observed in SCNT-clones of cattle livestock, there have been few reports of physically healthy cloned cattle which have even gone to conceive the pregnancy and also gave birth to healthy offspring (Lanza et al. 2001; Pace et al. 2002).

Some abnormalities are even seen in the cloned animals subsequent to their birth; frequencies of these abnormalities are not concerned with the type of organ that actually donated the nuclear material. Such abnormalities may include defects in the cardiovascular system, diseases of the musculoskeletal and nervous systems, and susceptibility to various kinds of infections and disorders of the digestive system. Most of these abnormalities result from wrong reprogramming of the donor chromosomal DNA of a somatic cell which goes into transition from directing cellular activities into the complex form of developmental pathways required to develop to form entirely new embryo. Among the cloned offspring, researchers have found abnormal gene expression behaviors and also the mistakes and abnormalities in imprinting as well as inactivation of the X-chromosome (Thibault 2003).

Food safety The major food-safety issue concerned with SCNT clones is whether the nuclear rearrangements that usually occur in cloning procedures may have influenced the chemical, biological, and physical characteristics of these animal food products, since there are no fundamental reasons to suspect that offspring derived through SCNT will produce some novel toxins and allergens.

In different studies aimed to compare the characteristics of SCNT clones as well as other clones of the dairy cattle with their all offspring, no clear differences could be observed regarding their performance, physical and biological attributes, and even milk composition (Takahashi and Ito 2004; Norman and Walsh 2004; Walsh et al. 2003; Tome et al. 2004; Tian et al. 2005). In the United States, the FDA Center for Veterinary Medicine is developing a strategy for the assessment of risks in order to specify various kinds of hazards originating from cloning and to identify food consumption risks (Rudenko et al. 2004). The center's report regarding the cloning processes of livestock states, "the current weight of evidence suggests that there are no biological reasons, either based on underlying scientific assumptions or empirical studies, to indicate that consumption of edible products from clones of cattle, pigs, sheep or goats poses a greater risk than consumption of those products from their nonclone counterparts" (FDA 2003). Even after all these findings, marketing of such cloned food products such as milk and meat obtained through SCNT clones as well as their progeny is subjected to the issue of prohibition on voluntary basis. The FDA report indicates, "additional data on the health status of progeny, and composition of milk and meat from clones and their progeny, would serve to further increase the confidence in these conclusions." So researchers and

organizations around the world are aggressively engaged in data collection of these types so as to minimize issues related to the awareness and social acceptance of such food products. Very less or even no investments can be seen from rest of the world other than the USA in this field from commercial organizations, especially from developing countries. Food safety issues must be brought to the knowledge of the common person for social acceptance of these foods.

Environmental concerns The National Academy of Sciences has published a report that states that environmental issues are the major concerns being faced in the animal biotechnology industry, which are based upon scientific findings (NRC 2002). The greatest concern so far is if this genetically engineered organism specifically including those of fish and insects escape from their confinement they can become dangerous. This report also suggested that interbreeding of these genetically modified organisms (GMO) can have some serious ecological consequences, such as having enhanced fitness characteristics such as sexual maturation at the time of relatively younger ages (Muir and Howard 1999, 2001, 2002).

The real threats to the environment exerted by each specific species/transgene combination would depend on so many factors, such as containment strategies, mobility of species, potential to become threat, environmental interaction of the genotype, and also the stability of recipient communities. Similarly, food safety issues regarding transgenic animals are also going to be case-specific based on the fundamental characteristics of the proteins that are recombinant in nature and if pharmaceutical in nature or industrial proteins or even food proteins. To encourage scientific as well as applied research "the U.S. Department of Agriculture's (USDA) Biotechnology Risk Assessment Grants Program" is currently providing $5,000,000 every year to support research. These research activities are aimed at identification and development of suitable strategies so as to reduce the risks of physical and also biological nature, which are related to genetically engineer animal species.

Animal welfare considerations The term animal welfare means the physical as well as psychological betterment of animals (Hewson 2003). They are the sentient, living creatures and due consideration should be given to them when used for human purposes (Hewson 2003). They might be more often treasured members of a family. Owing to our own different personal beliefs, some persons are usually against their human use for specific purposes; still many others have concerns regarding the implications and consequences which GE and cloning together might have exerted on animal health and welfare. Some even consider it specifically hurting to terminologies such as "transgenic animal bioreactors" which are employed in industries and are used to introduce genetically engineered organisms that produce human therapeutic products and industrial proteins. Both animal cloning and transgenic technologies create issues regarding the welfare of animals, most of which is related to the poor efficiency of these newly developed techniques. This obviously results in the use of more and more animals than those it would actually be required if these were more refined so as to achieve high rates of success. Procedures like embryo transfer and superovulation can bring pain and discomfort to animals. Such manipulations involving these procedures are essential

to produce the genetically engineered organisms and also the clones. However, traditional livestock breeders have been employing such techniques for so many years that such concerns are not new or even unique specified only with the techniques of biotechnology. In bovine embryo cultures in which in vitro embryo culture techniques are commonly used (such as SCNT clones), it is commonly experienced that the resulting young calves may have high birth weights along with long gestational ages. This phenomenon lead to higher rates of caesarian sections for the dam and is termed as "large offspring syndrome" and results in some calving difficulties. More specifically, poorly managed gene expression of this newly introduced gene is the major welfare concern for these engineered animals. Different types of growth-related abnormalities are observed in these genetically engineered animals, which express specifically for a transgene growth hormone (Pursel et al. 1989; Devlin et al. 1995).

If animal biotechnology can increase the efficiency of production of farm animals, then this may have been the effect of harsh conditions which some already observe as not acceptable and were found in exclusive animal-feeding processes. This particular issue is also not new or even unique while employing these techniques of GE because of the fact that any genetic selection program exclusively directed toward the efficiency of higher yields has raised welfare concerns for these farm animals, irrespective of the technologies employed to achieve that objective. Animal biotechnology could also be employed for improving specific characteristics of interest such as resistance against specific diseases, which would ultimately have its role in reducing animal diseases. It may also be more likely that GE is employed in increasing agricultural yields; it seems possible that these techniques are used in future for biomedical applications. In such cases, the genetic manipulations are not required to bring about the changes with physiological effects but usually can raise some concerning welfare issues for these animals (NRC 2002).

Some unique concerns are still there, like premature lactational shutdowns which have been seen in some animals whose mammary glands express specific recombinant proteins (Shamay et al. 1992). Moreover, particular pathogen-free housing needs for animals are needed to produce therapeutic agents for humans and even organs for the use of organ transplantation in humans can also have potential to compromise the behavioral needs of an animal.

Biotechnological developments have recently resulted in innumerable benefits for man but apart from these benefits there are also some serious concerns with unintended environmental issues, social acceptance, and health-related consequences. While taking all these impacts into consideration, researchers should not only take into account the consequences of any scientific development but also accept the major responsibility for the appropriate application of these scientific results.

In the field of biotechnology, most critics tend to argue that "it will jeopardize even our past achievements and add numerous incalculable risks to our future. The human body will be commoditized and objectified, becoming a source of patentable raw materials which can be combined to produce tissues and living organisms that cannot develop naturally" (Andrew 1993).

Hans Jonas was the first who expressed such an idea in his seminal work "The Imperative of Responsibility" (Jonas 1984). He was of the view that animal biotechnology has the potential for environmental and health risks and hence can exert negative impact on other species of the world.

Emerging technologies Various technologies have been developed and improved in the field of animal biotechnology. Biotechnologies provide an excellent opportunity for man. These technologies make it possible to produce foods that have more nutritious value, medicine, and also the chance to develop different ways for growing more food in saline water, almost drought lands, and also in stressed conditions. Despite all these contributions of animal biotechnology, various controversies have also been raised in this area. All of them bring forth various ethical challenges. What is the environmental impact of biotechnology? Another ethical issue is much concerned with animal's welfare and also to human health. During the last couple of decades different kinds of arguments have been discussed in this regard. Is animal biotechnology violating the concepts of 'animal integrity' or otherwise? The Dutch National Committee on Animal Biotechnology presented its argument that biotechnology does have the potential for negative impacts on animals. For the purpose of benefits to humans, these technologies have changed the characteristics and attributes of animals by means of genetic modification, being considered to be the "violation of the integrity of the animal." Before discussing the objections against the animal biotechnology applications, it needs to be clarified the concept of "animal integrity." Such a concept was not developed in the context of these applications of biotechnology. In fact this concept was adapted from the field of ethics to discuss the impact assessment of animal biotechnology as well as the genetic modifications of these animals. A large number of ethicists and veterinarians in Utrecht University gave definition to the term, "animal integrity." In this definition, they emphasized the fact that all animals bear

(i) "wholeness and completeness,"
(ii) "species-specific balance of an animal," and
(iii) Animals possess their own capacity to maintain themselves independently in the environments well suited to the species (Vries 2006; Rutgers and Heeger 1999, pp. 41–51; Heeger 1997, pp. 243–252).

The notion of "animal integrity" signifies that we should not apply any kind of interference among all these features possessed by animals. This definition implies the fact that all animals have their own "physical intactness," which should not be compromised or interfered. However, the techniques of GE and also transgenesis processes involve the introduction of "a gene foreign to the species to a gamete, the wholeness and completeness of the animal is altered at its most fundamental level, the genome" (Vries 2006).

Now it is understood that animal biotechnology in any of its forms is rather a sort of interference in the "wholeness and completeness" of the animal. For example, a chicken or a goat has specific features of its own that should not be altered by modifying its original physical intactness. However, the breeding and transgenic processes used in broiler chickens have certainly violated the concept of wholeness

and completeness of animals. Rutgers and Heegers described the second feature of animal integrity as: "species-specific capacities." They believe that the violation of the second feature of the definition is actually the violation of principle concept of animal integrity. After producing a broiler chicken, for example, it has the capability to grow very fast but at the same time it is unable to move naturally. It rather grows at an abnormal pace and its biological fitness and attributes are not well suited for the environment it grows in. All of these things upset the biological balance of these animals. Rutgers and Heeger claim that "the more the animals lose its species-specific capacities and characteristics, the more serious the integrity violation" (Rutgers and Heeger 1999).

Environmental suitability remains the third characteristic feature for the definition of animal integrity of the specific animal species. Animal integrity also signifies the best role for the moral status of animals, being a prime issue.

Many different kinds of questions may also be asked in this case:

What remains the ethical status of animal species?

Are there some more ethically important species than those of other species?

Controversies and animal biotechnology Controversies are even observed among researchers on the point if animals do have moral status or otherwise. Besides, in what sense could these animals be considered morally? The answer to this question was given by Heeger thus: "animals have well of their own" and "they have interests, namely in everything that contributes to the realization of their good" (Vries 2006). Biotechnological interventions involve the whole or intact body of any given animal and also the ability to threaten its necessary characteristics and biological attributes.

In the previous century, a large number of scientific discoveries marked the opening of a new era related to different scientific advancements. Advent of information technology (IT) and GE are the major notable discoveries of the past couple of decades. During the past few decades, biotechnology had ushered int-different kinds of technologies a few of which are:

(i) Bio-processing, which refers to in vitro manipulation of individual cells,
(ii) Recombinant DNA technology
(iii) Monoclonal antibody.

Despite potential capabilities which animal biotechnology has already got, there is increasing number of controversies related to the different areas of applications of animal biotechnology. If one concentrates only on the benefits that man has obtained in terms of consequences of animal biotechnology, then possibly we will not be able to find problems in it. There is also the dark side to biotechnology, similar to animal biotechnology, which cannot be ignored whatsoever. A large number of ethical experts have started realizing the adverse effects of this technology. Drastic implications and the social impacts of biotechnology have been compared by Gerhald Becker as "the splitting of the atom and the technological exploitation of nuclear power. As with nuclear technology, biotechnology has put enormous power in our hands" (Becker 1996).

Nuclear power has contributed positively for the welfare and well-being of man but at the same time, it has the ability to destroy innumerable lives of mankind as

mass destruction. Similarly, biotechnology has also the same potential for such evil purposes as well and hence could cause "incalculable risks for human integrity, well-being and freedom" (Becker 1996).

Experts such as Thomson (1996) are of the opinion that these potential risks laid down by these techniques can also be transformed into moral concerns. Thomson concentrates on the "unintended consequences" and "ethical concerns" of this modern biotechnology and which is "inherently unethical" (Thomson 1996).The developments in the field of biotechnology in the past decades have increasingly raised concerns of ethical controversy. Critics have formulated different kinds of arguments while opposing biotechnology. For the sake of convenience these can be divided into two kinds: (Kaiser 2005).

(1) Intrinsic arguments
(2) Extrinsic arguments

Intrinsic arguments being against these technologies suggest that biotechnology is "objectionable in itself" (Comstock 2000), while extrinsic argument concentrates mainly on the "allegedly harmful consequences of making GMOs" (Comstock 2000). Animal biotechnology, in this sense, remains ethically problematic as "it is unnatural to genetically engineer plants, animals and foods" (Comstock 2002).

These arguments put forwardthat animal biotechnology is a kind of "redesigning an animal" referred to as "Playing with God". There are also fears that these technologies have also the potential for breakdown of boundaries of these natural species. In the context of extrinsic arguments, the technologies employed by animal biotechnology are ethically problematic in the sense that they may contain negative consequences over mankind, animals, and even on the environment as a whole.

Anticipated benefits of animal biotechnology Currently, the world population is around 7 billion and it is not possible to feed this population; yet it is growing at a decent pace and is expected to reach 8 billion in 2020, using traditional techniques employed in livestock and agriculture at the beginning of the previous century. Similarly, all the traditional techniques used in the early and mid-1900s could not fulfill the food requirements of today; the technologies being employed today will not be sufficient to feed the 8 billion world population expected in 2030. In fact, some estimates argue that if population growth is simply stopped at this stage, even then the current demands of global agricultural production will have to be doubled in order to meet the requirements of our current world population in the year 2030. These calculations can be best interpreted in terms of affluence which is currently following upward trends. The current demands for food commodities such as meat, milk, and eggs is also increasing day-by-day due to this fact.

The developed western countries have rarely experienced any food shortage as do the people of Africa and other underdeveloped countries. According to some estimates 20 % of the world population is considered to be malnourished. According to the Food and Agricultural Organization (FAO) of the United States, Asian countries can hardly produce 17 g of animal proteins per person each day to feed their 4 billion citizens. In the developed western countries these figures are high, for example in the USA, American citizens consume around 65–70 g;

similarly other European countries are also increasing their consumption. Internationally, it can be speculated that agricultural researchers will be required to produce an estimated 55 g of animal proteins per person/day by the year 2030.

Recent economic crises prevailing in the western world has affected affluence patterns as well as the global demands for agricultural food products, especially in Asian countries. Thus it is the need of the hour to develop or design new strategies for the production of food products, rather than refining food production of animal sources to bring about economic and political stability. The universal law also emphasizes to adapt or die so that these methods for food production may be best exploited without endangering the future of man.

While it is easier to draw the terrible picture of a hungry population, in reality our current global food supplies have outstripped our demand, which is in-fact fortunate in the short run. Oversupplies followed by decreased demands and domestic support programs of these commodities have led to livestock markets in the most disheartening phases in recent years. Producers able to withstand such kinds of extreme economic hardships will continue to face genuine issues concerned with air as well as water pollution regulations, issues regarding health and well-being of animals, inflating production investments, and food safety. Nevertheless, the end user of these products that are continuously in increasing numbers, will keep on expecting to get not only safe but economical food products at the same time. Such producers whose ranks are not good and are diminishing will definitely face the challenges to meet these requirements under such harsh circumstances, which will allow them to realize fair profit margins. In order to achieve that goal, production efficiencies will have to be enhanced or improved, but the question that arises is how are we going to do this? One approach toward the solution, being followed aggressively in this complicated economic, regulatory, and political environment, is the development as well as application of biotechnologies that will emphasize the need to engineer the GMO or the generation of such superiororganisms that have enhanced metabolic activity, both plant and animal. This will lead to the goal of designing efficient, robust, socially acceptable, highly productive agricultural system with more profitability.

Genetic engineering is already technically feasible, if not yet practically, in the case of large animals of economic importance. Using these GMOs engineered to enhance their efficiency in terms of growth and reproduction can gain more resistance against specific diseases, harbor extreme environmental conditions, produce food with higher value of nutrients, and even yield all molecules that are of value for human as well as veterinary medicines. These could also provide a way out to many of the challenges being faced by mankind. To exploit these technologies in an efficient manner it is imperative to gain support for aggressive research and development (R&D) activities and also to the attract the best and bright minds of the younger generation in the field of agriculture research. Moreover, it will also be necessary to recognize the role of effective educational programs to be implemented aggressively, which will ultimately help in public understanding and beliefs in the value of this work. By this way it can also generate social acceptance, and the potential and safety of these technologies along with food items obtained from it.

In the later years of the previous century (1980s onwards), biotechnology emerged like a distinct discipline. Although the beginning of biotechnology is traced back to prehistoric civilizations of man when they started using microorganisms in processes like "Fermentation, Preparation of Cheese from milk, and of Vinegar from molasses, Tanning of leather, Production of Beer and Dairy products, selective breeding and domestication of the animals of interest," etc.

In the last couple of decades we have seen rapid growth in the number of animal and plant scientists who used techniques for cell, tissue, organ culture aimed at the improvement of animal species and plants of interest. In the twenty-first century the discovery of restriction enzymes is considered to be a milestone in scientific revolution which leads to the advent of a variety of gene technologies. Hence, the future of biotechnology looks rather bright, safe, and strong. Scientists have started to realize that biotechnology is a boon for mankind and has the capability to solve so many issues. Now scientists do believe that this biotechnology will provide us with some wonderful tools to solve many of the problems mentioned above and that in the coming decades we are going to benefit from too many things from the origin of biotechnology.

Public concerns and animal biotechnology In 2005 a survey was conducted in the USA to record the public awareness and attitudes about genetically engineered food products. No such surveys are available from other parts of the world, especially in Asian countries. The results of this survey showed that knowledge regarding these foods was limited as only 6 % of the respondents had actually declared that they had enough knowledge of applying these biotechnologies on animals, 45 % answered that they did not know anything about these animal biotechnology techniques. In this survey, approximately half the respondents opposed the use of such cloned or engineered products and also opposed research on GMO, the main reasons for opposition being ethical or religious grounds rather than concerns of food safety (Gallup 2005).

Consumers may be least willing to accept the practices of genetically modifying animals than those of plants; some argue while observing that people relate these animals differently. Many do recognize animals as sentient, living creatures. Some critics have also expressed concerns that cloning of farm animals will ultimately lead to not only pet cloning but also cloning of humans in the coming years. Still some other observers are of the view that such animal modifications just to save the lives of humans through xeno-transplantation or producing some important drug agents is more acceptable to them rather than production of cheaper foods through these methods.

Food industry producers seem more sensitive about the consumer's issues of acceptance of animal biotechnology as well as cloning. Most companies are not willing to process and market meat and milk from these origins. They believe that consumers will view these food products as less safe than those of conventional food products, being produced by traditional methods. The food industry is wary of the fact that it does not overstep any such widely held ethical issues as well as moral concerns regarding these newly developed technologies (Kochhar et al. 2005).

Some observers believe that segregation and labeling of the food products of biotechnology, such as meat and milk, will help enable the consumers to decide whether to purchase these products or otherwise. This could also contribute to public assumption that these food commodities are inferior, less safe, or different in a negative sense, which will lead ultimately to sort of policy decisions having some contradictions (Foreman 2003).

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